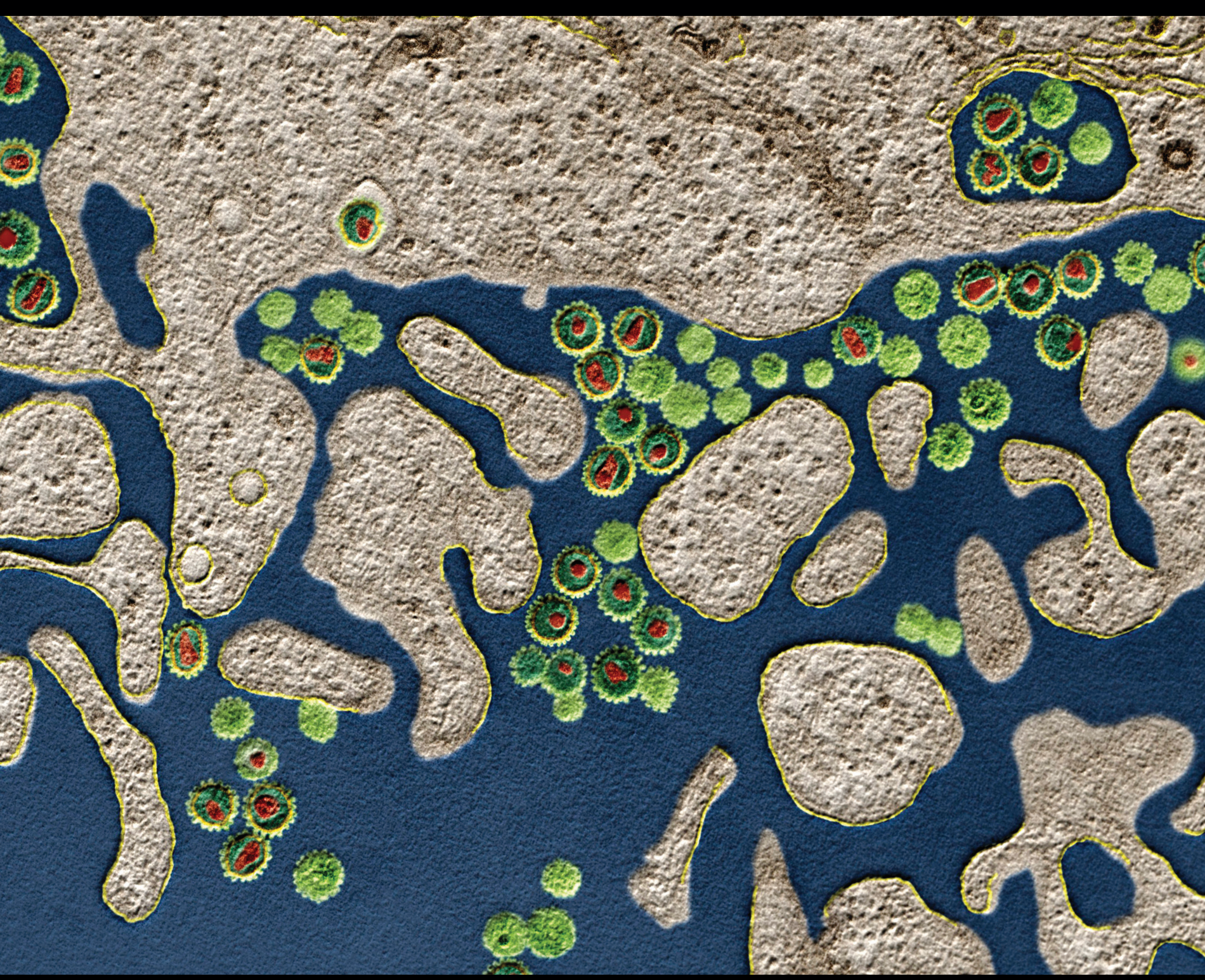
Journal of Immunology Research

Immune and Inflammatory-Mediated Disorders: From Bench to Bedside

Lead Guest Editor: Marcella Reale
Guest Editors: Lucia Conti and Diana Velluto



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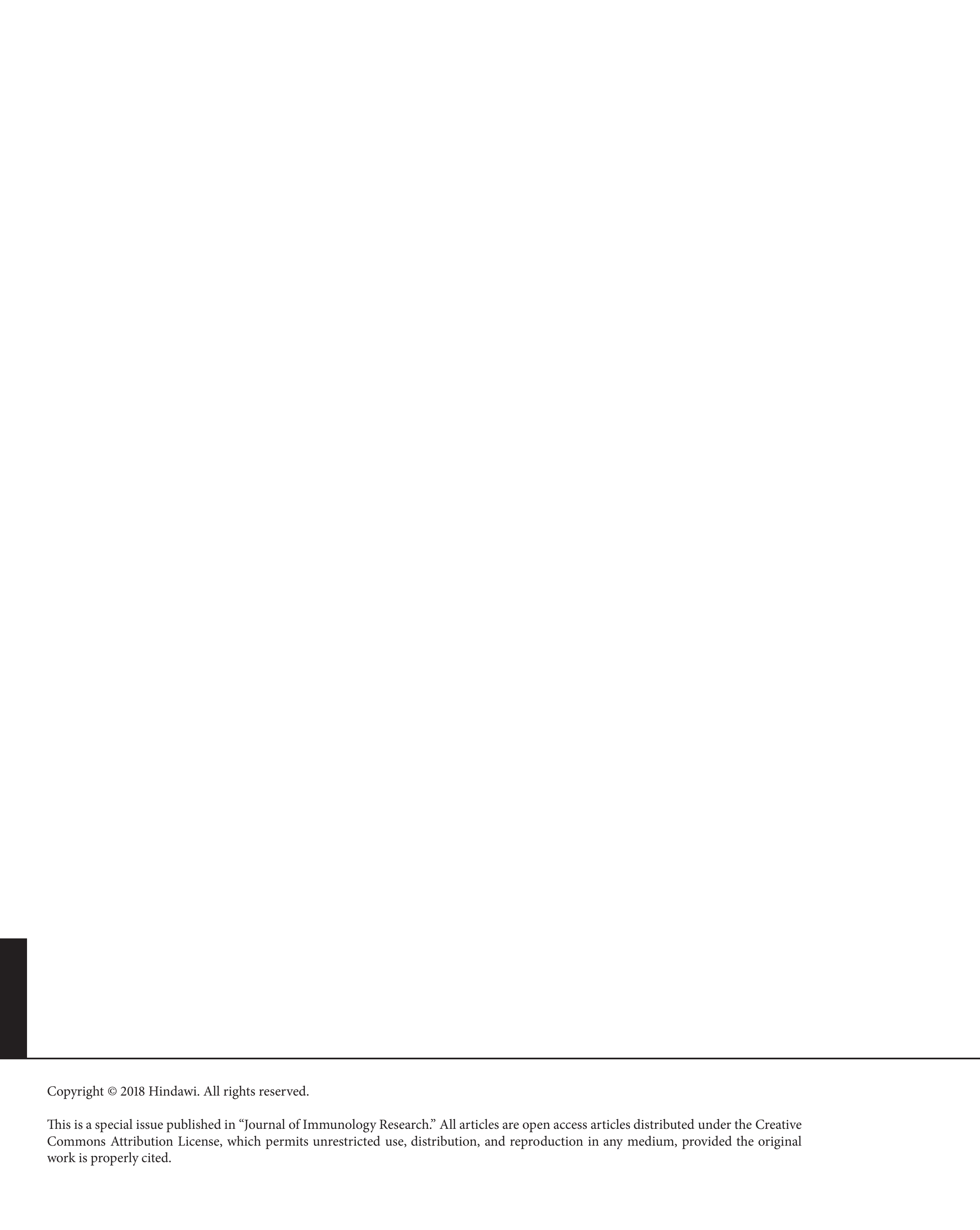

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Editorial

Immune and Inflammatory-Mediated Disorders: From Bench to Bedside

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Immune responses play a key role in maintaining tissue homeostasis, influencing nearly all organs and systems of the body including skin, gut, lungs, brain, and the cardiovascular system. Excessive or dysregulated immune responses and chronic inflammation represent a central driving force in many disorders, including infectious, inflammatory, and autoimmune diseases, as well as cancer. Cells of the mononuclear phagocyte lineage act as innate sentinels and are actively involved in regulating the balance between homeostasis and inflammation, thus ultimately contributing to the maintenance of the health condition. Age- and gender-related differences in immune response as well as in gut microbiota are emerging as important contributors in increasing the complexity in the diagnosis, treatment, and prevention of immune-mediated disorders.

In four original investigation articles, different autoimmune diseases were studied to find new biomarkers that could help explain the aetiology and pathogenesis of the diseases and be useful for new targeted therapy.

Myasthenia gravis (MG) is an antibody-mediated disease affecting the neuromuscular junction, caused by antibodies against the nicotinic acetylcholine receptor (AChR, AChR-Ab). Thanks to short half-life serum levels, free immunoglobulin light chains (FLCs) can be considered an instantaneous marker of B cell activity. In their study, U. Basile et al. showed an increase in free k chains in both AChR- and muscle-specific tyrosine kinase (MuSK-) MG while free λ chain levels were increased only in AChR-MG. Thus, they suggest that at least k chains can be considered a very sensitive circulating biomarker of B cell activation and humoral autoimmune response. This may represent a preliminary important study for a more detailed multicenter analysis.

It is clinically known that patients with one autoimmune disease tend to develop additional autoimmune diseases, and recently an increased prevalence of neuromyelitis optica (NMO) in patients with MG has been reported. To explain the exacerbation or increased susceptibility of patients with one autoimmune disease to developing an additional autoimmune syndrome, T. Mizrachi et al. established an animal model for both NMO and MG, using EAMG mice immunized with Torpedo AChR and then subjected to passive transfer of NMO-IgG or to immunization with AQP4-derived peptide. This study shows that injection of either AQP4 peptide or NMO-Ig to naïve mice caused increased fatigability and when the same molecules were injected into EAMG mice, the disease severity mediated by muscle weakness significantly increased.

In the course of primary Sjögren's syndrome (pSS), inflammatory cell infiltration consists mainly of lymphocytes infiltrating exocrine glands, which leads to their impaired function. The characteristic feature is generalized dryness. The disease develops slowly, and months can pass before a patient presents full spectrum of clinical symptoms. Insufficient treatment without inhibiting the autoimmune response leads to severe complications. A. Sebastian et al. attempted to answer the question whether it is possible to distinguish between patients with pSS and individuals with dryness caused by other pathologies without applying invasive diagnostic methods. The study included 68 patients with pSS

and 43 healthy controls with dryness. They found that chronic fatigue syndrome is more common in pSS patients and can be a subjective distinguishing factor in the group of people with dryness.

E. Dziadkowiak et al. have planned their study to establish whether in patients with pSS without central nervous system (CNS) involvement, the function of the central portion of the sensory pathway can be challenged. The authors, by measuring somatosensory evoked potentials (SEP) to evaluate the function of afferent sensory pathways, confirmed dysfunction of the central sensory neuron, which indicates subclinical damage to the CNS in pSS patients.

Behcet's disease (BD) is an autoimmune and autoinflammatory disorder which origin is unknown, although both genetic and environmental factors play a role. Several genes have been found to be associated with the disease. Transcriptional profiling of PBCs, obtained from patients with active BD, to evaluate the role of the immune system in the pathogenesis of the disease was performed by A. Puccetti et al. The authors found up- and downregulated transcripts. By performing Gene Ontology analysis, they evidenced that most of the regulated transcripts can be related to inflammation, immune response, apoptosis, blood coagulation, vascular damage, and cell proliferation pathways, all playing a key role in BD.

The mechanisms contributing to the chronic inflammatory condition underlying some immunomediated disorders have been investigated or reviewed in a number of articles.

Lobular inflammation and mixed portal/periportal inflammation were observed in recurrent hepatitis C virus (HCV) infection and in acute cellular rejection (ACR), respectively. The aim of the research by A. I. Gomaa et al. was to evaluate whether the origin of macrophages and the immune mediator CXCR3 could help in differentiating between acute recurrent HCV infection and ACR after liver transplantation. Analyzing the expression of CD68 and CXCR3 in the postliver transplant biopsy in cases of recurrent HCV infection and cases of ACR, the authors found that CD68 was expressed in both recurrent HCV infection and ACR, and in patients suffering from recurrent HCV, stronger CD11b deposits in liver biopsies were also detected. On the other hand, CXCR3 is a marker and plays a considerable role in acute rejection following liver transplantation. The authors concluded that macrophages infiltrating the liver tissue after transplantation can distinguish between ACR by upregulation of CXCR3 and recurrent HCV infection by predominantly expressing CD11b.

H. Li et al. have reviewed the role of the innate immune system, inflammatory cells, immunoglobulins, immune-mediated mechanisms, and key cytokines in the pathogenesis of abdominal aortic aneurysm (AAA), a common degenerative cardiovascular disease. Reviewed studies demonstrate that immune-inflammatory reactions play a key role in AAA formation, development, and progression opening the door to the individuation of molecular targets and that, although a good deal of strategies have been proposed, the clinical practice is still lacking a valuable test.

G. Gelders et al. have reported current concepts of neuroinflammation and its involvement in Parkinson's disease- (PD-) associated neurodegeneration and interventions that could modify the pathological immune response in PD. In particular, the potential link among α-synuclein, activated microglia, increased expression of Toll-like receptors (TLRs), and several proinflammatory mediators, which consequently activate peripheral immune response, might open novel therapeutic options to modulate disease progression and outcome.

Among the recognized chronic inflammatory disorders is atherosclerosis, that represents a major threat to public health worldwide as is the main cause of cardiovascular diseases. Atherosclerosis is induced by oxidized low-density lipoprotein (ox-LDL) accumulation in the arterial intima under hypercholesterolemic conditions, which generates a state of chronic vascular inflammation. Multiple innate cell types contribute to this pathophysiological process, with macrophages playing a major role. In their original research paper, Y. Wu et al. reported, in the mouse macrophage model RAW264.7, that Toll/IL-1R domain-containing adaptor-inducing IFN-β (TRIF), a key adaptor of TLR3/TLR4-mediated signaling, plays an important role in regulating the ox-LDL-induced inflammatory response. They specifically demonstrated that TRIF modulates the expression of BIC/miR-155 and the downstream SOCS1-STAT3-NF-κB signaling pathway via ERK1/2 activation, highlighting the potential role of TRIF as a novel therapeutic target for atherosclerosis. Y. Hao et al. investigated the mechanisms of the chronic inflammatory response contributing to visceral hyperalgesia (VH), providing new insight into the mechanisms involved in the antinociceptive effect of protease-activated receptor 4 (PAR4) activation. VH characterizes subjects with irritable bowel syndrome (IBS) who perceive excessive pain during abdominal distension. Activated colonic mucosal mast cells (MC) have been shown to play a crucial role in this process, through the release of proinflammatory mediators like tryptase, iNOS, IL-1β, and P2X7. The authors confirmed the expression of PAR4 in this cell type in a rat model of VH and its role in blocking the induction of proinflammatory mediators, suggesting that, in the gastrointestinal tract, the antinociceptive effects of PAR4 activation are mediated, directly or indirectly, by MC. They also hypothesized that nociceptive receptors could represent additional targets for modifying pain in gastrointestinal disorders such as IBS and inflammatory bowel diseases.

Myocarditis, mostly induced by viral infections, is an immune-mediated disorder resulting from both direct virus-triggered damage and indirect lesions induced by the host immune system. In particular, dilated cardiomyopathy, a complication of myocarditis, results from host immune response-induced killing of virus-infected and virus-uninfected cardiomyocytes and can lead to death. L. Zhao and Z. Fu summarized in their review article the specific role of host immunity (autoimmunity) in the development of viral myocarditis and in dilated cardiomyopathy.

Regarding cardiovascular diseases, in another review article, W. Zhou et al. reported on a novel mediator, the NLRP3. The NLRP is a subfamily of the nucleotide-binding and oligomerization domain- (NOD-) like receptors (NLRs) containing a pyrin domain. The NLRP mainly participates

in inflammasome formation that is linked to many cardiovascular diseases. In this review, the authors presented the current knowledge about the function of NLRP in vascular disease, ischemic and nonischemic heart disease, and they discussed the potential therapeutic options targeting the NLRP3 inflammasome.

Human gut microbiota, as well as microbiota associated with other body sites (i.e., oral cavity, airways, and skin), is increasingly recognized for its important role in health. Microbiota and host mutually affect each other, and this intimate relationship strongly contributes to the maintenance of homeostasis. Disruption of this equilibrium has been associated with several chronic inflammatory diseases.

In this special issue, F. A. Salzano et al. have reviewed recent studies documenting the emerging role of nasal microbiota in reactive nasal inflammatory conditions, where the effects of allergens and environmental agents are mediated by host factors, including innate and adaptive immune responses. The critical role of nasal microbiota in coordinating these components and the contribution of microbial composition in affecting the onset and progression of allergic or nonallergic inflammation were discussed. Likewise, R. Yang et al. discussed the role of structural changes in gut microbiota composition in inducing immunological changes and in sustaining chronic hepatitis B virus infection and liver inflammation. The role of innate immunity components such as TLRs in linking intestinal flora and liver immunity is also reviewed.

Given the need of improving cancer prognosis, Y. Gidron et al. have studied the influence of the vagus nerve on tumorigenesis and observed that the vagus nerve may slow tumor progression because it inhibits inflammation. They have examined the relationship between a new vagal neuroimmunomodulation (NIM) index and survival in pancreatic cancer and in non-small-cell lung cancer. They found that the NIM index, reflecting vagal modulation of inflammation, may be a new independent prognostic biomarker in fatal cancers.

An interesting study on ventilator-induced lung injury (VILI) in preterm newborns has been also published in this issue. Here, the authors, C. Gutiérrez Carvalho et al., analyzed any association between the oxygen levels at blood sampling and plasma levels of the interleukins IL-6, IL-1β, IL-10, and IL-8 and TNF-α in preterm newborns under mechanical ventilation (MV) in their first two days. The study was conducted on 20 neonates (gestational age 32.2 $\pm$ 3 weeks) with severe respiratory distress. Blood samples were collected right before and 2 hours after invasive MV. The newborns were separated according to oxygen requirement: low-oxygen ($\leq$30%) and high-oxygen (>30%) groups. In the high-oxygen group, IL-6, IL-8, and TNF-α plasma levels increased significantly after two hours under MV. Despite the small sample studied, data showed that there is some relationship between VILI, proinflammatory cytokines, and oxygen-induced lung injury, but a study considering oxidative marker measurements is needed. It seems that less oxygen may keep safer saturation targets playing a less harmful role.

Finally, a large amount of evidence has demonstrated that neuroinflammation plays a significant role in both acute and chronic neurodegenerative disorders including Parkinson's disease, Alzheimer's disease, multiple sclerosis, stroke, and traumatic brain injury. Y. Fu et al. reviewed the role of excessive microglial activation inducing inflammation-mediated neuronal damage and degeneration. They explored new herbal compounds that are able to suppress neurotoxicity via inhibiting microglial activation. The therapeutic targets and pharmacological mechanisms of these compounds have also been discussed in the review.

This special issue is a collection of original or review articles submitted by investigators representing eight countries across Europe, Asia, Africa, and South America, to highlight some of the objectives achieved in basic, translational, and clinical immunology. It provides a glimpse on some selected immune-mediated disorders highlighting the cell types and molecular mechanisms involved in the damage triggered by host immune responses either directly or following virus infections or changes in commensal flora composition.

Authors' Contributions

Marcella Reale and Lucia Conti contributed equally to this work.

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Review Article

The Immunologic Role of Gut Microbiota in Patients with Chronic HBV Infection

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Hepatitis B can cause acute or chronic liver damage due to hepatitis B virus (HBV) infection. Cirrhosis or hepatocellular carcinoma (HCC) caused by chronic HBV infection often leads to increased mortality. However, the gut and liver have the same embryonic origin; therefore, a close relationship must exist in terms of anatomy and function, and the gut microbiota plays an important role in host metabolic and immune modulation. It is believed that structural changes in the gut microbiota, bacterial translocation, and the resulting immune injury may affect the occurrence and development of liver inflammation caused by chronic HBV infection based on the in-depth cognition of the concept of the “gut-liver axis” and the progress in intestinal microecology. This review aims to summarize and discuss the immunologic role of the gut microbiota in chronic HBV infection.

1. Introduction

Chronic hepatitis B virus (HBV) infection is a global problem that threatens human health. Chronic HBV infection can easily lead to some severe complications, such as liver failure, cirrhosis, and even hepatocellular carcinoma (HCC), which can be life-threatening if not treated in a timely and normative manner. According to the latest report from the World Health Organization (WHO), there are approximately 257 million people with chronic HBV infection worldwide, and of these cases, 887,000 people die due to complications caused by chronic HBV infection [1]. Although the application of the hepatitis B vaccine and antiviral drugs has caused the rate of HBV infection to decrease yearly, chronic HBV infection still remains a heavy economic burden and health threat for many families [2, 3].

Researchers have persisted in finding better ways to control this disease. In recent years, studies on intestinal flora have provided new targets for the prevention and treatment of chronic HBV infection. Studies have demonstrated that changes in the gut microbiota play important roles in inducing and promoting the development of liver diseases, and the diversity of the gut microbiota has been associated with alcoholic liver disease, autoimmune liver disease, chronic hepatitis B (CHB), cirrhosis and HCC; moreover, changes in the intestinal flora are dependent on the various pathogeneses that are present [4–8]. Furthermore, scholars from Taiwan have indicated that the gut microbiota may determine acute hepatitis B and CHB attacks. Specifically, the composition of the gut microbiota affects the host immune response to HBV, and the infection can easily transform into a chronic infection when the intestinal flora is in an abnormal state [9]. Accordingly, the fact that immune injuries caused by structural changes in the gut microbiota have a significant influence on the development of chronic HBV infection cannot be ignored.

2. Chronic HBV Infection and Innate Immunity

Adults generally experience acute onset after infection with HBV. However, most people are able to clear the virus via a healthy immune system, and only a few individuals with impaired immunity or other liver diseases progress to chronic HBV infection. Most chronic HBV infections occur in infants and young children due to their immature immune systems and unstable intestinal flora [10, 11]. Therefore, the

occurrence and development of chronic HBV infection not only depend on the viral load, virulence, and invasion path of the HBV but also are associated with the immune function and intestinal flora of the host. It is currently believed that the cause of liver injury is not HBV replication in liver cells but rather the immune response caused by the HBV. The immune response is considered a key factor in the development of CHB. Various immune cells and cytokines not only are involved in the initiation and regulation of the immune response but also can activate downstream signaling pathways that directly or indirectly inhibit HBV replication. It can be concluded that the host immune response caused by HBV infection exerts a significant influence on the prognosis of hepatitis B and the treatment effect of antiviral drugs [12]. The immune response against HBV mainly includes innate immunity and adaptive immunity; however, the effective innate immune response not only can eliminate the virus directly but also can exert a significant influence on HBV-specific immunity [13].

Toll-like receptors (TLRs) are important protein molecules involved in innate immunity. Acting as a bridge connecting nonspecific immunity and specific immunity, they recognize molecules with conserved structures derived from microorganisms. After the breach of the body's physical barriers, such as the skin or mucous membranes by microorganisms, TLRs identify molecules and become activated, leading to immune responses [14]. TLRs are expressed in multiple liver immune cells, including plasmacytoid dendritic cells (pDCs), monocytes, macrophages, Kupffer cells, hepatic stellate cells, and liver cells [12]. Experimental studies have indicated that the activation of cell signaling pathways by TLRs and the release of antiviral cytokines are the main mechanisms by which HBV replication in liver cells is suppressed. When the host is infected by HBV, the virus is identified by the relevant TLRs, and the mechanisms of antiviral regulation are activated. These mechanisms include TLR recognition, the release of interferon (IFN), activation of NK and NKT cells, and the production of proinflammatory cytokines [14]. For example, the activation of the HBV-TLR3 signaling pathway causes the production of IFN-α/β, and TLR2/TLR4 can initiate the MAPK and PI3K/Akt signaling pathways [15]. However, another research has demonstrated that HBV can reduce the expression of TLR2 in liver Kupffer cells and mononuclear cells in patients with chronic HBV infection and can also attenuate intracellular signaling pathways to enable the evasion of the immune response [16]. A recent study also found that the HBV DNA viral load is negatively correlated with TLR7 expression in biopsy samples, which indicates that expression of TLR7 on liver cells can be inhibited by HBV [17]. Obviously, the TLR-mediated signaling pathways induce immune responses to HBV infection, but the HBV virus itself downregulates the expression of TLRs on the immune cells. The latter mechanism may be one of the main causes of progression to chronic HBV infection in patients. Studies on the gut-liver axis have revealed that the intestine and liver have the same embryonic origin and are linked by the portal venous system. The intestinal tract has been proposed to exert a regulatory effect on the development of chronic HBV infection. The intestinal flora is an important mediator of the interaction between the intestine and the liver and the portal vein functions as a bridge connecting these structures [8].

3. Gut Microbiota

As the name suggests, the gut microbiota includes microorganisms that have colonized and coexist in the human intestinal tract. Due to host genetic, diet, and environmental factors and use of antibiotics, certain differences exist in the components of the human intestine among individuals, but the human body will gradually establish a stable intestinal flora structure and regulate and maintain the body's health under the influence of these factors [18–20]. Tens of thousands of microbes live in the intestinal tracts of normal adults, and most of these microbes are located in the colon. The number of bacteria located in the colon of the human body is estimated to be approximately 3.8×10^{13} according to the identified bacterial content and colon volume. There are approximately 40,000 types of microbes in the gut and can be divided into two main categories, that is, *Bacteroides* and the thick-walled bacteria. The most common bacteria are *Bifidobacteria*, *Lactobacillus*, *Clostridium*, and *Streptococcus* [21]. These intestinal bacteria are considered beneficial, and their roles in metabolism, immunity, and nutrition can prevent invasion of the host by disease-causing pathogens [22]. Thus, the intestinal immune function cannot be neglected. Data of experiments showed that imbalances of the intestinal flora easily lead to disorders of the intestinal immune system and cause a variety of diseases such as irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), autoimmune liver disease, and CHB [23–26]. Moreover, the gut microbiota is involved in nonalcoholic fatty liver disease (NAFLD) and associated with the progression of NAFLD to nonalcoholic steatohepatitis, cirrhosis, or HCC [19, 27].

4. Immune Function of Gut Microbiota in the Development of Chronic HBV Infection

In recent years, many scholars, especially domestic scholars, have studied the relationship between gut microbiota and chronic HBV infection because of the high proportion of HBV-infected people worldwide. In 2006, Xing et al. found that liver ischemia and reperfusion can reduce the numbers of intestinal *Bifidobacteria* and *Lactobacillus* and increase the numbers of Enterobacteriaceae and *Enterococcus*; these changes are associated with the loss of intestinal microvilli, the widening of the intestinal mucosal space and intestinal bacterial translocation [28]. Subsequently, the same changes were found in the intestinal flora of chronic HBV carriers, CHB patients, and hepatitis B-induced cirrhosis patients; that is, the structures and abundances of the bacterial groups were obviously different. Specifically, patients with CHB and cirrhosis exhibit dramatically decreased *Bifidobacteria* and *Lactobacillus* levels, while *Enterococcus* and Enterobacteriaceae levels are significantly increased compared to healthy people. The progression of liver diseases, particularly liver cirrhosis, is caused by bacterial products from the intestine [29, 30]. Chou et al. found that HBV could not be detected

after six weeks of HBV infection in adult mice without intestinal flora (treated by antibiotics), and 60% of adult mice with intestinal flora (no antibiotics) still exhibited HBV. These data imply that gut microbes play a critical role in immunity against HBV [9]. Wang et al. studied the intestinal flora of CHB patients and healthy people, and the data revealed that the *Bacteroides* level was decreased in CHB patients compared to healthy people based on sequencing the V3-V4 region of the 16S rRNA gene of the intestinal flora. Additionally, these authors also discovered that the intestinal microflora structure of the CHB patients had changed compared to that before severe liver injury, which indicated that the structural changes in the intestinal flora played a potential pathogenic role in patients with chronic HBV infection [31]. In summary, an imbalance exists in the intestinal microbiota of patients with CHB and cirrhosis. Similarly, overgrowth of harmful bacteria in the intestinal tract leads to increased mucosal permeability, which causes harmful bacteria to travel through the portal vein into the liver and thus activate the liver's innate immune system. The structural changes in the intestinal microflora and the severity of liver disease are mutually causal, and to a certain extent, they affect the transformation process of CHB to liver fibrosis or liver failure [12]. Therefore, we suggest that, in chronic HBV infection, the injury to hepatocytes not only originates from the cellular immune response caused by HBV invasion but also is caused by the natural immune response elicited by pathogen-associated molecular patterns (PAMPs) produced by the intestinal microbes with structural disorders. TLRs are the main pattern recognition receptors in the natural immune system and play a crucial role in the immune response [12]. It is now known that the intestinal PAMPs associated with chronic HBV infection are mainly composed of lipopolysaccharide (LPS), unmethylated CpG DNA, bacterial cell wall components, and bacterial DNA/RNA. The related immune mechanisms are described below.

4.1. The LPS-TLR4 Pathway. LPS is the main component of the outer membrane of Gram-negative bacteria and is an endotoxin mainly released by Enterobacteriaceae. Research has demonstrated a high level of LPS in the blood of patients with chronic HBV liver failure, which indicates that LPS may be related to the severity of the disease [32]. Another study found that LPS in the intestinal tract can downregulate the expression of tight junction proteins (ZO-1 and closed protein), increase the permeability of the intestinal mucosa, and enter the blood flow through the portal venous system [33, 34]. LPS can be recognized by TLR4, and TLR4 is mainly expressed in mononuclear macrophages [32]. Chou et al. [9] found that mice, regardless of age, subjected to TLR4 silencing are able to clear HBV and obviously produce antibodies in 8 weeks. In other words, TLR4-silenced mice are not immune to LPS but can clear HBV. Conversely, LPS-induced immune responses may contribute to the progression of chronic HBV infection. Researches showed that LPS binds to LPS-binding protein, and this combination can be identified by TLR4 on the surface of mononuclear macrophages. This recognition then stimulates $CD14^+$ Kupffer cells, triggers the inflammatory cascade effect, activates the NF-κB-related pathway, and produces inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), IL-1, and IL-6, and thus causes acute liver injury [35, 36]. Simultaneously, this pathway also induces Kupffer cells to release immunosuppressive mediators, such as IL-10, which can inhibit the release of inflammatory mediators of mononuclear macrophages and HBV-specific immune responses, in turn inhibiting the efficient clearing of bacteria and HBV [37]. Additionally, hepatic stellate cells also express TLR4 and can release a large number of extracellular matrix proteins in a LPS-TLR4 pathway-dependent manner. These proteins are involved in the fibrotic process and may also be among the factors that cause chronic HBV infection to develop into liver fibrosis [38].

4.2. The Unmethylated CpG DNA-TLR9 Pathway. Unmethylated CpG DNA is an important immune adjuvant that can activate TLR9. TLR9 is mainly expressed on mononuclear cells, B cells, $CD4^+$ T cells, pDCs, and Treg cells [39]. The CpG-TLR9 signaling pathway not only activates the innate immune response but also adjusts the adaptive immune response. The CpG-TLR9 signaling pathway plays an important role in the prevention and treatment of infectious diseases [40]. Researches have highlighted that CpG-TLR9 and MyD88 form compounds (MyD88, IRAK4, and TRAF6), and TRAF6 phosphorylates IRAK1 to trigger the NF-κB and MAPK signaling pathways, which may activate DCs to express and secrete cytokines and chemokines to further promote the proliferation and differentiation of B and T cells, resulting in the secretion of proinflammatory cytokines and IFN [40, 41]. However, different bacteria have different CpG DNA levels. In the intestinal flora of animals, unmethylated CpG DNAs are abundant in *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Bifidobacteria*, Proteobacteria, and Bacteroidetes [40]. As mentioned above, the intestinal floras of chronic HBV-infected patients with aggravated conditions are highly maladjusted, and *Lactobacillus* and *Bifidobacteria* with richer unmethylated CpG DNA levels are greatly reduced, causing the weakening of the CpG DNA-TLR9 pathway, decreasing of the production of protective cytokines, especially IFN, and diminishing of the immune effect on HBV.

4.3. Other Pathways. Other components of intestinal bacteria can also be identified by liver immune cells, such as the following cell wall components: teichoic acid (TA), peptidoglycan (PGN), and specialized proteins (flagellin). TA and PGN are recognized by TLR2, and flagellin mainly activates TLR5 [42, 43]. TLR3 can combine with dsRNA in bacteria, and ssRNA can activate TLR7 and TLR8 receptors [44]. A series of protective immune responses depend on the MyD88-TRIF pathway, which triggers the downstream signal and then initiates the activation of related immune cells to release proinflammatory cytokines [45]. Nevertheless, this process will also aggravate liver injury if the immune response persists for a long time or reaches an excessive intensity, which then results in the production of a large number of cytokines in the body [32].

5. Summary and Prospects

In conclusion, studies have confirmed that imbalances of the intestinal flora play an important role in promoting the development of chronic HBV infection. The PAMPs of intestinal bacteria are transferred into the liver through the portal vein and identified by TLRs in the immune cells, which causes a series of immune responses, the release of various cytokines (IL, TNF, and IFN), and further liver cell damage. Among these processes, TLRs play an essential role as an important link between the intestinal flora and the liver immune reaction.

Although antiviral drugs and IFN have significant efficacy in the treatment of chronic HBV infection, for various reasons, it seems inevitable that chronic HBV infection develops into liver cirrhosis, liver failure, or liver cancer. Currently, studies on the gut microbiota and its products have provided a new therapeutic target. An article proposed that the application of synthetic unmethylated CpG DNA is a promising measure for the treatment of infectious diseases [41]. Additionally, the physiological indexes (BMI) and serum metabolites of the patients with chronic HBV infection also correlated with the structure of the intestinal flora and liver fibrosis [31]. Certain physical exercises may actively regulate the intestinal floras of patients with chronic diseases [46].

In recent years, probiotics have been widely used in the treatment of intestinal diseases. Many studies have demonstrated that compound probiotics can improve the abnormal state of the gut microbiota, as well as chronic inflammation in chronic liver diseases, but short-term probiotic treatment still has no effect on adjusting intestinal permeability or liver function [47, 48]. With the in-depth study of gut microbiota, someone suggested that fecal microbiota transplantation (FMT) will be a promising treatment for chronic HBV infection. FMT refers to the infusion of a faecal suspension from healthy people into the intestinal tract of patients to cure a specific disease [32]. More and more researches showed that FMT has been used to treat *Clostridium difficile* infection, IBD, IBS, and various liver diseases with some effects [49]. A 2015 case report described that FMT can reduce blood ammonia, increase cognitive abilities, and improve hepatic encephalopathy for a 57-year-old male with cirrhosis secondary to both alcohol and the hepatitis C virus [50]. A report on HBeAg-positive CHB therapy in patients with ongoing ETV/TDF therapy showed that FMT can induce HBeAg clearance in a significant proportion of cases who have persistent positive HBeAg even after long-term antiviral treatment. Although only 5 patients participated in the FMT group, the statistics varied significantly ($P = 0.0001$) and these dramatic results were particularly encouraging for patients with HBeAg-positive CHB who could not cease oral antiviral therapy [51]. Therefore, FMT may be as a potential immunomodulator in improving the intestinal microenvironment and alleviating the damage to the liver caused by harmful intestinal bacteria [32]. However, there are also associated ethical, legal, and social problems; we should establish a balance of scientific research, health, and marketing regarding the study of the intestinal flora [52]. And the data on the field of FMT treatment related to HBV-related diseases is still limited. So there will be a lot of work to do on FMT and chronic HBV infection for us in the future [32].

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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References

[1] World Health Organization, *Global Hepatitis Report 2017*, WHO, 2017, http://www.who.int/hepatitis.

[2] S. Beckebaum, M. Malagó, O. Dirsch et al., “Efficacy of combined lamivudine and adefovir dipivoxil treatment for severe HBV graft reinfection after living donor liver transplantation,” *Clinical Transplantation*, vol. 17, no. 6, pp. 554–559, 2003.

[3] C. Castelnau, F. le Gal, M. P. Ripault et al., “Efficacy of peginterferon alpha-2b in chronic hepatitis delta: relevance of quantitative RT-PCR for follow-up,” *Hepatology*, vol. 44, no. 3, pp. 728–735, 2006.

[4] P. Hartmann, W. C. Chen, and B. Schnabl, “The intestinal microbiome and the leaky gut as therapeutic targets in alcoholic liver disease,” *Frontiers in Physiology*, vol. 3, p. 402, 2012.

[5] R. Lin, L. Zhou, J. Zhang, and B. Wang, “Abnormal intestinal permeability and microbiota in patients with autoimmune hepatitis,” *International Journal of Clinical and Experimental Pathology*, vol. 8, no. 5, pp. 5153–5160, 2015.

[6] M. Bhat, B. M. Arendt, V. Bhat, E. L. Renner, A. Humar, and J. P. Allard, “Implication of the intestinal microbiome in complications of cirrhosis,” *World Journal of Hepatology*, vol. 8, no. 27, pp. 1128–1136, 2016.

[7] M. Grąt, K. M. Wronka, M. Krasnodębski et al., “Profile of gut microbiota associated with the presence of hepatocellular cancer in patients with liver cirrhosis,” *Transplantation Proceedings*, vol. 48, no. 5, pp. 1687–1691, 2016.

[8] D. E. Fouts, M. Torralba, K. E. Nelson, D. A. Brenner, and B. Schnabl, “Bacterial translocation and changes in the intestinal microbiome in mouse models of liver disease,” *Journal of Hepatology*, vol. 56, no. 6, pp. 1283–1292, 2012.

[9] H. H. Chou, W. H. Chien, L. L. Wu et al., “Age-related immune clearance of hepatitis B virus infection requires the establishment of gut microbiota,” *Proceedings of the National Academy of Sciences*, vol. 112, no. 7, pp. 2175–2180, 2015.

[10] A. J. Prendergast, P. Klenerman, and P. J. R. Goulder, “The impact of differential antiviral immunity in children and adults,” *Nature Reviews Immunology*, vol. 12, no. 9, pp. 636–648, 2012.

[11] K. N. Tsai, C. F. Kuo, and J. H. J. Ou, “Mechanisms of hepatitis B virus persistence,” *Trends in Microbiology*, vol. 26, no. 1, pp. 33–42, 2018.

[12] D. Xu, Y. Huang, and J. Wang, "Gut microbiota modulate the immune effect against hepatitis B virus infection," *European Journal of Clinical Microbiology & Infectious Diseases*, vol. 34, no. 11, pp. 2139–2147, 2015.

[13] A. Busca and A. Kumar, "Innate immune responses in hepatitis B virus (HBV) infection," *Virology Journal*, vol. 11, no. 1, p. 22, 2014.

[14] E. Zhang and M. Lu, "Toll-like receptor (TLR)-mediated innate immune responses in the control of hepatitis B virus (HBV) infection," *Medical Microbiology and Immunology*, vol. 204, no. 1, pp. 11–20, 2015.

[15] Z. Ma, E. Zhang, D. Yang, and M. Lu, "Contribution of Toll-like receptors to the control of hepatitis B virus infection by initiating antiviral innate responses and promoting specific adaptive immune responses," *Cellular & Molecular Immunology*, vol. 12, no. 3, pp. 273–282, 2015.

[16] K. Visvanathan, N. A. Skinner, A. J. V. Thompson et al., "Regulation of Toll-like receptor-2 expression in chronic hepatitis B by the precore protein," *Hepatology*, vol. 45, no. 1, pp. 102–110, 2007.

[17] D. Das, I. Sengupta, N. Sarkar et al., "Anti-hepatitis B virus (HBV) response of imiquimod based toll like receptor 7 ligand in hbv-positive human hepatocelluar carcinoma cell line," *BMC Infectious Diseases*, vol. 17, no. 1, p. 76, 2017.

[18] I. Sekirov, S. L. Russell, L. C. M. Antunes, and B. B. Finlay, "Gut microbiota in health and disease," *Physiological Reviews*, vol. 90, no. 3, pp. 859–904, 2010.

[19] H. Fukui, "Gut microbiota and host reaction in liver diseases," *Microorganisms*, vol. 3, no. 4, pp. 759–791, 2015.

[20] K. Korpela, A. Salonen, L. J. Virta et al., "Intestinal microbiome is related to lifetime antibiotic use in Finnish pre-school children," *Nature Communications*, vol. 7, article 10410, 2016.

[21] R. Sender, S. Fuchs, and R. Milo, "Revised estimates for the number of human and bacteria cells in the body," *PLoS Biology*, vol. 14, no. 8, article e1002533, 2016.

[22] K. Atarashi and K. Honda, "Microbiota in autoimmunity and tolerance," *Current Opinion in Immunology*, vol. 23, no. 6, pp. 761–768, 2011.

[23] M. Rajilić-Stojanović, D. M. Jonkers, A. Salonen et al., "Intestinal microbiota and diet in IBS: causes, consequences, or epiphenomena?," *The American Journal of Gastroenterology*, vol. 110, no. 2, pp. 278–287, 2015.

[24] S. Michail, M. Durbin, D. Turner et al., "Alterations in the gut microbiome of children with severe ulcerative colitis," *Inflammatory Bowel Diseases*, vol. 18, no. 10, pp. 1799–1808, 2012.

[25] L.-M. Haag and B. Siegmund, "Intestinal microbiota and the innate immune system – a crosstalk in Crohn's disease pathogenesis," *Frontiers in Immunology*, vol. 6, p. 489, 2015.

[26] J. D. Forbes, G. van Domselaar, and C. N. Bernstein, "The gut microbiota in immune-mediated inflammatory diseases," *Frontiers in Microbiology*, vol. 7, article 1081, 2016.

[27] F. R. Ponziani, S. Bhoori, C. Castelli et al., "Hepatocellular carcinoma is associated with gut microbiota profile and inflammation in non-alcoholic fatty liver disease," *Hepatology*, 2018.

[28] H. C. Xing, L. J. Li, K. J. Xu et al., "Protective role of supplement with foreign Bifidobacterium and Lactobacillus in experimental hepatic ischemia-reperfusion injury," *Journal of Gastroenterology and Hepatology*, vol. 21, no. 4, pp. 647–656, 2006.

[29] H. Lu, Z. Wu, W. Xu, J. Yang, Y. Chen, and L. Li, "Intestinal microbiota was assessed in cirrhotic patients with hepatitis B virus infection. Intestinal microbiota of HBV cirrhotic patients," *Microbial Ecology*, vol. 61, no. 3, pp. 693–703, 2011.

[30] D. W. Jun, K. T. Kim, O. Y. Lee et al., "Association between small intestinal bacterial overgrowth and peripheral bacterial DNA in cirrhotic patients," *Digestive Diseases and Sciences*, vol. 55, no. 5, pp. 1465–1471, 2010.

[31] J. Wang, Y. Wang, X. Zhang et al., "Gut microbial dysbiosis is associated with altered hepatic functions and serum metabolites in chronic hepatitis B patients," *Frontiers in Microbiology*, vol. 8, p. 2222, 2017.

[32] Y. Kang and Y. Cai, "Gut microbiota and hepatitis-B-virus-induced chronic liver disease: implications for faecal microbiota transplantation therapy," *Journal of Hospital Infection*, vol. 96, no. 4, pp. 342–348, 2017.

[33] P. Brun, I. Castagliuolo, V. D. Leo et al., "Increased intestinal permeability in obese mice: new evidence in the pathogenesis of nonalcoholic steatohepatitis," *American Journal of Physiology-Gastrointestinal and Liver Physiology*, vol. 292, no. 2, pp. G518–G525, 2007.

[34] T. H. Frazier, J. K. DiBaise, and C. J. McClain, "Gut microbiota, intestinal permeability, obesity-induced inflammation, and liver injury," *Journal of Parenteral and Enteral Nutrition*, vol. 35, 5_Supplement, pp. 14s–20s, 2011.

[35] E. Seki and B. Schnabl, "Role of innate immunity and the microbiota in liver fibrosis: crosstalk between the liver and gut," *The Journal of Physiology*, vol. 590, no. 3, pp. 447–458, 2012.

[36] M. Bermudez-Brito, S. Muñoz-Quezada, C. Gomez-Llorente et al., "Cell-free culture supernatant of *Bifidobacterium breve* CNCM I-4035 decreases pro-inflammatory cytokines in human dendritic cells challenged with *Salmonella typhi* through TLR activation," *PLoS One*, vol. 8, no. 3, article e59370, 2013.

[37] A. Borrelli, P. Bonelli, F. M. Tuccillo et al., "Role of gut microbiota and oxidative stress in the progression of non-alcoholic fatty liver disease to hepatocarcinoma: current and innovative therapeutic approaches," *Redox Biology*, vol. 15, pp. 467–479, 2018.

[38] Y. H. Paik, R. F. Schwabe, R. Bataller, M. P. Russo, C. Jobin, and D. A. Brenner, "Toll-like. Receptor 4 mediates inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells," *Hepatology*, vol. 37, no. 5, pp. 1043–1055, 2003.

[39] L. Lin and J. Zhang, "Role of intestinal microbiota and metabolites on gut homeostasis and human diseases," *BMC Immunology*, vol. 18, no. 1, p. 2, 2017.

[40] K. Gao, L. Liu, and H. Wang, "Advances in immunomodulation of microbial unmethylated CpG DNA on animal intestinal tract–a review," *Acta Microbiologica Sinica*, vol. 55, no. 5, pp. 543–550, 2015.

[41] N. Cools, A. Petrizzo, E. Smits et al., "Dendritic cells in the pathogenesis and treatment of human diseases: a Janus Bifrons?," *Immunotherapy*, vol. 3, no. 10, pp. 1203–1222, 2011.

[42] M. U. Cinar, M. A. Islam, M. Pröll et al., "Evaluation of suitable reference genes for gene expression studies in porcine PBMCs in response to LPS and LTA," *BMC Research Notes*, vol. 6, no. 1, p. 56, 2013.

[43] B. Lemaitre and S. E. Girardin, "Translation inhibition and metabolic stress pathways in the host response to bacterial

pathogens," *Nature Reviews Microbiology*, vol. 11, no. 6, pp. 365–369, 2013.

[44] T. Kawasaki, T. Kawai, and S. Akira, "Recognition of nucleic acids by pattern-recognition receptors and its relevance in autoimmunity," *Immunological Reviews*, vol. 243, no. 1, pp. 61–73, 2011.

[45] A. Ignacio, C. I. Morales, N. O. S. Câmara, and R. R. Almeida, "Innate sensing of the gut microbiota: modulation of inflammatory and autoimmune diseases," *Frontiers in Immunology*, vol. 7, p. 54, 2016.

[46] R. Codella, L. Luzi, and I. Terruzzi, "Exercise has the guts: how physical activity may positively modulate gut microbiota in chronic and immune-based diseases," *Digestive and Liver Disease*, vol. 50, no. 4, pp. 331–341, 2018.

[47] Y. Liang, S. Liang, Y. Zhang et al., "Oral administration of compound probiotics ameliorates HFD-Induced gut microbe dysbiosis and chronic metabolic inflammation via the G protein-coupled receptor 43 in non-alcoholic fatty liver disease rats," *Probiotics and Antimicrobial Proteins*, pp. 1–11, 2018.

[48] D. S. Kwak, D. W. Jun, J. G. Seo et al., "Short-term probiotic therapy alleviates small intestinal bacterial overgrowth, but does not improve intestinal permeability in chronic liver disease," *European Journal of Gastroenterology & Hepatology*, vol. 26, no. 12, pp. 1353–1359, 2014.

[49] R. D. Heath, C. Cockerell, R. Mankoo, J. A. Ibdah, and V. Tahan, "Fecal microbiota transplantation and its potential therapeutic uses in gastrointestinal disorders," *Northern Clinics of Istanbul*, vol. 5, no. 1, pp. 79–88, 2018.

[50] T.-C. D. Shen, L. Albenberg, K. Bittinger et al., "Engineering the gut microbiota to treat hyperammonemia," *The Journal of Clinical Investigation*, vol. 125, no. 7, pp. 2841–2850, 2015.

[51] Y. D. Ren, Z. S. Ye, L. Z. Yang et al., "Fecal microbiota transplantation induces hepatitis B virus e-antigen (HBeAg) clearance in patients with positive HBeAg after long-term antiviral therapy," *Hepatology*, vol. 65, no. 5, pp. 1765–1768, 2017.

[52] Y. Ma, H. Chen, C. Lan, and J. Ren, "Help, hope and hype: ethical considerations of human microbiome research and applications," *Protein & Cell*, vol. 9, no. 5, pp. 404–415, 2018.

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Research Article

TRIF Regulates BIC/miR-155 via the ERK Signaling Pathway to Control the ox-LDL-Induced Macrophage Inflammatory Response

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Toll/IL-1R-domain-containing adaptor-inducing IFN-β (TRIF) is an important adaptor for TLR3- and TLR4-mediated inflammatory signaling pathways. Recent studies have shown that TRIF plays a key role in vessel inflammation and atherosclerosis; however, the precise mechanisms are unclear. We investigated the mechanisms of the TRIF-regulated inflammatory response in RAW264.7 macrophages under oxidized low-density lipoprotein (ox-LDL) stimulation. Our data show that ox-LDL induces TRIF, miR-155, and BIC expression, activates the $ERK_{1/2}$ and SOCS1-STAT3-NF-κB signaling pathways, and elevates the levels of IL-6 and TNF-α in RAW264.7 cells. Knockdown of TRIF using TRIF siRNA suppressed BIC, miR-155, IL-6, and TNF-α expression and inhibited the $ERK_{1/2}$ and SOCS1-STAT3-NF-κB signaling pathways. Inhibition of $ERK_{1/2}$ signaling also suppressed BIC and miR-155 expression. These findings suggest that TRIF plays an important role in regulating the ox-LDL-induced macrophage inflammatory response and that TRIF modulates the expression of BIC/miR-155 and the downstream SOCS1-STAT3-NF-κB signaling pathway via $ERK_{1/2}$. Therefore, TRIF might be a novel therapeutic target for atherosclerosis.

1. Introduction

Atherosclerosis (AS) is a chronic arterial disease and a major threat to public health worldwide, as it is a main cause of cardiovascular disease (CVD), ischemic stroke, and local thrombosis [1]. AS is now recognized as a chronic inflammatory disorder that is induced by oxidized low-density lipoprotein (ox-LDL) accumulation and inflammation in the arterial intima under hypercholesterolemic conditions [2]. Multiple cells, such as macrophages, lymphocytes, endothelial cells, and smooth muscle cells, contribute to the occurrence and development of AS [3]. Macrophages play especially important roles in this pathophysiological process, as they are the major effector cells that stimulate the vascular inflammatory response through various inflammatory mediators and form foam cells in atherosclerotic lesions, thereby promoting plaque formation and impacting plaque stability [4, 5]. Therefore, it is important to explore novel mechanisms underlying the ox-LDL-induced macrophage inflammatory response.

Toll/IL-1R-domain-containing adaptor-inducing IFN-β (TRIF) is a Toll/IL-1R- (TIR-) domain-containing adaptor [6, 7]. TRIF plays a pivotal role following the activation of Toll-like receptor (TLR) 3 and 4 signaling, leading to the production of inflammatory mediators through the activation of several transcription factors, including NF-κB, IRF3, and AP-1 [8–11]. Vorkapic E et al. showed that knockout of TRIF suppressed angiotensin (Ang) II-induced aneurysm formation and vascular inflammation *in vivo* [12]. Lundberg and colleagues reported that TRIF deficiency in hematopoietic cells reduced atherogenic diet-induced vascular inflammation and protected against atherosclerosis, as shown in $Ldlr^{-/-}$ mice after receiving a bone marrow transfer from TRIF-deficient mice [13]. Another study revealed that $Ldlr^{-/-}$ mice with a loss-of-function mutation in TRIF (Lps2) were significantly protected from atherosclerosis and exhibited reduced cytokine secretion from peritoneal macrophages under hyperlipidemic conditions [14]. Overall, the above studies indicate that TRIF plays a key role in vessel

inflammation and atherosclerosis; however, its precise mechanism is unclear.

MicroRNA (miRNA) is an endogenous, short length (~22 nucleotides) noncoding RNA. Recently, several reports have shown that miRNAs, especially miR-155, play a pivotal role in the regulation of inflammatory responses in AS by binding to the 3′-untranslated region (UTR) of target mRNAs [15]. There was a previous report by Tian et al. that miR-155 was upregulated in macrophages by ox-LDL stimulation. They also reported that miR-155 is involved in ox-LDL-induced macrophage inflammatory response, including expression of inflammatory factors IL-6 and TNF-α. Other studies have also demonstrated that elevated miR-155 promotes foam cell formation and atherosclerosis by repressing its downstream target genes, including Bcl-6, SOCS1, HMG box-transcription protein 1 (HBP1), and mitogen-activated protein kinase 10 (MAP3K10) [16–19]. Although ox-LDL-induced miR-155 play a key role for AS occurrence and progress, the potential mechanism is still unknown.

In the present study, we investigated the potential mechanism of ox-LDL-induced miR-155 and inflammation response in macrophages and found that ox-LDL induced TRIF expression and activated $ERK_{1/2}$ signal, then enhanced the expression of B-cell integration cluster (BIC, miR-155 host gene)/miR-155, thus promoting inflammation mediator production.

2. Materials and Methods

2.1. Materials. Raw264.7 macrophages were purchased from CellBio (Shanghai, China). Oxidized low-density lipoprotein (ox-LDL) was purchased from Peking Union-Biology Co. Ltd. (Beijing, China). TriPure Isolation Reagent, X-tremeGENE siRNA Transfection Reagent, X-tremeGENE HP DNA Transfection Reagent, the Transcriptor First Strand cDNA Synthesis Kit, and FastStart Universal SYBR Green Master Mix were purchased from Roche (Switzerland). The following primary antibodies were used in this study: rabbit antisuppressor of cytokine signaling 1 (SOCS1) (Abcam, UK), rabbit antiphosphorylation-signal transducer and activator of transcription 3 (p-STAT3) (Cell Signaling Technology, USA), rabbit antiphosphorylated-protein kinase (MAPK)/extracellular signal-regulated $kinase_{1/2}$ (p-$ERK_{1/2}$) and $ERK_{1/2}$ (Cell Signaling Technology, USA), NFκB p65 (Cell Signaling Technology, USA), and rabbit anti-β-actin (Cell Signaling Technology, USA). Horseradish peroxidase-(HRP-) conjugated AffiniPure goat anti-rabbit IgG was purchased from Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd. (Beijing, China). NF-κB luciferase reporter plasmid (pNFκB-luc) was purchased from Beyotime Inc. (Jiangsu, China). High-glucose Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (USA). ERK inhibitor, SCH772984, was purchased from MedChem Express (USA). TRIF siRNA sequences have been designed and synthesized by GenePharma Co. Ltd. (Shanghai, China). All other reagents were commercially available and used as received.

2.2. Cell Culture and Treatment. RAW264.7 cells were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin. These cells were treated with 20 μg/mL ox-LDL for 0, 6, 12, and 24 h and then underwent further study.

2.3. Transfection with TRIF siRNA. TRIF siRNA and negative control (NC) had been transfected into RAW264.7 cells as previously described [20]. The sequences of TRIF siRNA are the following: (1) 5′-GCU AUG UAA CAC ACC GCU GTT-3′; (2) 5′-GGA CAU ACG UUA CAC UCC ACC AACA GTT-3′; (3) 5′-GGU CAA ACG UGA CAC UCa ACC UGC GTT-3′; and NC sequence: 5′-ACG UGA CAC GUU CGG AGA ATT-3′.

2.4. RT-qPCR Analysis of miR-155 and B-Cell Integration Cluster (BIC), IL-6, and TNF-α mRNA Expression. Total RNA was isolated from treated RAW264.7 cells, and cDNA was synthesized using a Transcriptor First Strand cDNA Synthesis Kit according to the manufacturer's instructions. The following primers were used in the quantitative PCR (qPCR) assay: miR-155 forward primer, 5′-ACACTC CAGCTGGGTTAATGCTAATCGTG-3′, miR-155 reverse primer, 5′-CTCAACTGGTGTCGTGGAGT-3′; U6 forward primer, 5′-GTGCTCGCTTCGGCAGCA-3a′, U6 reverse primer, 5′-CAAAATATGGAACGCTTC-3′; BIC forward primer, 5′-CAAACCAGGAAGGGGAAGTGT-3′, BIC reverse primer, 5′-TAGGAGTCAGTCAGAGGCCAA-3′; TRIF forward primer, 5′-GCAGGCAGCACAAGTACAA C-3′, TRIF reverse primer, 5′-GTGCTCGGTTTCAGGC AATG-3′; TNF-α forward primer, 5′-GACCCTCACAC TCAG ATCATC-3′, TNF-α reverse primer: 5′-GAACCT GGGAGTAGATAAGG; IL-6 forward primer, 5′-GTAT GAACAACGATGATGCACTTG3′, IL-6 reverse primer, 5′-ATGGTACTCCAGAAGACCAGAGGA-3′; and β-actin forward primer, 5′-CACGGCATCGTCACCAACT-3′, β-actin reverse primer, 5′-GTCCTACGGAAAACGGCAGA-3′. PCR amplification was performed under the following conditions: 95°C for 10 min followed by 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s for 35 cycles. Data analysis was performed using the $2^{-\triangle\triangle CT}$ method to determine the relative level of target gene expression, which was normalized to U6 or β-actin expression.

2.5. Evaluation of TRIF, SOCS1, p-STAT3, NFκB p65, p-$ERK_{1/2}$, and $ERK_{1/2}$ Protein Levels by Western Blot. Western blot was performed as previously described [21]. Briefly, total protein was isolated from treated cells. Each protein sample was separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. PVDF membranes were then incubated with antibodies, including TRIF, SOCS1, p-STAT3, p-$ERK_{1/2}$, and $ERK_{1/2}$ antibodies. The protein bands were detected by an enhanced chemiluminescence detection system.

2.6. Analysis of NF-κB Promoter Activity by Dual-Luciferase Reporter Assay. RAW264.7 cells were seeded onto 24-well tissue culture plates and cultured overnight. The cells were

cotransfected with 100 ng of pNFκB-luc and 100 ng of pRL-TK as a control using X-tremeGENE HP DNA Transfection Reagent for 6 h. Then, TRIF siRNA or negative control (NC) was transfected into these cells. After 24 h, the cell culture media were replaced with DMEM containing 10% FBS, and the cells were treated with 20 μg/mL ox-LDL for 24 h. The treated cells were harvested in the lysis buffer, and luciferase activity was analyzed [22].

2.7. Statistical Analysis. Data are presented as the mean ± S.E. Statistical comparisons among groups were performed using one-way ANOVA. $p < 0.05$ was considered statistically significant.

3. Results

3.1. ox-LDL Induced TRIF Expression in a Time-Dependent Manner. To explore the effect of TRIF on the ox-LDL-induced inflammatory macrophage inflammatory response, we investigated the expression of TRIF protein after RAW264.7 cells were treated with 20 μg/mL ox-LDL for 24 h. As shown in Figures 1(a) and 1(b), TRIF protein expressions were significantly increased at 6, 12, and 24 h compared to those at 0 h ($p < 0.05$). Moreover, the level of TRIF mRNA was higher at 6, 12, and 24 h than at 0 h ($p < 0.05$). Therefore, ox-LDL increased TRIF levels in a time-dependent manner. These data showed that ox-LDL gradually increased the expression of TRIF protein and mRNA over time, suggesting that TRIF might be involved in the ox-LDL-induced inflammatory response.

3.2. ox-LDL Induced miR-155 Expression and the Macrophage Inflammatory Response. In this study, we analyzed the expression of miR-155 and BIC following the exposure of RAW264.7 cells to ox-LDL. These data demonstrated that the expression of BIC RNA was markedly increased at 6, 12, and 24 h compared to that at 0 h ($p < 0.05$) (Figure 2(a)). Similarly, the expression of miR-155 increased with prolonged ox-LDL treatment time compared to that at the 0 h time point ($p < 0.05$) (Figure 2(b)). We further studied the effect of ox-LDL on the miR-155-mediated inflammatory signaling pathway. These data showed that the level of SOCS1 protein, a target of miR-155, was lower at 6, 12, and 24 h after ox-LDL exposure than at 0 h ($p < 0.05$). In contrast, the expression of p-STAT3 was significantly elevated after the RAW264.7 cells were treated with ox-LDL for 6, 12, and 24 h compared to the 0 h time point ($p < 0.05$). The expression of NFκB p65 protein was similar with p-STAT3. (Figures 2(c) and 2(d)). NF-κB promoter activity and the level of IL-6 and TNF-α mRNA expression were also increased when RAW264.7 cells were exposed to 20 μg/mL ox-LDL (Figures 2(e)–2(g)). Additionally, the expression of IFN-β mRNA was lower at 6 h than at 0 h ($p < 0.05$), and the IFN-β mRNA expression at 12 h and 24 h was not significantly different compared to that at 0 h ($p > 0.05$) (Figure 2(h)). These data suggested that ox-LDL not only induced miR-155 generation but also activated the inflammatory signaling pathway via miR-155.

3.3. Inhibition of TRIF by TRIF siRNA Suppressed ox-LDL-Induced miR-155 Generation. To explore the role of TRIF in ox-LDL-induced inflammation, we silenced TRIF mRNA and protein using TRIF siRNA. The data showed that the three TRIF siRNA oligos markedly suppressed TRIF mRNA and protein expression ($p < 0.05$) and that the TRIF siRNA-1 and siRNA-2 oligo was more effective in silencing TRIF expression than the other siRNA oligos (Figures 3(a)–3(c)). Interestingly, the expression of BIC RNA was significantly decreased when the Raw264.7 cells were preincubated with TRIF siRNA-1 and siRNA-2, then exposed to ox-LDL compared to cells that were preincubated with NC followed by ox-LDL exposure ($p < 0.05$) (Figure 3(d)). Similarly, miR-155 expression was lower in the ox-LDL/TRIF siRNA group than that in the ox-LDL/NC group ($p < 0.05$) (Figure 3(e)). However, the levels of BIC and miR-155 remained higher in the ox-LDL/TRIF siRNA group than those in the control group ($p < 0.05$). These data demonstrated that TRIF knockdown could partly reverse ox-LDL-induced miR-155 generation and suggested that TRIF modulates miR-155 generation. Additionally, given to TRIF siRNA-1 was better to suppress TRIF expression; therefore, it was used to knock down TRIF expression in subsequent experiments.

3.4. TRIF Silencing Inactivated the miR-155-Mediated Inflammatory Pathway. TRIF plays an important role in the MyD88-independent inflammatory pathway. Given that TRIF could upregulate the generation of miR-155, we hypothesized that TRIF knockdown could suppress the miR-155-mediated inflammatory pathway. Our data demonstrated that the level of SOCS1 protein expression was decreased after RAW264.7 cells were challenged with ox-LDL in the ox-LDL/NC group and partly restored in the ox-LDL/TRIF siRNA group. In contrast to the expression of p-STAT, NFκB p65 was significantly inhibited in the ox-LDL/TRIF siRNA group compared to that in the ox-LDL/NC group ($p < 0.05$) (Figures 4(a) and 4(b)). The promoter activity of NF-κB was higher in the ox-LDL, ox-LDL/NC, and ox-LDL/TRIF siRNA groups than that in the control group ($p < 0.05$). Moreover, NF-κB activity was notably suppressed in the ox-LDL/TRIF siRNA group compared to that in the ox-LDL/NC group ($p < 0.05$) (Figure 4(c)). Meanwhile, the expressions of IL-6 and TNF-α mRNA were consistent with the NF-κB promoter activity (Figures 4(d) and 4(e)). These data revealed that TRIF knockdown inactivated the miR-155-mediated inflammatory pathway, suggesting that TRIF was a novel and important target for the inhibition of the ox-LDL-induced macrophage inflammatory response.

3.5. $ERK_{1/2}$ Signaling Is Involved in TRIF-Mediated miR-155 Generation. $ERK_{1/2}$ signaling plays a key role in the pathophysiology of AS [23–25]. In our study, we investigated the role of $ERK_{1/2}$ in ox-LDL-induced miR-155 generation in RAW264.7 cells. As shown in Figures 5(a) and 5(b), the level of p-$ERK_{1/2}$ was elevated at 12 and 24 h after macrophages were treated with 20 μg/mL ox-LDL compared to that at the 0 h time point ($p < 0.05$). Subsequent experiments showed that TRIF silencing by TRIF siRNA significantly

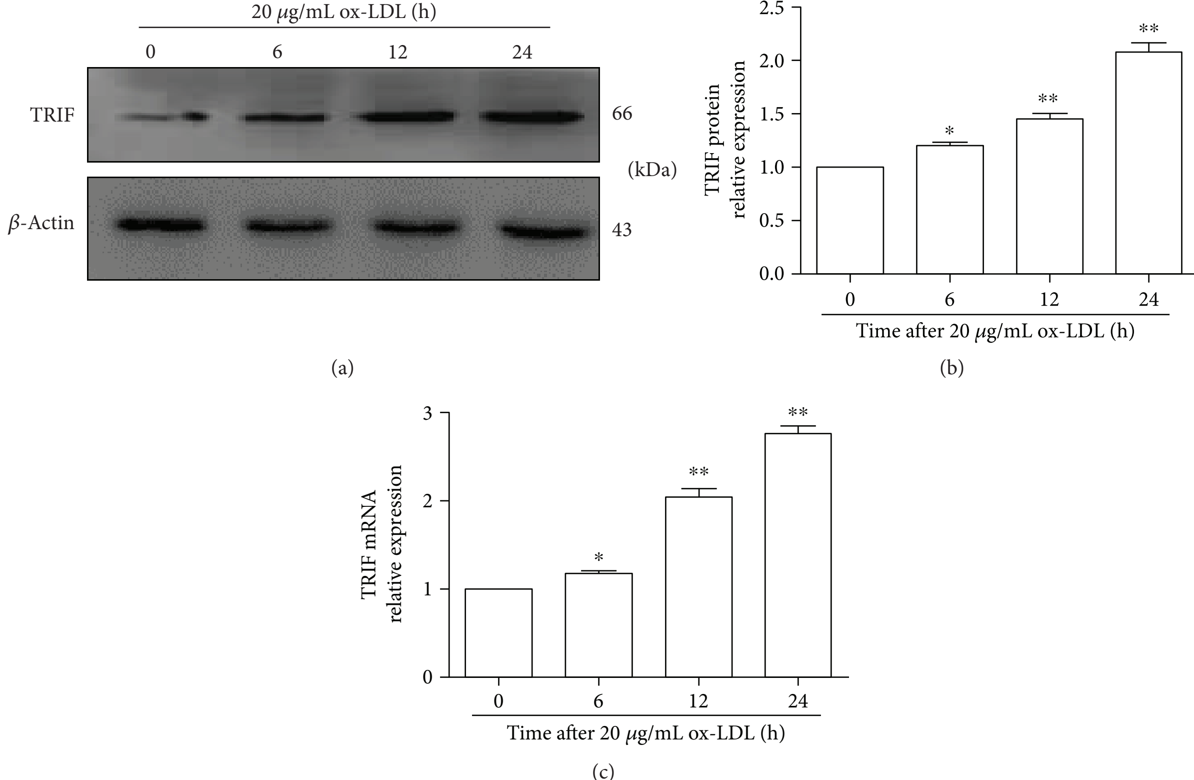

Figure 1: Effect of ox-LDL on TRIF expression in RAW264.7 cells. (a) TRIF protein expression was evaluated by Western blot. RAW264.7 cells were treated with 20 μg/mL ox-LDL for the indicated times. Representative bands show the expression of TRIF protein (upper panel) and β-actin protein (lower panel). (b) Histograms illustrate TRIF protein expressions, which were normalized to β-actin expression. The data represent the mean ± S.E. of three independent experiments. (c) qPCR was used to detect the level of TRIF mRNA. The data represent the mean ± S.E. of four independent experiments. $^*p < 0.05$ and $^{**}p < 0.01$ versus the 0 h time point.

inhibited the expression of p-ERK$_{1/2}$ compared to the NC group ($p < 0.05$). Furthermore, the level of p-ERK$_{1/2}$ expression was lower in the TRIF siRNA/ox-LDL group than that in the ox-LDL group ($p < 0.05$) (Figures 5(c) and 5(d)). Moreover, the level of p-ERK$_{1/2}$ expression was lower in the SCH772984 group than that in the control group ($p < 0.05$) and lower in the ox-LDL/SCH772984 group than that in the ox-LDL group ($p < 0.05$) (Figures 5(e) and 5(f)). Additionally, the expressions of BIC and miR-155 were suppressed in the ox-LDL/SCH772984 group compared with that in the ox-LDL group ($p < 0.05$) (Figures 5(g) and 5(h)). These data suggest that ERK$_{1/2}$ signaling is involved in TRIF-mediated miR-155 generation.

4. Discussion

Herein, we determined that ox-LDL induced TRIF, miR-155, and BIC (the precursor of miR-155) expression. Knockdown of TRIF expression partly reversed ox-LDL-induced BIC and miR-155 expression, inactivated the miR-155-modulated SOCS1-STAT3-NF-κB pathway, and reduced the production of inflammatory mediators. Moreover, we showed that ERK$_{1/2}$ signaling is involved in the induction of mediated miR-155 generation by TRIF. These data suggest that TRIF promotes the ox-LDL-induced macrophage inflammatory response by inducing miR-155 generation.

Recent studies have shown that TRIF plays a critical role in modulating the progression of AS and vessel inflammation in animal models [13, 14] following the activation of TLR 3 and 4 in the endosomes [26]. Our data showed that ox-LDL induced the expression of TRIF in macrophages; furthermore, activation of NK-κB signaling and upregulated TNF-α and IL-6 expression were also been found [21, 27]. While knockdown of TRIF using TRIF siRNA partly suppressed ox-LDL-induced NK-κB activation and inflammatory mediator expression, our data suggest that upregulation of TRIF by ox-LDL promotes the macrophage inflammatory response and that TRIF is a novel target for blocking inflammatory mediator release from macrophages. Additionally, the expression of IFN-β was decreased when RAW264.7 cells were exposed to ox-LDL for 6 h and then gradually restored to baseline. This finding might be due to the induction of TRAF family member-associated NF-κB activator (TANK) monoubiquitination by ox-LDL and the subsequent suppression of TRIF-dependent IFN-β expression [28].

BIC was first identified in avian leukosis virus-induced B lymphomagenesis as a collaborator with c-myc even though it is a nonprotein-coding RNA due to the lack of a large open reading frame (ORF) [29–31]. Lagos-Quintana et al. identified the miR-155 foldback precursor sequence within a conserved region of BIC [32]. Eis and

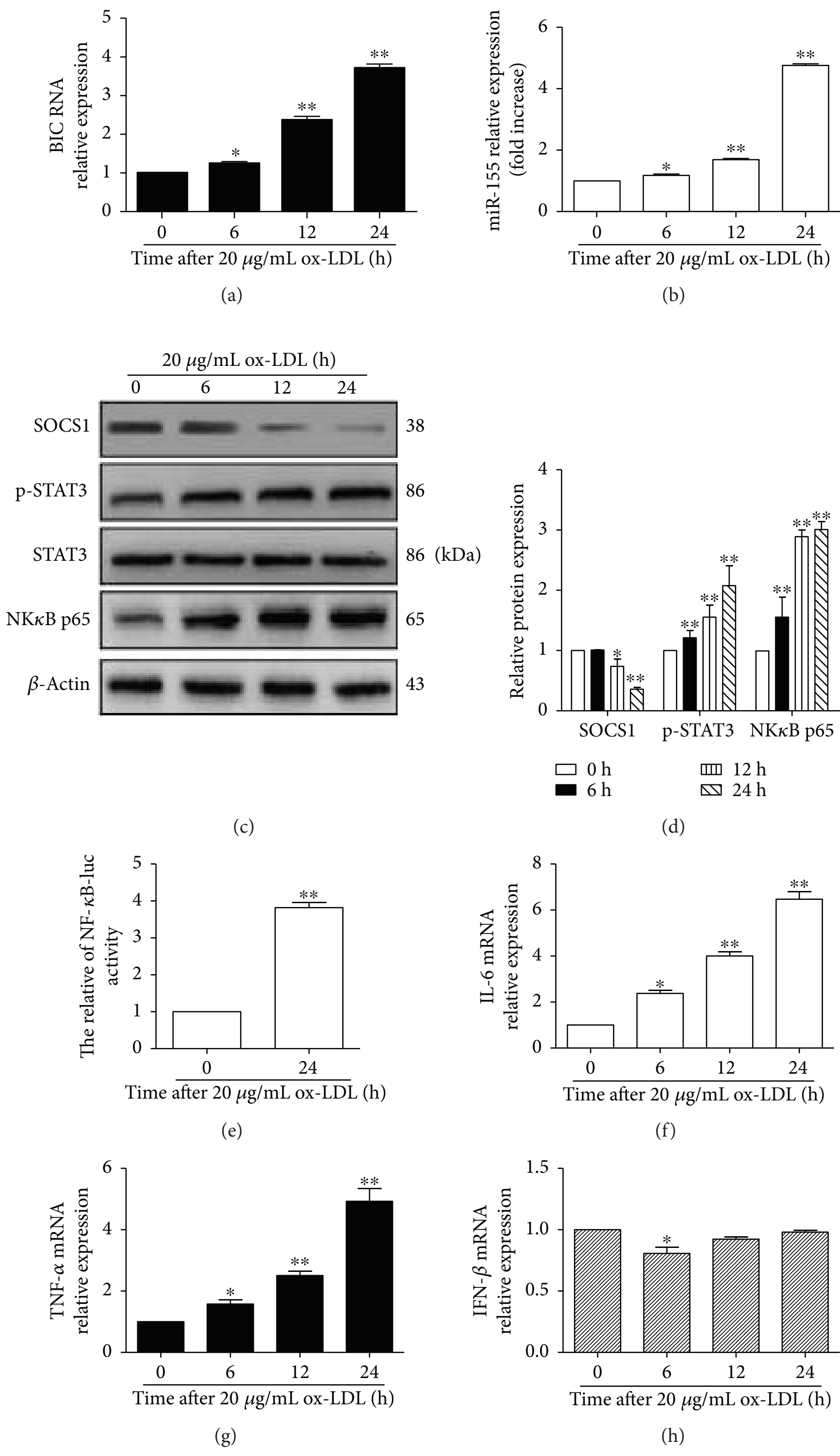

Figure 2: ox-LDL induced miR-155 generation and the macrophage inflammatory response. RAW264.7 cells were treated with 20 μg/mL ox-LDL for the indicated times. (a and b) qPCR was used to detect the expression of miR-155HG (BIC) and miR-155 in macrophages. (c) SOCS1, NFκB p65, STAT3, and p-STAT3 protein expression was evaluated using Western blot. Representative bands show the expression of SOCS1 protein (upper panel), p-STAT3 (second panel), STAT3 (third panel), NFκB p65 (fourth panel), and β-actin protein (lower panel). (d) Histograms illustrated the SOCS1, NFκB p65, and p-STAT3 protein expressions, which were normalized to β-actin expression. The data represented the mean ± S.E. of three independent experiments. (e) The promoter activity of NF-κB was analyzed by dual-luciferase reporter assay. (f, g, h) The expressions of IL-6, TNF-α, and IFN-β mRNA were measured using qPCR. The data represent the mean ± S.E. of four independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus the 0 h time point.

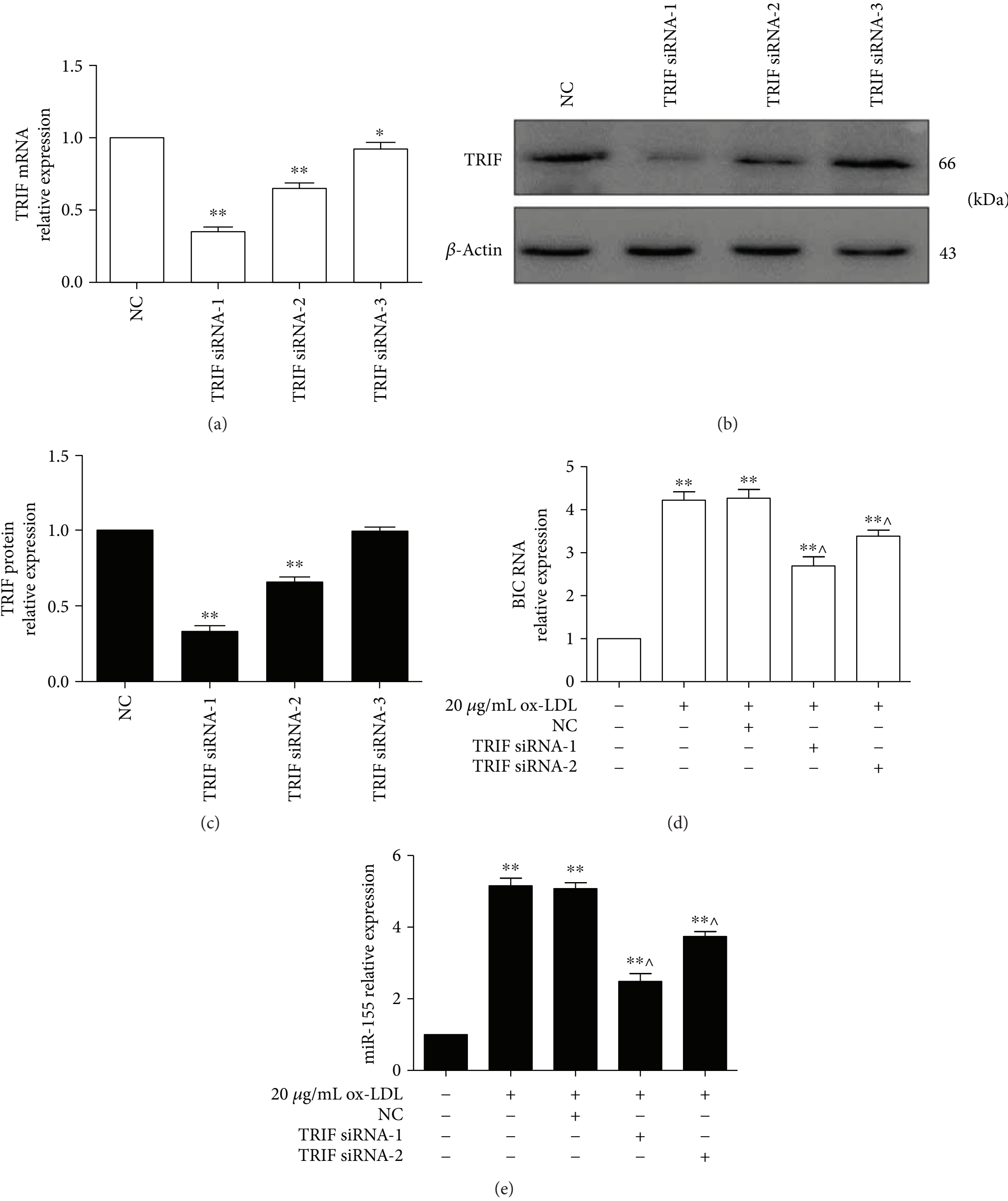

Figure 3: TRIF silencing suppressed BIC and miR-155 expression. (a and b) TRIF mRNA and protein expression was inhibited by TRIF siRNA. Three TRIF siRNA oligos and negative control (NC) were separately transfected into RAW264.7 cells for 24 h, and then, the cells were harvested for detection of TRIF mRNA and protein expressions by qPCR and Western blot, respectively. Representative bands in B show the expression of TRIF protein (upper panel) and β-actin protein (lower panel). (c) Histograms illustrate the TRIF protein expressions, which were normalized to β-actin expression. The data represent the mean ± S.E. of three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus the NC group. (d and e) BIC RNA and miR-155 expression was evaluated by qPCR. RAW264.7 cells were preincubated with TRIF siRNA-1 or siRNA-2 for 24 h followed by treatment with 20 μg/mL ox-LDL for 24 h. The data represent the mean ± S.E. of four independent experiments. $^{**}p < 0.01$ versus the control group and $^{\wedge}p < 0.05$ versus the 20 μg/mL ox-LDL/NC group.

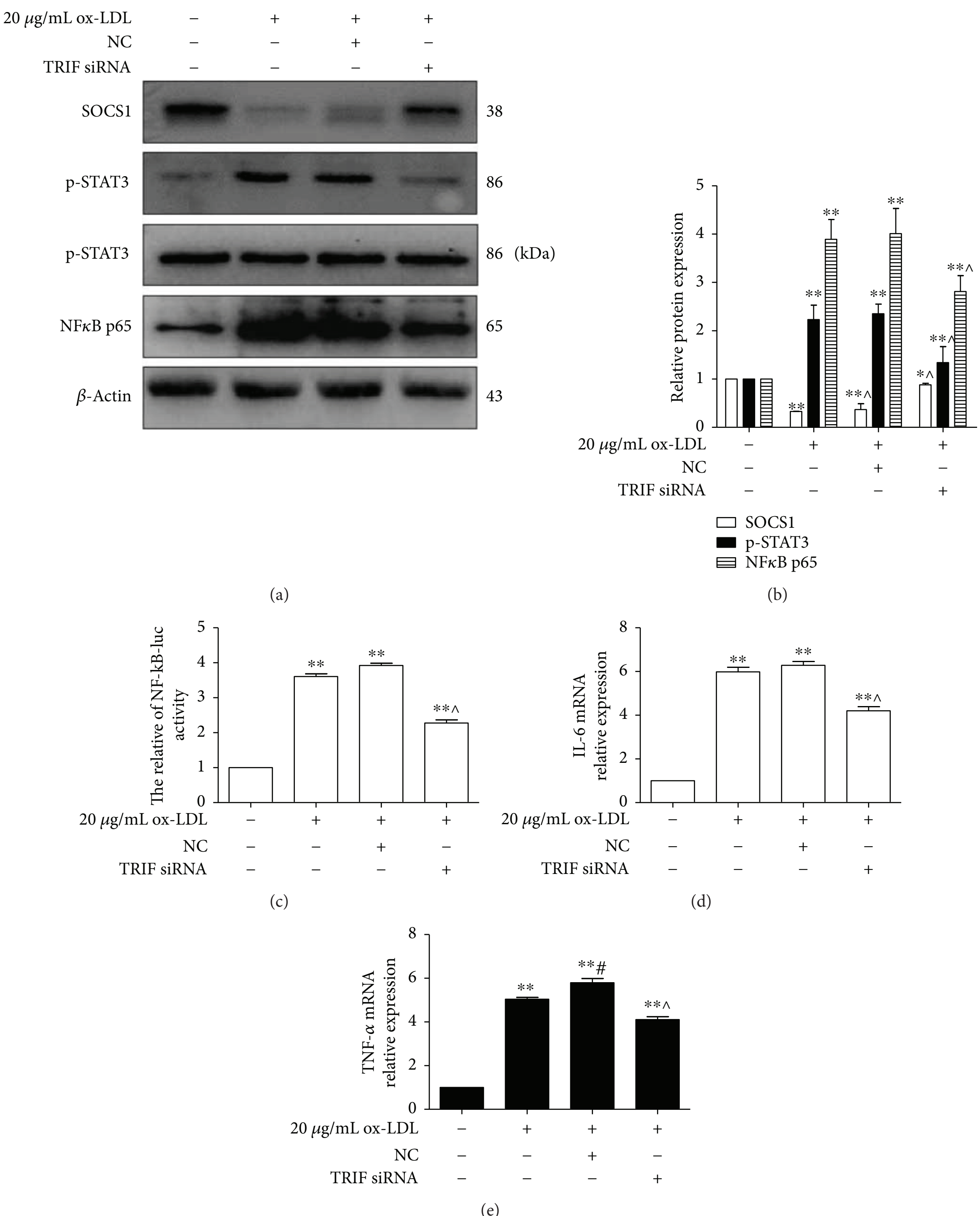

Figure 4: Knockdown of TRIF suppressed the miR-155-mediated inflammatory pathway. RAW264.7 cells were preincubated with TRIF siRNA for 24 h followed by treatment with 20 μg/mL ox-LDL for 24 h. (a) The levels of SOCS1 NFκB p65, STAT3, and p-STAT3 protein were evaluated using Western blot. Representative bands showed the levels of SOCS1 protein (upper panel), p-STAT3 (second panel), STAT3 (third panel), NFκB p65 (fourth panel), and β-actin protein (lower panel). (b) Histograms illustrated the SOCS1, NFκB p65, and p-STAT3 protein expressions, which were normalized to β-actin expression. (c) NF-κB promoter activity was analyzed using a dual-luciferase reporter assay. (d and e) The expressions of IL-6 and TNF-α mRNA were measured using qPCR. The data represent the mean ± S.E. of four independent experiments. $^{*}p < 0.05$ versus the control group, $^{**}p < 0.01$ versus the control group, $^{\#}p < 0.05$ versus the 20 μg/mL ox-LDL group, and $^{\wedge}p < 0.05$ versus the 20 μg/mL ox-LDL/NC group.

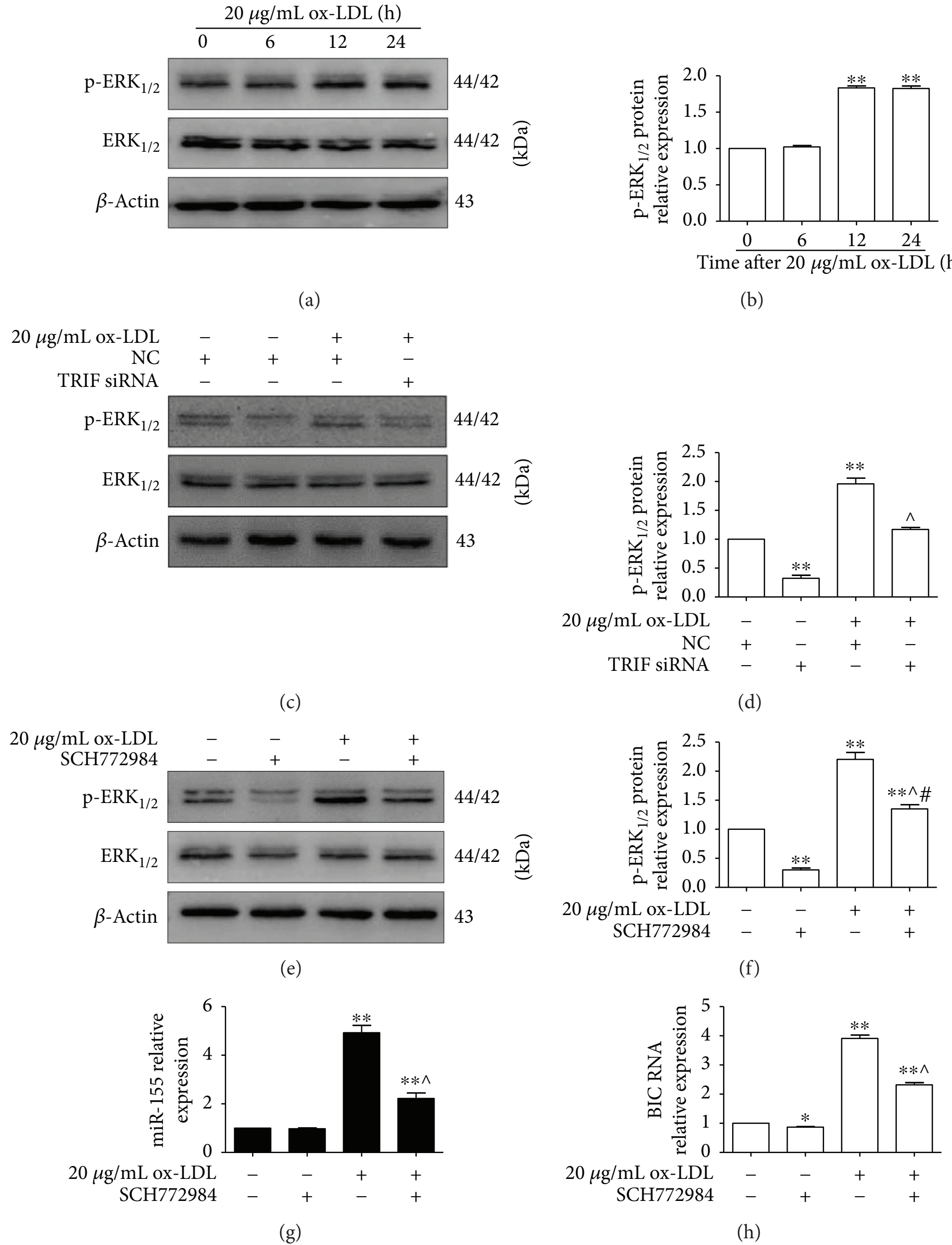

Figure 5: $ERK_{1/2}$ is involved in the regulation of BIC and miR-155 expression by TRIF. (a) The expression of p-$ERK_{1/2}$ protein was evaluated by Western blot. RAW264.7 cells were treated with 20 μg/mL ox-LDL for the indicated times. Representative bands showed the expression of p-$ERK_{1/2}$ protein (upper panel), p-$ERK_{1/2}$ protein (middle panel), and β-actin protein (lower panel). (b) Histograms illustrated the p-$ERK_{1/2}$ protein level, which was normalized to the $ERK_{1/2}$ level. The data represented the mean ± S.E. of three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus the 0 h time point. (c) TRIF silencing suppressed p-$ERK_{1/2}$ expression. RAW264.7 cells were transfected with NC or TRIF siRNA for 24 h followed by treatment with 20 μg/mL ox-LDL for 24 h. Representative bands show the expression of p-$ERK_{1/2}$ protein (upper panel), p-$ERK_{1/2}$ protein (middle panel), and β-actin protein (lower panel). (d) Histograms illustrate the p-$ERK_{1/2}$ protein expression, which was normalized to the $ERK_{1/2}$ expression. The data represent the mean ± S.E. of three independent experiments. $^{**}p < 0.01$ versus the NC group and $^{\wedge}p < 0.05$ versus the TRIF siRNA/ox-LDL group. (e) The level of p-$ERK_{1/2}$ expression was suppressed by SCH772984 ($ERK_{1/2}$ inhibitor). RAW264.7 cells were preincubated with 1 μM SCH772984 for 2 h followed by treatment with 20 μg/mL ox-LDL for 24 h. (f) Histograms illustrate the p-$ERK_{1/2}$ protein expression, which was normalized to the $ERK_{1/2}$ expression. (g and h) The expressions of miR-155 and BIC RNA were measured using qPCR. RAW264.7 cells were preincubated with 1 μM SCH772984 for 2 h followed by treatment with 20 μg/mL ox-LDL for 24 h. The data represent the mean ± S.E. of three independent experiments. $^{**}p < 0.01$ versus the control group, $^{\wedge}p < 0.05$ versus the 20 μg/mL ox-LDL group, and $^{\#}p < 0.05$ versus the SCH772984 group.

colleagues subsequently found that the level of miR-155 expression was dependent on the level of BIC RNA and the regulation of pre-miR-155 generation [33]. Recently, O'Connell et al. demonstrated that activation of TLRs by their ligands upregulated miR-155 through either a MyD88-dependent or MyD88-independent (TRIF) signaling pathway in THP-1 cells. Moreover, BIC RNA was shown to be involved in TLR ligand-induced miR-155 expression [34]. Similarly, our study found that ox-LDL induced BIC and miR-155 expression in RAW264.7 cells, thereby suppressing the target gene SOCS1 and activating the STAT3-NF-κB signaling pathway. Knockdown of TRIF suppressed the expression of BIC and miR-155, partly inactivated the STAT3-NF-κB signaling pathway, and downregulated the expression of inflammatory mediators, thus suggesting that TRIF induces miR-155 expression and activates its downstream signaling pathways by regulating the level of BIC RNA.

It is well known that activation of the $ERK_{1/2}$ signaling pathway plays an important role in controlling several cellular biological responses, including cell cycle arrest, cell survival, and apoptosis [35–37]. Recently, studies have shown that $ERK_{1/2}$ activation is involved in ox-LDL-induced endothelial cell injury [38–40], vascular smooth muscle cell (VSMC) proliferation and migration [41], and the macrophage inflammatory response [42, 43]. Consistent with the above research, our study also demonstrated that $ERK_{1/2}$ signaling pathways are activated in macrophages following stimulation with ox-LDL. Interestingly, we found that knockdown of TRIF hampered the ox-LDL-mediated activation of $ERK_{1/2}$ signaling pathways, suggesting that activation of $ERK_{1/2}$ signaling is dependent on TRIF when macrophages are exposed to ox-LDL. Luan et al. showed that knockdown of TRIF-related adaptor molecule (TRAM) using TRAM siRNA suppressed Broncho-Vaxom-induced $ERK_{1/2}$ activation in RAW264.7 cells [44]. Other studies have revealed that lipopolysaccharide (LPS) induces $ERK_{1/2}$ activation through TRIF and MyD88- and TRIF-dependent signaling *in vivo* and *in vitro* [45–47]. According to the results of the above research, activation of $ERK_{1/2}$ might be at least partly dependent on TRIF signaling when macrophages are stimulated by ox-LDL. In addition, our results suggest that $ERK_{1/2}$ plays an important role in the regulation of BIC and miR-155 expression. Our findings are consistent with those of the previous report that the activation of $ERK_{1/2}$ and c-Jun N-terminal kinase (JNK) pathways could upregulate BIC and miR-155 expression [48]. However, the exact mechanism requires additional investigation.

In conclusion, these findings illustrate that TRIF pathways play an important role in the regulation of the ox-LDL-induced macrophage inflammatory response. The elevation in TRIF leads to $ERK_{1/2}$ signal activation, which in turn enhances the expression of BIC/miR-155, thus promoting inflammation mediator production. This study precisely defined an important mechanism underlying the ox-LDL-induced macrophage inflammatory response and showed that TRIF is a novel therapeutic target for atherosclerosis.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Yaxi Wu and Jinshan Ye contributed equally to this work.

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References

[1] W. Herrington, B. Lacey, P. Sherliker, J. Armitage, and S. Lewington, "Epidemiology of atherosclerosis and the potential to reduce the global burden of atherothrombotic disease," *Circulation Research*, vol. 118, no. 4, pp. 535–546, 2016.

[2] B. Zarzycka, G. A. F. Nicolaes, and E. Lutgens, "Targeting the adaptive immune system: new strategies in the treatment of atherosclerosis," *Expert Review of Clinical Pharmacology*, vol. 8, no. 3, pp. 297–313, 2015.

[3] S. Pant, A. Deshmukh, G. S. GuruMurthy et al., "Inflammation and atherosclerosis—revisited," *Journal of Cardiovascular Pharmacology and Therapeutics*, vol. 19, no. 2, pp. 170–178, 2014.

[4] G. K. Hansson and A. Hermansson, "The immune system in atherosclerosis," *Nature Immunology*, vol. 12, no. 3, pp. 204–212, 2011.

[5] J. L. Stöger, P. Goossens, and M. P. de Winther, "Macrophage heterogeneity: relevance and functional implications in atherosclerosis," *Current Vascular Pharmacology*, vol. 8, no. 2, pp. 233–248, 2010.

[6] K. A. Jenkins and A. Mansell, "TIR-containing adaptors in Toll-like receptor signalling," *Cytokine*, vol. 49, no. 3, pp. 237–244, 2010.

[7] T. Ve, N. J. Gay, A. Mansell, B. Kobe, and S. Kellie, "Adaptors in toll-like receptor signaling and their potential as therapeutic targets," *Current Drug Targets*, vol. 13, no. 11, pp. 1360–1374, 2012.

[8] M. Yamamoto, S. Sato, H. Hemmi et al., "Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway," *Science*, vol. 301, no. 5633, pp. 640–643, 2003.

[9] H. Oshiumi, M. Matsumoto, K. Funami, T. Akazawa, and T. Seya, "TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction," *Nature Immunology*, vol. 4, no. 2, pp. 161–167, 2003.

[10] K. A. Fitzgerald, D. C. Rowe, B. J. Barnes et al., "LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF," *The Journal of Experimental Medicine*, vol. 198, no. 7, pp. 1043–1055, 2003.

[11] K. Hoebe, X. du, P. Georgel et al., "Identification of *Lps2* as a key transducer of MyD88-independent TIR signalling," *Nature*, vol. 424, no. 6950, pp. 743–748, 2003.

[12] E. Vorkapic, A. M. Lundberg, M. I. Mäyränpää, P. Eriksson, and D. Wågsäter, "TRIF adaptor signaling is important in abdominal aortic aneurysm formation," *Atherosclerosis*, vol. 241, no. 2, pp. 561–568, 2015.

[13] A. M. Lundberg, D. F. J. Ketelhuth, M. E. Johansson et al., "Toll-like receptor 3 and 4 signalling through the TRIF and TRAM adaptors in haematopoietic cells promotes atherosclerosis," *Cardiovascular Research*, vol. 99, no. 2, pp. 364–373, 2013.

[14] M. R. Richards, A. S. Black, D. J. Bonnet et al., "The *LPS2* mutation in TRIF is atheroprotective in hyperlipidemic low density lipoprotein receptor knockout mice," *Innate Immunity*, vol. 19, no. 1, pp. 20–29, 2013.

[15] B. Laffont and K. J. Rayner, "MicroRNAs in the pathobiology and therapy of atherosclerosis," *The Canadian Journal of Cardiology*, vol. 33, no. 3, pp. 313–324, 2017.

[16] Y. Yang, L. Yang, X. Liang, and G. Zhu, "MicroRNA-155 promotes atherosclerosis inflammation via targeting SOCS1," *Cellular Physiology and Biochemistry*, vol. 36, no. 4, pp. 1371–1381, 2015.

[17] M. Nazari-Jahantigh, Y. Wei, H. Noels et al., "MicroRNA-155 promotes atherosclerosis by repressing Bcl6 in macrophages," *The Journal of Clinical Investigation*, vol. 122, no. 11, pp. 4190–4202, 2012.

[18] J. Ye, R. Guo, Y. Shi, F. Qi, C. Guo, and L. Yang, "miR-155 regulated inflammation response by the SOCS1-STAT3-PDCD4 axis in atherogenesis," *Mediators of Inflammation*, vol. 2016, Article ID 8060182, 14 pages, 2016.

[19] J. Zhu, T. Chen, L. Yang et al., "Regulation of microRNA-155 in atherosclerotic inflammatory responses by targeting MAP3K10," *PLoS One*, vol. 7, no. 11, article e46551, 2012.

[20] B. Huang, D. W. Park, and S. H. Baek, "TRIF is a regulator of TLR2-induced foam cell formation," *Molecular Medicine Reports*, vol. 14, no. 4, pp. 3329–3335, 2016.

[21] F. J. Tian, L. N. An, G. K. Wang et al., "Elevated microRNA-155 promotes foam cell formation by targeting HBP1 in atherogenesis," *Cardiovascular Research*, vol. 103, no. 1, pp. 100–110, 2014.

[22] R. H. Du, E. G. Li, Y. Cao, Y. C. Song, and R. X. Tan, "Fumigaclavine C inhibits tumor necrosis factor α production via suppression of toll-like receptor 4 and nuclear factor κB activation in macrophages," *Life Sciences*, vol. 89, no. 7-8, pp. 235–240, 2011.

[23] N. Li, J. E. McLaren, D. R. Michael, M. Clement, C. A. Fielding, and D. P. Ramji, "ERK is integral to the IFN-γ-mediated activation of STAT1, the expression of key genes implicated in atherosclerosis, and the uptake of modified lipoproteins by human macrophages," *Journal of Immunology*, vol. 185, no. 5, pp. 3041–3048, 2010.

[24] I. Lázaro, R. Ferré, L. Masana, and A. Cabré, "Akt and ERK/Nrf2 activation by PUFA oxidation-derived aldehydes upregulates FABP4 expression in human macrophages," *Atherosclerosis*, vol. 230, no. 2, pp. 216–222, 2013.

[25] S. Thatcher, "Commentary for Clancy, P et al., ARBs and ERK activation: new insights on human atherosclerosis," *Atherosclerosis*, vol. 236, no. 1, pp. 131-132, 2014.

[26] M. O. Ullah, M. J. Sweet, A. Mansell, S. Kellie, and B. Kobe, "TRIF-dependent TLR signaling, its functions in host defense and inflammation, and its potential as a therapeutic target," *Journal of Leukocyte Biology*, vol. 100, no. 1, pp. 27–45, 2016.

[27] C. Liang, X. Wang, J. Hu et al., "PTPRO promotes oxidized low-density lipoprotein induced oxidative stress and cell apoptosis through toll-like receptor 4/nuclear factor κB pathway," *Cellular Physiology and Biochemistry*, vol. 42, no. 2, pp. 495–505, 2017.

[28] N. Tzieply, A. M. Kuhn, D. Morbitzer et al., "OxLDL inhibits LPS-induced IFNβ expression by Pellino3- and IRAK1/4-dependent modification of TANK," *Cellular Signalling*, vol. 24, no. 6, pp. 1141–1149, 2012.

[29] B. E. Clurman and W. S. Hayward, "Multiple proto-oncogene activations in avian leukosis virus-induced lymphomas: evidence for stage-specific events," *Molecular and Cellular Biology*, vol. 9, no. 6, pp. 2657–2664, 1989.

[30] W. Tam, S. H. Hughes, W. S. Hayward, and P. Besmer, "Avian bic, a gene isolated from a common retroviral site in avian leukosis virus-induced lymphomas that encodes a noncoding RNA, cooperates with c-myc in lymphomagenesis and erythroleukemogenesis," *Journal of Virology*, vol. 76, no. 9, pp. 4275–4286, 2002.

[31] W. Tam, "Identification and characterization of human BIC, a gene on chromosome 21 that encodes a noncoding RNA," *Gene*, vol. 274, no. 1-2, pp. 157–167, 2001.

[32] M. Lagos-Quintana, R. Rauhut, A. Yalcin, J. Meyer, W. Lendeckel, and T. Tuschl, "Identification of tissue-specific microRNAs from mouse," *Current Biology*, vol. 12, no. 9, pp. 735–739, 2002.

[33] P. S. Eis, W. Tam, L. Sun et al., "Accumulation of miR-155 and BIC RNA in human B cell lymphomas," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 10, pp. 3627–3632, 2005.

[34] R. M. O'Connell, K. D. Taganov, M. P. Boldin, G. Cheng, and D. Baltimore, "MicroRNA-155 is induced during the macrophage inflammatory response," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 5, pp. 1604–1609, 2007.

[35] S. Meloche and J. Pouysségur, "The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition," *Oncogene*, vol. 26, no. 22, pp. 3227–3239, 2007.

[36] K. Balmanno and S. J. Cook, "Tumour cell survival signalling by the ERK1/2 pathway," *Cell Death and Differentiation*, vol. 16, no. 3, pp. 368–377, 2009.

[37] A. von Kriegsheim, D. Baiocchi, M. Birtwistle et al., "Cell fate decisions are specified by the dynamic ERK interactome," *Nature Cell Biology*, vol. 11, no. 12, pp. 1458–1464, 2009.

[38] Y. Luo, S. Lu, X. Dong, L. Xu, G. Sun, and X. Sun, "Dihydromyricetin protects human umbilical vein endothelial cells from injury through ERK and Akt mediated Nrf2/HO-1 signaling pathway," *Apoptosis*, vol. 22, no. 8, pp. 1013–1024, 2017.

[39] L. Yang, J. Liu, and G. Qi, "Mechanism of the effect of saikosaponin on atherosclerosis in vitro is based on the MAPK signaling pathway," *Molecular Medicine Reports*, vol. 16, no. 6, pp. 8868–8874, 2017.

[40] H. F. Deng, X. L. Wang, H. Sun, and X. Z. Xiao, "Puerarin inhibits expression of tissue factor induced by oxidative low-density lipoprotein through activating the PI3K/Akt/eNOS pathway and inhibiting activation of ERK1/2 and NF-κB," *Life Sciences*, vol. 191, pp. 115–121, 2017.

[41] W. Li, W. Zhi, F. Liu, J. Zhao, Q. Yao, and X. Niu, "Paeoniflorin inhibits VSMCs proliferation and migration by arresting cell cycle and activating HO-1 through MAPKs and NF-κB pathway," *International Immunopharmacology*, vol. 54, pp. 103–111, 2018.

[42] M. X. Hao, L. S. Jiang, N. Y. Fang et al., "The cannabinoid WIN55,212-2 protects against oxidized LDL-induced

inflammatory response in murine macrophages," *Journal of Lipid Research*, vol. 51, no. 8, pp. 2181–2190, 2010.

[43] Q. Shao, L. H. Shen, L. H. Hu et al., "Nuclear receptor Nur77 suppresses inflammatory response dependent on COX-2 in macrophages induced by oxLDL," *Journal of Molecular and Cellular Cardiology*, vol. 49, no. 2, pp. 304–311, 2010.

[44] H. Luan, Q. Zhang, L. Wang et al., "OM85-BV induced the productions of IL-1β, IL-6, and TNF-α via TLR4- and TLR2-mediated ERK1/2/NF-κB pathway in RAW264.7 cells," *Journal of Interferon & Cytokine Research*, vol. 34, no. 7, pp. 526–536, 2014.

[45] M. Z. Xia, Y. L. Liang, H. Wang et al., "Melatonin modulates TLR4-mediated inflammatory genes through MyD88- and TRIF-dependent signaling pathways in lipopolysaccharide-stimulated RAW264.7 cells," *Journal of Pineal Research*, vol. 53, no. 4, pp. 325–334, 2012.

[46] B. A. Watts III, T. George, E. R. Sherwood, and D. W. Good, "Monophosphoryl lipid A induces protection against LPS in medullary thick ascending limb through a TLR4-TRIF-PI3K signaling pathway," *American Journal of Physiology. Renal Physiology*, vol. 313, no. 1, pp. F103–F115, 2017.

[47] L. Sun, T. T. Pham, T. T. Cornell et al., "Myeloid-specific gene deletion of protein phosphatase 2A magnifies MyD88- and TRIF-dependent inflammation following endotoxin challenge," *Journal of Immunology*, vol. 198, no. 1, pp. 404–416, 2017.

[48] Q. Yin, X. Wang, J. McBride, C. Fewell, and E. Flemington, "B-cell receptor activation induces BIC/miR-155 expression through a conserved AP-1 element," *The Journal of Biological Chemistry*, vol. 283, no. 5, pp. 2654–2662, 2008.

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Review Article

Modulation of Immune-Inflammatory Responses in Abdominal Aortic Aneurysm: Emerging Molecular Targets

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Abdominal aortic aneurysm (AAA), a deadly vascular disease in human, is a chronic degenerative process of the abdominal aorta. In this process, inflammatory responses and immune system work efficiently by inflammatory cell attraction, proinflammatory factor secretion and subsequently MMP upregulation. Previous studies have demonstrated various inflammatory cell types in AAA of human and animals. The majority of cells, such as macrophages, CD4+ T cells, and B cells, play an important role in the diseased aortic wall through phenotypic modulation. Furthermore, immunoglobulins also greatly affect the functions and differentiation of immune cells in AAA. Recent evidence suggests that innate immune system, especially Toll-like receptors, chemokine receptors, and complements are involved in the progression of AAAs. We discussed the innate immune system, inflammatory cells, immunoglobulins, immune-mediated mechanisms, and key cytokines in the pathogenesis of AAA and particularly emphasis on a further trend and application of these interventions. This current understanding may offer new insights into the role of inflammation and immune response in AAA.

1. Introduction

Abdominal aortic aneurysm (AAA) is a common degenerative cardiovascular disease. This disease is generally caused by smoking, genetic diversity or variants, and atherosclerosis [1–3]. The majority of AAAs are detected in the infrarenal aorta, proximal to the aortic bifurcation [4]. AAA is a potentially lethal disease due to the risk of rupture [5]. Clinically, AAAs can be repaired using open surgical technique only when the diameter of aorta has surpassed 5.5 cm with a substantially increased risk of rupture [6]. Understanding the potential mechanism of AAA development and developing therapeutic strategies that modify the disease process of AAA is very important.

Vascular inflammation is the main initial factor of aortic aneurysm. In this process, a large number of exogenous immune cells, including lymphocytes, macrophages, mast cells, neutrophils, and natural killer cells, infiltrate into the tissue from adventitia to intima gradually, evoking a series of inflammatory response [7–11]. Infiltration of inflammatory cells and cellular elements produce and stimulate smooth muscle cells (SMC) to secret matrix metalloproteinases (MMPs), which are considered key enzymes directly related to AAA formation and progression [12, 13]. These enzymes destroy the stability and mechanical property of the aortic walls by modulating interstitial elastin and collagen [14–16], resulting in loss of smooth muscle cells in the aortic media and destruction of extracellular matrix (ECM) [17]. Inflammation is an important component of the immune system. The adaptive and innate immune systems have a great role in the initiation and propagation of the inflammatory response in aortic tissue. Recent increased knowledge suggests that immunological processes are involved in the pathogenesis of AAA [18–20]. In this view, we will discuss

phenotypes of inflammatory cells, innate immune system, immunoglobulins, and key cytokines in the AAA disease and provide novel mechanistic insight for the development of immune-targeted therapies.

2. Innate Immunity

Innate immune system, also known as the nonspecific immune system, is the first line of defense against pathogenic invasion. In the pathological process of aortic aneurysm, a series of changes in the innate immune system including upregulation of TLRs (Toll-like receptors), activation of chemokine receptors, and deposition of complements were involved. We will show the most recent research progress in these areas and discuss particularly in the following paragraph.

2.1. TLRs in AAA. TLRs play a fundamental role in several of inflammatory response and innate immunity process. As the initiating gate of innate immunity, pattern recognition receptor (PRR) activation is a start of all the subsequent immune responses [21, 22]. One of the transmembrane subtypes of PRRs, TLR, is a researching hotspot in recent years on the pathological mechanism of AAAs. TLRs are expressed on inflammatory cells (such as macrophages, monocytes, and B lymphocytes), endothelial cells, and SMCs, and all of these types of cells contribute to the inflammatory response of aortas [23]. In general, myeloid differentiation primary response gene-88 (MyD88) and TRIF as the intracellular signaling adaptors were involved in the proinflammatory process initiated by TLR activation. Most TLRs, including TLR2 and TLR4, signal through MyD88. But TLR3 signals through TRIF. Only TLR4 signals through both MyD88 and TRIF [24]. Till now, about 9 kinds of TLRs were discovered [25, 26] and some of these subtypes work actively in AAA (Figure 1).

2.1.1. TLR2. TLR2 is mainly implicated in the initiation and maintenance of the inflammatory responses of autoimmune diseases. Upregulation of TLR2 contributes to immune reactivity and aggravates the inflammatory response [19]. TLR2 pathway displays a strong proinflammation action in aorta. TLR2 deficiency will decrease the concentrations of proinflammatory cytokines, whereas anti-inflammatory interleukin 10 (IL-10) was elevated [27, 28]. In atherosclerosis, TLR2 was involved in the process of inflammation and matrix degradation. Recently, activation of the TLR2 pathway has also been confirmed accelerating AAA formation [29], and a series of reactions coinciding with the crucial pattern of how the AAAs generate proinflammatory and MMP secretion followed. However, blocking TLR2 decreased the expression of endogenous ligands interacting with TLR2, and consecutively decreased chronic inflammation, activity of MMP2/9, and vascular remodeling of AAA [30]. Compared with their inhibitors of MMPs and anti-inflammatory agents, TLR2 blocking may provide a new therapeutic method in AAA treatment.

2.1.2. TLR4. TLR4 is recognized as a vital traffic joint in AAA progression in recent years [31–35]. TLR4 can promote AAA formation directly by upregulating the expression of MMP-2 and MMP-9. In the indirect pathway, TLR4 induces the progression of AAA by elevating proinflammatory chemokine like IL-6 and MCP-1, proinflammatory cells like M1 macrophages, and the c-Jun NH2-terminal protein kinase (JNK) pathway [36–38]. JNK-signaling pathway can regulate inflammatory responses and is mainly activated by a series of phosphorylation [39]. TLR4 is an upstream of the JNK-promoting pathway [33, 38, 40]. The JNK pathway enhances MCP-1 expression and inflammatory cell infiltration [41]. Our investigation and other groups show that blockade of this pathway by its inhibitor, SP600125 or curcumin, can inhibit secretion of MCP-1, MCP-2, and MMP-9, thereby attenuating aortic aneurysm formation [42–44]. Shang et al. found MyD88-dependent TLR4 pathway participants in AAA progression. In this experiment, tanshinone IIA significantly decreased the overexpression of TLR-4, MyD88, phosphorylated nuclear factor κB (pNF-κB), and phosphorylated IκBα (pIκBα) in AAA induced by elastase perfusion [32]. Nevertheless, Owens et al. report that MyD88 slows down AAA formation independent of signal from TLR4 or TLR2. Given the critical roles of MyD88 and TLR4 in AAA, bone marrow transplantation is performed to determine whether the effect of MyD88 or TLR4 deficiency on AngII-induced AAA is mediated by cells of the hematopoietic lineage. MyD88 deficiency in bone marrow-derived cells profoundly reduces AngII-induced AAA. However, TLR4 deficiency in bone marrow-derived cells has no effect on AAA [45]. The difference appears probably due to the different inducer for the AAA model in use. In the future, a more detailed upstream and downstream of the TLR4 in AAA are needed to explore.

2.1.3. TLR3. TLR2 and TLR4 have been shown to significantly induce atherosclerotic lesion and AAA by promoting macrophage recruitment and expression of inflammatory factors. However, TLR3 deficiency has no effect on aortic cytokine/chemokine expression [24, 46]. Ishibashi et al. discover that matrix-degrading action of TLR3 is partly mediated by modulation macrophage MMP-2 and -9 activities. The study highlighted that TLR3 signaling may increase MMP-2 activity by the p38/MAPK pathway [46]. However, collagen type I as an important structural component of plaque caps was not studied. In the future study, the role of TLR3 on collagen type I degradation should be investigated.

2.2. Chemokine Receptors. Chemokines are critical for the function of the innate immune, which own the ability to induce chemotaxis of immune cells after activation of the innate immune system [47]. According to the chemokine subclass, chemokine receptors, a large family of G protein-coupled receptors (GPCRs) [48], are classified into CR, CCR, CXCR, and CX_3CR with a large variety of distribution and function in AAA [49]. Chemokines start a series of inflammatory reaction in AAAs. CXCR4 is believed to contribute to the AAA formation. When the receptor is blocked, the progression of AAA is attenuated [50, 51].

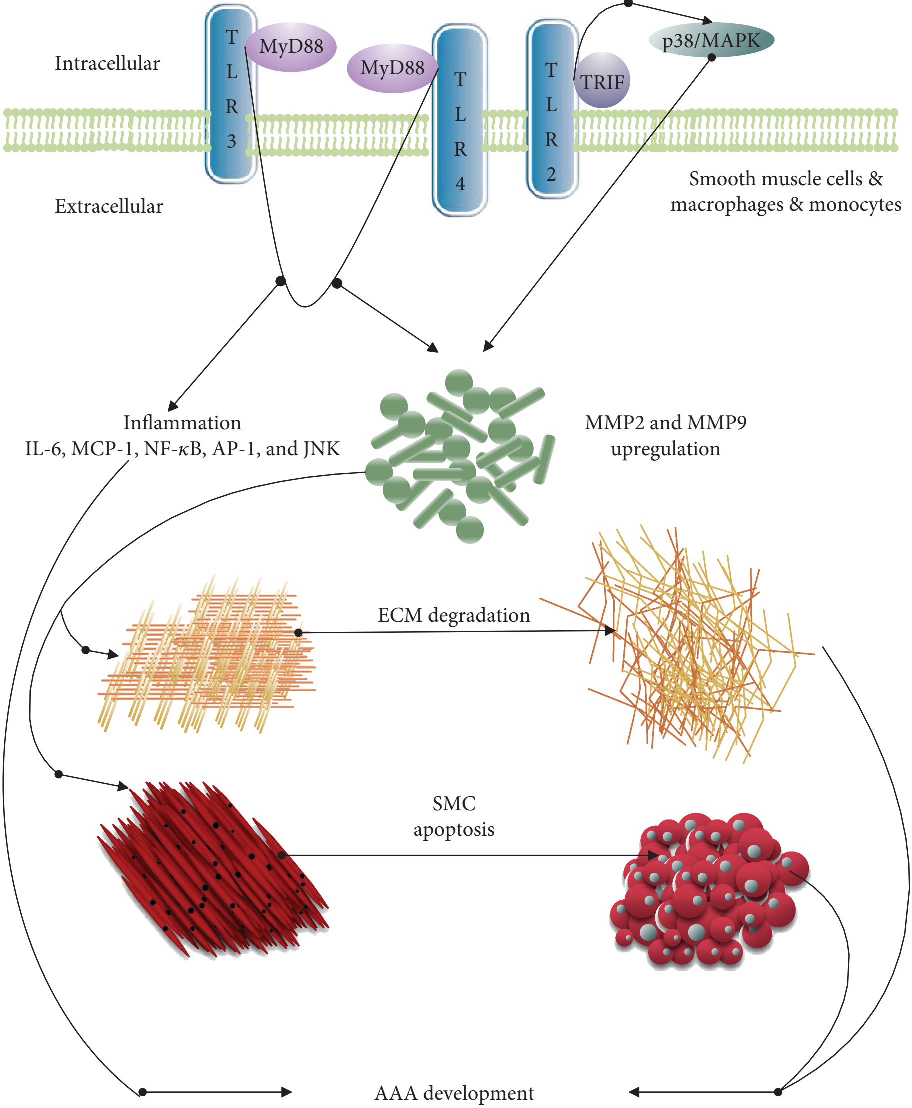

FIGURE 1: Possible mechanisms of TLRs in promotion of AAA development. The schematic diagram shows that TLR2 and TLR4 promote inflammation and MMP expression, and TLR3 promotes MMP expression in the aortic wall during aneurysm development.

However, increased expression of CXCR4 in bone mesenchymal stem cells (BMSCs) might improve the treatment of AAA. Further studies are required to clear the detailed mechanism [52]. In TAA patients, a high concentration of CXCR3 ligand chemokines is detected in plasma. CXCR3 are the proinflammation chemokine receptor in AAA by attracting CD45-positive cell infiltration [53, 54]. Blockade of C-X-C motif ligand 1 (CXCL1) receptor, CXCR2, will attenuate tissue damage through inhibition of neutrophil recruitment [55, 56]. In aortas, CXCR2-neutralizing antibody obviously prevented the expansion and rupture of the dissected aorta by preventing neutrophil infiltration and reducing IL-6 expression [57]. As to another important chemokine receptor, CCR2, activation of CCR2 mediates the inflammation in AAA [58, 59] and this may be achieved by attracting mast cells to the tissue. One of its ligands, CCL2 (MCP-1), plays a crucial role in macrophage chemotaxis [60].

2.3. Complement. Complement factors are the major proinflammatory components of the innate immune system. Although as one of the nonclassical complement pathway, the complement alternative pathway contributes a lot to the AAA formation. C3 deposition is recognized as a cause of the subsequent reactions in AAA [61]. Zhou et al. discovered that IgG antibodies in plasma were able to activate the complement alternative pathway by inducing C3 deposition in AAA [62]. In their later study, they find that the IgG antibodies binding to fibrinogen can lead to AAA formation by activating the complement lectin pathway [63]. Another lectin pathway activator, ficolin-3, was also demonstrated to contribute to AAAs [64]. This may provide the evidence that the C3-inducing AAAs are not specific, and some proper anti-C3 drugs may work in attenuating the development of AAAs. Another complement component C4d, however, shows a protective role in some inflammatory aortic disease such as aortic dissection [65].

3. Immune Cell Infiltration in AAA

3.1. Macrophages. Macrophages play an important role in the innate and adaptive immune responses. Macrophage infiltrating into aortic tissue and secreting matrix degradable substance directly contributes to AAA formation [8, 66, 67]. Macrophages may recruit to the AAA area through an "outside-in" pattern which means infiltration initiates from the adventitia [68].

Macrophages have a great number of subtypes in which M1 phenotype and M2 phenotype play a major role in AAA. The M1/M2 ratio imbalance can promote the AAA development. M1 macrophages are proinflammatory, while M2 macrophages are anti-inflammatory [69–71]. M2 macrophages may achieve the anti-inflammation effect by release of IL-10 and profibrotic factors such as TGF-beta [72]. The protective effect of TGF-beta also involves a critical role in the control of excessive monocyte/macrophage activation, as monocyte depletion inhibits AAA formation [73]. M1 or M2 macrophage polarization plays an important effect in regulating chronic inflammatory process. The infiltrating M2 macrophage will convert to M1 macrophage and vice versa in certain circumstances [74]. CD4(+)CD25(+)Treg cells play a key role in the macrophage-to-M2 switching [75, 76]. So, intervention of preventing M2 to M1 transition or promoting macrophage switching into M2 may help a lot in AAA treatment.

3.1.1. Cytokines Modulating Macrophage Infiltration. Granulocyte-macrophage colony-stimulating factor (GM-CSF) was once reported being able to change mesothelial cells into macrophage-like cells by an autocrine pattern [77]. GM-CSF gene expression was also associated with macrophage densities in the arterial wall [78]. Constantly increasing secretion of GM-CSF may trigger aortic aneurysm [79]. The study shows that GM-CSF is the key regulator of AAA. If the GM-CSF pathway is blocked, macrophage infiltration and MMP-9 secretion will decrease [80]. CD4+ T cells secrete more GM-CSF in smad3−/− mice compared with WT mice. Deficiency of smad3 in genes contributes to aortic aneurysm maybe through GM-CSF pathway [81].

MCP-1 (monocyte chemotactic protein-1), also called CCL-2, is a kind of c-c chemokine secreted mainly by inflammatory cells and endothelial cells [82]. It is positively correlated with macrophage infiltration into aortic walls and acts as a promoter of AAA formation and development [78, 83, 84]. Usually upregulation of MCP-1 occurs early than the chronic inflammatory responses [85]. Our previous study found that MCP-1 was involved in aortic aneurysm development. MCP-1 secreted by SMCs could promote AAA progression by enhancing MMP-9 production [86, 87]. The apoptotic SMCs attract monocytes and other leukocytes by producing MCP-1. However, MCP-1-primed macrophages will further elicit aortic SMC apoptosis [88, 89]. MCP-1-promoted AAA may also be achieved by enhancing macrophage infiltration and cytotoxicity as well as promoting SMC phenotype transformation and apoptosis [85, 90, 91]. IL-6 is a proinflammatory cytokine that can contribute to SMC apoptosis and modulate extracellular matrix by MMP enhancement [92, 93]. There is a regulatory loop between IL-6 and MCP-1. Recent study shows that IL-6 can promote macrophages secreting MCP-1 and, in turn, MCP-1 has a positive feedback on IL-6 through the p38 pathway [93]. In the AngII-induced mouse model, IL-6 and MCP-1 are upregulated. Lacking either IL-6 or MCP-1 receptor CCR2 will reduce the early onset of aortic dissections. The enhancement of MCP-1 and IL-6 can promote macrophage secreting CD14 and CD11b, which in turn can induce MCP-1 and MMP-9 expression [94]. At the same time, researchers identify that CD14 plays the crucial role in promoting the macrophage precursor recruitment in early AAA walls [67].

3.2. T Cells

3.2.1. Th Cells. CD4+ Th cells include two main types, Th1 cells and Th2 cells. Th1 cells mainly secrete cytokines including IL-2 and INF-γ, while Th2-characteristic cytokines include IL-4 and IL-5 [95, 96]. Both types of cells regulate each other by these cytokines, and the Th1/Th2 ratio is dependent on the environment and inflammatory response [96]. Both Th1 cells and Th2 cells can contribute to vascular inflammation [97]. In most cases, Th1 cells play an anti-inflammatory role and Th2 cells play a proinflammatory role [98, 99]. A high ratio of Th2 cells in AAA was observed, and a dysfunctional IL-4 expression will reduce AAA formation [100, 101]. Th2 polarizing induced by CD19 treatment in mice alleviates the macrophage infiltration and vascular inflammation [102]. INF-γ secreted by Th1 cells may attenuate AAA formation and development [103]. Blockade of the INF-γ pathway will lead to sequential severe AAA formation with increased expression of MMP-2 and MMP-9 [100]. Cytokines of Th2 cells may promote collagenolytic and elastolytic activation while Th1-characteristic cytokine reduces MMP expression. On the contrary, Galle et al. find that Th1 cells are the prominent in fresh T cells isolated from AAA tissue with high expression of INF-γ, which suggests that INF-γ contributes to AAA formation [104]. Another study also demonstrates that INF-γ deletion attenuates MMP expression and inhibits aneurysm development. The attenuation function of INF-γ may come from other coacting signaling in Th1 cells [105]. The differences are potentially attributable to different animal models studied. The INF-γ effect on the development of AAA should be further explored in the same animal models, disease stage, and anatomical areas in the future study. The regulation of Th cell polarization will be an investigation direction in AAA treatment.

3.2.2. Regulatory T (Treg) Cells. Treg cells as the T cell subpopulation are engaged in sustaining immunological self-tolerance and homeostasis, which are essential for preventing autoimmune diseases and limiting chronic inflammatory diseases [106]. The transcription factor Foxp3 helps Treg cells complete the specification to control immune responses. Foxp3(+) Tregs may prevent AAA formation by inhibiting local inflammation in the aortic wall. Genetic depletion of Foxp3(+) Tregs significantly increases the mortality of AAA [107]. It is always thought that functional Treg cells limit AAA development by secreting the inhibitory

cytokines [108–110], such as IL-10, which plays key effect in reduction of cell death, inhibition of vascular smooth muscle cell proliferation, inhibition of macrophage function, and reduction in inflammatory cell recruitment. Cytokine IL-10 secreted by the Treg cells acts in the pathogenesis of AAA and suppresses inflammatory response [111–113]. TGF-beta, secreted by Treg cells, is confirmed that it can protect AAA progression [73]. Genetic variation of the TGF-beta pathway leads to AAA development and contributes to multiple syndromic presentations of aortic aneurysm [114, 115]. In contrast, the recent study also reports that deficiency of TGF-beta signal prevents AAA formation [116]. To understand the concreted mechanism, the studies should be to investigate the TGF-beta isoform involved in AAA formation and delete it in a cell-specific manner in mice.

Some cytokines can act back on Treg cells and regulate their function. A recent study finds that impaired secretion of TGF-beta results in number loss of Treg cells. Once monocytes and B cells have an impaired capacity in inducing Foxp3 upregulation of Treg cells, exogenous TGF-beta can rescue the function [117]. Flores-García et al. also find that Treg cells have an immunosuppressive activity on CD4+ T cell-dependent TGF-beta [118]. By releasing IL-10, IL-10-producing B cells are able to enhance Treg cell function and convert T effector cells into Treg cells [119]. Other cytokines such as IL-33, in collaboration with IgE, can also stimulate expansion of Treg cells [120, 121].

Recently, Balmert et al. have already succeeded in prohibiting allergic contact dermatitis by Treg induction. They use degradable microparticles containing TGF-beta, IL-2, and rapamycin to sustain a microenvironment to promote Treg cell differentiation [122]. The above evidence shows that the enhancing expansion and differentiation of Treg cells stimulated by cytokines may be a new therapeutic goal for AAA.

3.2.3. CD8+ T Cells. CD8+ T cells are important in cell-mediated toxicity. Cytotoxic CD8+ T cells have been implicated in targeting vascular endothelial and smooth muscle cells [123]. Yet the study on their role in AAA is few. In one study, CD8+ T deficiency significantly promotes elastase-induced AAA formation [124]. Another report shows that modulation of the function CD8+ T cells through reducing macrophage infiltration and Th17 cell polarization can attenuate the AAA induced by AngII.

3.3. B Cells. B cells can be divided into two developmentally distinct groups, B1 and B2 cells. B1 cells play crucial roles in the process of innate immunity, while B2 cells are the conventional players in adaptive humoral immunity [125]. In AAA, IgM, IgG, and C3c deposits are detected in the fibrous zone, which indicates that pathogenic B cell response is involved in the pathogenesis of AAA [126]. B cells in AAA are mainly specifically recruited to the adventitia of the aortic wall after stimulation [127]. B2 cells are the largest constituent of B cells in mouse AAA [128]. In atherosclerosis, B1 cells are protective via production of natural antibodies IgM, whereas B2 cells are proatherogenic via activation/proliferation of T cells. The recent study found that B cell deficiency could increase Treg cell infiltration in AAA tissue and inhibit AAA formation. In their study after anti-CD20 treatment, both wild type and apolipoprotein in E-knockout mouse model appear significant B1 and B2 depletion. Sequentially, higher number of dendritic cells appeared in aortas. Treg cell number is increased, but proinflammation genes are downregulated [128]. Another study also supports this result, which demonstrates that angiotensin II mobilizes monocytes from spleen to aorta in a B cell-dependent manner and promotes AAA formation in the apolipoprotein E KO mice [129]. However, one group finds that B2 cells from spleen of 8- to 10-week-old wild-type mice could suppress experimental aortic aneurysm of muMT mice by upregulating Treg cells and decreasing the number of aortic-infiltrating mononuclear cells [130]. It is possible that B2 cell transplantation might produce the protective antibodies. The differences of above results are needed to further explore the paradox immune response in muMT and anti-CD20 antibody-mediated B cell depleted mice.

3.4. NK Cells. Natural killer cells have shown the role in the development of chronic inflammatory responses. Apart from macrophages, T cells, and B cells, NK cells were significantly increased in the peripheral blood in AAA patients, which resulted in the increasing of cytotoxic activity and contributing the AAA formation [10]. NK cells can produce the proinflammatory cytokines such as IL-2 and INF-γ [131, 132]. Evidence indicates that the NK pathway is activated in AAA. One study shows that TNFα level is increased in AAA patients, and T cells isolated from AAA patients produce more TNFα [133, 134]. Recently, the protein expression of the NK cytotoxic signaling pathway is identified. In AAA tissues, two important NK pathway proteins (HCST and GRZB) are found expressed in CD8+ T cell and macrophage that participating in this pathway [135]. However, the exact role of NK cells in AAAs is still unclear.

3.5. Mast Cells. Mast cells are implicated in a number of inflammatory diseases through releasing of inflammatory mediators, serglycin and other proteoglycans, and proteases [136]. In human and animal AAA, the mast cells have been identified [9]. Interventions of mast cells such as tryptase deficiency, chymase deficiency, and mast cell functional substance antagonists attenuated the formation of AAA [137, 138]. In a recent human AORTA trial, three doses of the mast cell inhibitor pemirolast are given to 326 patients and AAA growth is monitored over 12 months; the result demonstrates that AAA growth rates are similar in patients receiving placebo and different doses of pemirolast, which concludes that pemirolast cannot retard the growth of medium-sized AAAs [139]. The effect of mast cells in the AAA and the validity of mast cell inhibition used to develop effective medications for AAA need to be cleared.

3.6. Neutrophils. Neutrophils have already been recognized as one of the initial contributors in AAA formation [11] via secreting some particular ECM-degrading enzymes such as neutrophil collagenase (MMP-8) and neutrophil protease [140, 141]. In adventitia neutrophil recruitment and

Table 1: Treatment effects of different immune therapeutic targets on the AAA progression.

Target	Treatment effect	Agent	Model	Reference
Immune system	Decrease aortic dilatation	Immunosuppressive agents	Elastase-induced rat aneurysm	[18]
TLR2	Decrease chronic inflammation, vascular remodeling and AAA formation	TLR2-neutralizing mAb	AngII-induced mouse aneurysm	[30]
TLR4	Repress aneurysm recurrence	Alginate oligosaccharide	Aneurysm patients	[31]
TLR4/MyD88	Attenuates AAA formation	Tanshinone IIA	Elastase-induced rat aneurysm	[32]
TLR4/JNK	Inhibit experimental AAA development	Rosiglitazone	AngII-induced mouse aneurysm	[33]
CXCR4	Suppress AAA formation and progression	AMD3100	$CaCl_2$-induced mouse aneurysm	[50]
CCR2 monocytes	Decrease aortic dilatation	Everolimus	Angiotensin II- (A2-) infused apolipoprotein E-deficient mouse	[58]
Complement alternative pathway	Prevent aneurysm formation	Properdin-free AP C3 convertase	Elastase-induced mouse aneurysm	[62]
M1/M2 macrophages polarization	Inhibit AAA formation	D-series resolvins	Elastase-induced mouse aneurysm	[70]
Foxp3(+) Tregs	Decrease incidence (52%) and mortality (17%) of AAA	Interleukin-2 complex	Apolipoprotein E-deficient mice fed a high-cholesterol diet with angiotensin II	[110]
B cells	Prevent experimental AAA formation	Anti-CD20 antibody	Elastase perfusion or angiotensin II infusion apolipoprotein E-knockout mouse	[128]
Mast cells	No difference with the placebo group	Pemirolast	Medium-sized AAA patient	[139]
Neutrophils	Inhibit experimental AAA formation	Antineutrophil antibody	Elastase-induced mouse aneurysm	[11]
NETs	Attenuate AAA formation	Cl-amidine, an inhibitor NET formation	Elastase-induced mouse aneurysm	[146]

activation, neutrophil-derived IL-6 enhances the adventitial inflammation that leads to aortic rupture [57]. Recent studies have detected an elevated level of neutrophil gelatinase-associated lipocalin (NGAL), a protein expressed by polymorphonuclear neutrophil which is considered an activated form of neutrophil [142]. NGAL is also a potential indicator for evaluation in aortic aneurysm repair [143]. Further studies are needed to understand the relationship between the NGAL level and AAA presence and growth.

Neutrophil extracellular traps (NETs) are originally identified as an innate immune response to bacterial infection [144]. In human AAA, neutrophil activation is also associated with NET formation in the intraluminal thrombus (ILT) [145]. IL-1β-induced NET formation promotes the development of AAA [146]. Neutrophil protease-mediated NET release contributes to elastase-induced AAA through plasmacytoid dendritic cell activation and type I interferon production [141].

4. ILT in AAA

In about 75% of clinically relevant AAA patients, the aneurysm lumen wall is covered by ILT [147]. ILT is a complex fibrin network and contains inflammatory cells, chemokines, and proinflammatory cytokines as well as ECM constituents [148–151]. ILT has been shown to be related with aortic wall weakening and a higher level of immunoinflammation in the AAA [152]. The volume of ILT is associated strongly with AAA size and growth in patients [153]. Recent studies demonstrate that the proinflammatory cytokines, reactive oxygen species, and proteases in the thrombus play a significant role in the development of human AAA [150, 151, 154]. In aortic aneurysms induced by AngII in the ApoE−/− mouse, the thrombus within the aortic wall is often observed [155]. The blood-ILT interface releases biological mediators which will activate the platelets and the coagulation cascade [151]. Anticoagulants fondaparinux treatment can reduce intramural thrombus formation, inflammation, and growth of experimental aortic aneurysm in the mouse model [154].

5. Immunoglobulins

B cells (and/or dendritic cells) present that antigen to T cells, activated T cells, and B cells interacts to promote the activation, proliferation, and differentiation of B cells. After activation, B cells in the germinal centers experience class switching and affinity maturation to become plasma cells that secrete large amounts of highly specific antibodies.

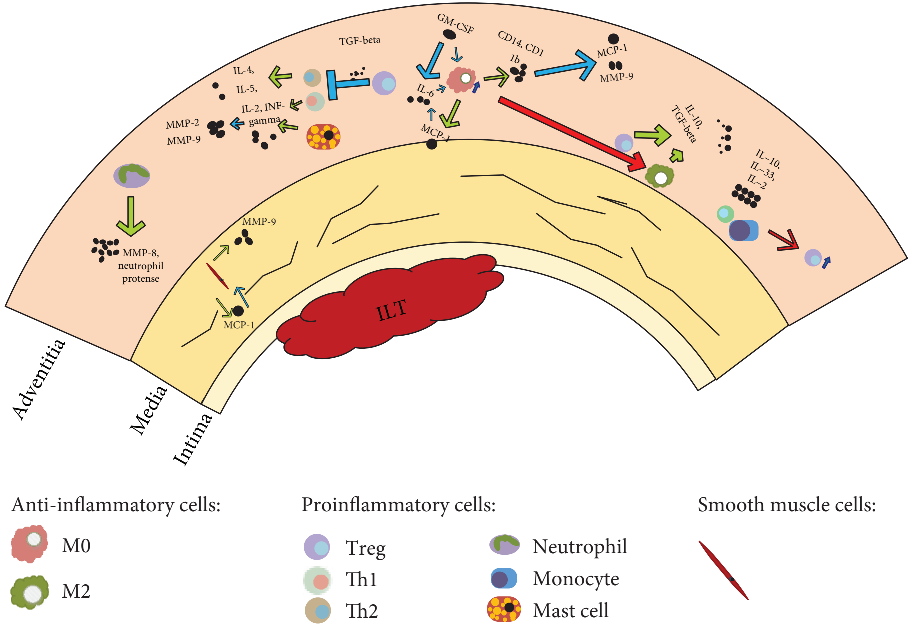

Figure 2: The roles of immune cells in the formation of AAA. The wall of AAA consists of plenty of inflammatory cell infiltration. Macrophages can be divided into two major phenotypes, M1 and M2 cells. M1 macrophages are proinflammatory. M2 macrophages are anti-inflammatory. CD4+ Th cells include two major types, Th1 and Th2 cells. Th1 cells mainly secrete cytokines including IL-2 and INF-γ, while Th2-characteristic cytokines includes IL-4 and IL-5. Neutrophils secrete collagenase (MMP-8) and neutrophil protease to degrade ECM. Mast cells produce cytokines, chemokines, and proteases, which further activate MMPs. The inflammatory cells release inflammatory mediators, which will result in breakdown of medial elastin and smooth muscle cell (SMC) apoptosis. Most aneurysms are covered by an intraluminal thrombus (ILT), and the presence of blood in the ILT is associated with AAA rupture.

5.1. IgG. IgG4 is the least abundant subclass among the IgG antibodies in the human body, but its level is associated with a series of vasculitis syndrome [156, 157]. One of the most common manifestations is inflammatory aortic aneurysm (IAA). Researchers find IgG4-positive plasma cell infiltration in the aortic wall of IgG4-related AAA [158–160]. The high level of serum IgG4 contributes to the aortic dilation [161]. IgG4-related AAA is one symptom of IgG4-related disease (IgG4-RD), a systemic inflammatory disease with a high level of serum IgG4, instead of an isolated disorder [162, 163]. Compared with the non-IgG4-related AAA, IAA shows a significant increase in the number of infiltrating IgG4-positive cells and the incidence of a disrupted follicular dendritic cell network in lymph follicles [164, 165], which indicates that IAA is not a simple inflammatory aorta. In general, patients with IgG4-related inflammatory aortic aneurysm have an allergic constitution [166]. Vasculitis caused by a high serum level of IgG4 is always treatable [158]. B cell depletion therapy acts well on IgG4-related AAA [128]. However, steroid therapy did not work well [167].

5.2. IgE. According to previous studies, high IgE concentration tends to promote coronary atherosclerosis and dilation [168–171]. Generally, it is always considered that IgE participates in artery inflammatory disease mainly through activating mast cells. However, a recent study shows that IgE participates the aortic aneurysm formation by acting on not only mast cells but also on CD4+ T cells and macrophages [9]. IgE induces CD4+ T cell production of IL-6 and IFN-γ but reduces the production of IL-10 [9]. This process may be similar to pulmonary inflammatory disease such as asthma [172–174].

5.3. IgM. In 2014, Villar et al. have found an intimate positive correlation between IgM and inflammatory disease [175]. Diepenhorst et al. also detected a promoting role of IgM antibodies in inflammation in acute myocardial infarction (AMI) patients [176]. In secreted IgM deficiency mouse model, atherosclerosis was facilitated by IgE [170]. However, natural IgM antibodies produced by B1 cells show a protective role in atherosclerosis and artery remodeling [177, 178]. IgM was also detected in the adventitia of AAA [126]. But its role in AAA formation and development is still unknown.

6. Immune Regulation Application

The outcome of targeting the inflammatory cells, innate immune system, and immunoglobulins in AAA has been reviewed (Table 1). Understanding and developing new strategies that regulate immunity will provide useful therapeutic targets for AAA.

Lots of studies have shown inflammatory cell infiltration in the AAA. The prominent of the cell types, such as macrophages and T cells, plays a significant role in the progression of AAA. Intervention of preventing M2 to M1 transition or promoting macrophage switching into M2 may help a lot

in AAA treatment. About T cells, regulation of Th cell polarization (Th1/Th2 ratio) can be an investigation direction in AAA treatment. Another kind of T cell, Treg cells have the anti-inflammation ability. The investigations provide data which are beneficial to the treatment of AAA. Enhancing expansion and differentiation of Treg cells stimulated by cytokines may be a new therapeutic method for AAA (Figure 2).

In normal physiological conditions, B cells play crucial roles in innate immunity and humoral immunity. Under pathological environments, T cells are activated and then stimulate B cells to produce the diseased antibodies in response to stimulations. The diseased antibodies take effect in the inflammatory process [179]. Recent studies refer that the B cells were involved in the AAA. Removing B cells prevents the development of AAA. However, delivery of B2 cells from the young wild-type mice to the AAA mice increases the number of Treg cells and also inhibits the formation of AAA. It seems that the results are paradoxical. B cell function in AAA might be impaired. The immunoglobulins secreted by B cells are pathological and lost the normal function. It is possible that B2 cell transplantation might produce the healthy protective antibodies. In the future studies, the B cell number and immunoglobulins should be monitored and analyzed in the development of AAA. Although the related experiments on B cell effect in AAA are few, modulation of B cells might bring a new field for AAA treatment.

Immunoglobulins in blood own an extensive variety of recognizing ligands and functions. They can greatly affect the functions and differentiation of immune cells [180–182]. A high level of serum IgG4 contributes to the aortic dilation. B cell depletion therapy will be a good method to treat the IgG4-related AAA. Intravenously applied normal polyclonal immunoglobulins (IVIg) have great therapeutic applications in the treatment of autoimmune, infectious, and inflammatory diseases [183]. Immunoglobulins can hopefully be a new therapy target in these aortic inflammation diseases. Taking good advantage of the effect among immunoglobulins in the immunologic therapy can be another task.

Innate immune system such as Toll-like receptors (TLRs), chemokine receptors and complements are recently shown to regulate immunological processes leading to the formation and progression of AAAs as well as to other cardiovascular pathologies. Most recent work highlights the significance of TLRs in AAA development. TLR2 and TLR4 promote the inflammation and matrix degradation by upregulation of MMP expression in AAA. Blockage of TLRs may serve as a potential therapeutic strategy for AAA.

7. Conclusions

As the previous study demonstrates, inflammation plays a vital role in AAA formation, development, and progression. The immune system also participates in regulation control of the AAA pathological process and has a profound effect on the AAA-related inflammatory reactions. Therefore, it is very important to understand the immune-inflammatory responses in abdominal aortic aneurysm and search the potential molecular targets in AAA. Although a good deal of strategies has been proposed, the clinical practicability is still lack of testing. The validity requires further clinical validation.

Abbreviations

AAA: Abdominal aortic aneurysm
MMPs: Matrix metalloproteinases
ECM: Extracellular matrix
TLRs: Toll-like receptors
PRRs: Pattern recognition receptors
MCP-1: Monocyte chemotactic protein-1
IL-10: Interleukin 10
IL-6: Interleukin 6
JNK: c-Jun NH2-terminal protein kinase
GM-CSF: Granulocyte-macrophage colony-stimulating factor
IRF: Interferon-regulatory factor
STAT: Signal transducer and activator of transcription
MyD88: Myeloid differentiation primary response gene-88
SMC: Smooth muscle cell
NGAL: Neutrophil gelatinase-associated lipocalin
AMI: Acute myocardial infarction
TNF-α: Tumor necrosis factor α
NK: Natural killer
CXCL1: C-X-C motif ligand 1
GPCRs: G protein-coupled receptors
Treg: Regulatory T cell
TGF-beta: Transforming growth factor-β
IFN-γ: Interferon-γ
IVIg: Intravenously applied normal polyclonal immunoglobulins
NETs: Neutrophil extracellular traps
ILT: Intraluminal thrombus.

Conflicts of Interest

The authors declare that they have no conflict of interests.

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References

[1] B. J. Toghill, A. Saratzis, and M. J. Bown, "Abdominal aortic aneurysm—an independent disease to atherosclerosis?," *Cardiovascular Pathology*, vol. 27, pp. 71–75, 2017.

[2] H. W. Kim and B. K. Stansfield, "Genetic and epigenetic regulation of aortic aneurysms," *BioMed Research International*, vol. 2017, Article ID 7268521, 12 pages, 2017.

[3] A. Ghosh, L. V. T. A. Pechota, G. R. Upchurch, and J. L. Eliason, "Cross-talk between macrophages, smooth muscle

cells, and endothelial cells in response to cigarette smoke: the effects on MMP2 and 9,” *Molecular and Cellular Biochemistry*, vol. 410, no. 1-2, pp. 75–84, 2015.

[4] K. W. Johnston, R. B. Rutherford, M. D. Tilson, D. M. Shah, L. Hollier, and J. C. Stanley, “Suggested standards for reporting on arterial aneurysms,” *Journal of Vascular Surgery*, vol. 13, no. 3, pp. 452–458, 1991.

[5] E. E. Joviliano, M. S. Ribeiro, and E. J. R. Tenorio, “MicroRNAs and current concepts on the pathogenesis of abdominal aortic aneurysm,” *Brazilian Journal of Cardiovascular Surgery*, vol. 32, no. 3, pp. 215–224, 2017.

[6] J. Golledge, J. Muller, A. Daugherty, and P. Norman, “Abdominal aortic aneurysm: pathogenesis and implications for management,” *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 26, no. 12, pp. 2605–2613, 2006.

[7] Y. W. Yan, J. Fan, S. L. Bai, W. J. Hou, X. Li, and H. Tong, “Zinc prevents abdominal aortic aneurysm formation by induction of A20-mediated suppression of NF-κB pathway,” *PLoS One*, vol. 11, no. 2, article e0148536, 2016.

[8] D. L. Rateri, D. A. Howatt, J. J. Moorleghen, R. Charnigo, L. A. Cassis, and A. Daugherty, “Prolonged infusion of angiotensin II in $apoE^{-/-}$ mice promotes macrophage recruitment with continued expansion of abdominal aortic aneurysm,” *American Journal of Pathology*, vol. 179, no. 3, pp. 1542–1548, 2011.

[9] J. Wang, J. S. Lindholt, G. K. Sukhova et al., “IgE actions on $CD4^+$ T cells, mast cells, and macrophages participate in the pathogenesis of experimental abdominal aortic aneurysms,” *EMBO Molecular Medicine*, vol. 6, no. 7, pp. 952–969, 2014.

[10] N. D. Forester, S. M. Cruickshank, D. J. A. Scott, and S. R. Carding, “Increased natural killer cell activity in patients with an abdominal aortic aneurysm,” *British Journal of Surgery*, vol. 93, no. 1, pp. 46–54, 2006.

[11] J. L. Eliason, K. K. Hannawa, G. Ailawadi et al., “Neutrophil depletion inhibits experimental abdominal aortic aneurysm formation,” *Circulation*, vol. 112, no. 2, pp. 232–240, 2005.

[12] G. M. Longo, W. Xiong, T. C. Greiner, Y. Zhao, N. Fiotti, and B. T. Baxter, “Matrix metalloproteinases 2 and 9 work in concert to produce aortic aneurysms,” *Journal of Clinical Investigation*, vol. 110, no. 5, pp. 625–632, 2002.

[13] G. M. Longo, S. J. Buda, N. Fiotta et al., “MMP-12 has a role in abdominal aortic aneurysms in mice,” *Surgery*, vol. 137, no. 4, pp. 457–462, 2005.

[14] R. J. Rizzo, W. J. McCarthy, S. N. Dixit et al., “Collagen types and matrix protein content in human abdominal aortic aneurysms,” *Journal of Vascular Surgery*, vol. 10, no. 4, pp. 365–373, 1989.

[15] W. B. Keeling, P. A. Armstrong, P. A. Stone, D. F. Bandyk, and M. L. Shames, “An overview of matrix metalloproteinases in the pathogenesis and treatment of abdominal aortic aneurysms,” *Vascular and Endovascular Surgery*, vol. 39, no. 6, pp. 457–464, 2005.

[16] E. M. Culav, C. H. Clark, and M. J. Merrilees, “Connective tissues: matrix composition and its relevance to physical therapy,” *Physical Therapy*, vol. 79, no. 3, pp. 308–319, 1999.

[17] N. Sakalihasan, P. Delvenne, B. V. Nusgens, R. Limet, and C. M. Lapière, “Activated forms of MMP2 and MMP9 in abdominal aortic aneurysms,” *Journal of Vascular Surgery*, vol. 24, no. 1, pp. 127–133, 1996.

[18] T. Yamaguchi, M. Yokokawa, M. Suzuki et al., “The effect of immunosuppression on aortic dilatation in a rat aneurysm model,” *Surgery Today*, vol. 30, no. 12, pp. 1093–1099, 2000.

[19] Y. Liu, J. Liao, M. Zhao et al., “Increased expression of TLR2 in $CD4^+$ T cells from SLE patients enhances immune reactivity and promotes IL-17 expression through histone modifications,” *European Journal of Immunology*, vol. 45, no. 9, pp. 2683–2693, 2015.

[20] V. P. Jagadesham, D. J. A. Scott, and S. R. Carding, “Abdominal aortic aneurysms: an autoimmune disease?,” *Trends in Molecular Medicine*, vol. 14, no. 12, pp. 522–529, 2008.

[21] G. Sellge and T. A. Kufer, “PRR-signaling pathways: learning from microbial tactics,” *Seminars in Immunology*, vol. 27, no. 2, pp. 75–84, 2015.

[22] S. Hontelez, A. Sanecka, M. G. Netea, A. B. van Spriel, and G. J. Adema, “Molecular view on PRR cross-talk in antifungal immunity,” *Cellular Microbiology*, vol. 14, no. 4, pp. 467–474, 2012.

[23] O. Pryshchep, W. Ma-Krupa, B. R. Younge, J. J. Goronzy, and C. M. Weyand, “Vessel-specific Toll-like receptor profiles in human medium and large arteries,” *Circulation*, vol. 118, no. 12, pp. 1276–1284, 2008.

[24] M. R. Richards, A. S. Black, D. J. Bonnet et al., “The *LPS2* mutation in TRIF is atheroprotective in hyperlipidemic low density lipoprotein receptor knockout mice,” *Innate Immunity*, vol. 19, no. 1, pp. 20–29, 2013.

[25] J. Xu and U. Sachdev, “The Toll of vascular insufficiency: implications for the management of peripheral arterial disease,” *Journal of Immunology Research*, vol. 2016, Article ID 8249015, 9 pages, 2016.

[26] B. Schreiner, J. Voss, J. Wischhusen et al., “Expression of toll-like receptors by human muscle cells in vitro and in vivo: TLR3 is highly expressed in inflammatory and HIV myopathies, mediates IL-8 release and up-regulation of NKG2D-ligands,” *The FASEB Journal*, vol. 20, no. 1, pp. 118–120, 2006.

[27] A. Koch, M. Pernow, C. Barthuber, J. Mersmann, K. Zacharowski, and D. Grotemeyer, “Systemic inflammation after aortic cross clamping is influenced by Toll-like receptor 2 preconditioning and deficiency,” *The Journal of Surgical Research*, vol. 178, no. 2, pp. 833–841, 2012.

[28] S. Kuwahata, S. Fujita, K. Orihara et al., “High expression level of Toll-like receptor 2 on monocytes is an important risk factor for arteriosclerotic disease,” *Atherosclerosis*, vol. 209, no. 1, pp. 248–254, 2010.

[29] N. Aoyama, J. I. Suzuki, M. Ogawa et al., “Toll-like receptor-2 plays a fundamental role in periodontal bacteria-accelerated abdominal aortic aneurysms,” *Circulation Journal*, vol. 77, no. 6, pp. 1565–1573, 2013.

[30] H. Yan, B. Cui, X. Zhang et al., “Antagonism of toll-like receptor 2 attenuates the formation and progression of abdominal aortic aneurysm,” *Acta Pharmaceutica Sinica B*, vol. 5, no. 3, pp. 176–187, 2015.

[31] Y. Yang, Z. Ma, G. Yang et al., “Alginate oligosaccharide indirectly affects toll-like receptor signaling via the inhibition of microRNA-29b in aneurysm patients after endovascular aortic repair,” *Drug Design, Development and Therapy*, vol. 11, pp. 2565–2579, 2017.

[32] T. Shang, F. Ran, Q. Qiao, Z. Liu, and C. J. Liu, “Tanshinone IIA attenuates elastase-induced AAA in rats via inhibition of

MyD88-dependent TLR-4 signaling," *VASA*, vol. 43, no. 1, pp. 39–46, 2014.

[33] G. Pirianov, E. Torsney, F. Howe, and G. W. Cockerill, "Rosiglitazone negatively regulates c-Jun N-terminal kinase and toll-like receptor 4 proinflammatory signalling during initiation of experimental aortic aneurysms," *Atherosclerosis*, vol. 225, no. 1, pp. 69–75, 2012.

[34] F. Atzeni, L. Boiardi, A. Vaglio et al., "TLR-4 and VEGF polymorphisms in chronic periaortitis," *PLoS One*, vol. 8, no. 5, article e62330, 2013.

[35] C. R. Balistreri, "Genetic contribution in sporadic thoracic aortic aneurysm? Emerging evidence of genetic variants related to TLR-4-mediated signaling pathway as risk determinants," *Vascular Pharmacology*, vol. 74, Supplement C, pp. 1–10, 2015.

[36] C. H. Lai, K. C. Wang, F. T. Lee et al., "Toll-like receptor 4 is essential in the development of abdominal aortic aneurysm," *PLoS One*, vol. 11, no. 1, article e0146565, 2016.

[37] Z. Qin, J. Bagley, G. Sukhova et al., "Angiotensin II-induced TLR4 mediated abdominal aortic aneurysm in apolipoprotein E knockout mice is dependent on STAT3," *Journal of Molecular and Cellular Cardiology*, vol. 87, pp. 160–170, 2015.

[38] Y. Q. Dong, C. W. Lu, L. Zhang, J. Yang, W. Hameed, and W. Chen, "Toll-like receptor 4 signaling promotes invasion of hepatocellular carcinoma cells through MKK4/JNK pathway," *Molecular Immunology*, vol. 68, no. 2, pp. 671–683, 2015.

[39] A. Kumar, U. K. Singh, S. G. Kini et al., "JNK pathway signaling: a novel and smarter therapeutic targets for various biological diseases," *Future Medicinal Chemistry*, vol. 7, no. 15, pp. 2065–2086, 2015.

[40] H. Li, Y. Li, D. Liu, and J. Liu, "LPS promotes epithelial–mesenchymal transition and activation of TLR4/JNK signaling," *Tumour Biology*, vol. 35, no. 10, pp. 10429–10435, 2014.

[41] F. Shang, J. Wang, X. Liu et al., "Involvement of reactive oxygen species and JNK in increased expression of MCP-1 and infiltration of inflammatory cells in pressure-overloaded rat hearts," *Molecular Medicine Reports*, vol. 5, no. 6, pp. 1491–6, 2012.

[42] K. Yoshimura, A. Nagasawa, J. Kudo et al., "Inhibitory effect of statins on inflammation-related pathways in human abdominal aortic aneurysm tissue," *International Journal of Molecular Sciences*, vol. 16, no. 12, pp. 11213–11228, 2015.

[43] Z. Z. Guo, Q. A. Cao, Z. Z. Li et al., "SP600125 attenuates nicotine-related aortic aneurysm formation by inhibiting matrix metalloproteinase production and CC chemokine-mediated macrophage migration," *Mediators of Inflammation*, vol. 2016, Article ID 9142425, 11 pages, 2016.

[44] J. Fan, X. Li, Y. W. Yan et al., "Curcumin attenuates rat thoracic aortic aneurysm formation by inhibition of the c-Jun N-terminal kinase pathway and apoptosis," *Nutrition*, vol. 28, no. 10, pp. 1068–1074, 2012.

[45] A. P. Owens, D. L. Rateri, D. A. Howatt et al., "MyD88 deficiency attenuates angiotensin II-induced abdominal aortic aneurysm formation independent of signaling through Toll-like receptors 2 and 4," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 31, no. 12, pp. 2813–2819, 2011.

[46] M. Ishibashi, S. Sayers, J. M. D'Armiento, A. R. Tall, and C. L. Welch, "TLR3 deficiency protects against collagen degradation and medial destruction in murine atherosclerotic plaques," *Atherosclerosis*, vol. 229, no. 1, pp. 52–61, 2013.

[47] C. L. Sokol and A. D. Luster, "The chemokine system in innate immunity," *Cold Spring Harbor Perspectives in Biology*, vol. 7, no. 5, article a016303, 2015.

[48] A. Marchese, "Endocytic trafficking of chemokine receptors," *Current Opinion in Cell Biology*, vol. 27, pp. 72–77, 2014.

[49] E. P. C. van der Vorst, Y. Döring, and C. Weber, "*Chemokines*," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 35, no. 11, pp. e52–e56, 2015.

[50] S. Michineau, G. Franck, O. Wagner-Ballon, J. Dai, E. Allaire, and M. Gervais, "Chemokine (C-X-C Motif) receptor 4 blockade by AMD3100 inhibits experimental abdominal aortic aneurysm expansion through anti-inflammatory effects," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 34, no. 8, pp. 1747–1755, 2014.

[51] F. Tanios, J. Pelisek, B. Lutz et al., "CXCR4: a potential marker for inflammatory activity in abdominal aortic aneurysm wall," *European Journal of Vascular & Endovascular Surgery*, vol. 50, no. 6, pp. 745–753, 2015.

[52] M. Y. Long, H. H. Li, X. Z. Pen, M. Q. Huang, D. Y. Luo, and P. S. Wang, "Expression of chemokine receptor-4 in bone marrow mesenchymal stem cells on experimental rat abdominal aortic aneurysms and the migration of bone marrow mesenchymal stem cells with stromal-derived factor-1," *Kaohsiung Journal of Medical Sciences*, vol. 30, no. 5, pp. 224–228, 2014.

[53] P. van den Borne, P. H. A. Quax, I. E. Hoefer, and G. Pasterkamp, "The multifaceted functions of CXCL10 in cardiovascular disease," *BioMed Research International*, vol. 2014, Article ID 893106, 11 pages, 2014.

[54] A. Gallo, A. Saad, R. Ali, A. Dardik, G. Tellides, and A. Geirsson, "Circulating interferon-γ-inducible Cys-X-Cys chemokine receptor 3 ligands are elevated in humans with aortic aneurysms and Cys-X-Cys chemokine receptor 3 is necessary for aneurysm formation in mice," *Journal of Thoracic and Cardiovascular Surgery*, vol. 143, no. 3, pp. 704–710, 2012.

[55] D. Cugini, N. Azzollini, E. Gagliardini et al., "Inhibition of the chemokine receptor CXCR2 prevents kidney graft function deterioration due to ischemia/reperfusion," *Kidney International*, vol. 67, no. 5, pp. 1753–1761, 2005.

[56] R. W. Chapman, J. E. Phillips, R. W. Hipkin, A. K. Curran, D. Lundell, and J. S. Fine, "CXCR2 antagonists for the treatment of pulmonary disease," *Pharmacology & Therapeutics*, vol. 121, no. 1, pp. 55–68, 2009.

[57] A. Anzai, M. Shimoda, J. Endo et al., "Adventitial CXCL1/G-CSF expression in response to acute aortic dissection triggers local neutrophil recruitment and activation leading to aortic rupture," *Circulation Research*, vol. 116, no. 4, pp. 612–623, 2015.

[58] C. S. Moran, R. J. Jose, J. V. Moxon et al., "Everolimus limits aortic aneurysm in the apolipoprotein E-deficient mouse by downregulating C-C chemokine receptor 2 positive monocytes," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 33, no. 4, pp. 814–821, 2013.

[59] E. McNeill, A. J. Iqbal, G. E. White, J. Patel, D. R. Greaves, and K. M. Channon, "Hydrodynamic gene delivery of CC chemokine binding Fc fusion proteins to target acute vascular inflammation in vivo," *Scientific Reports*, vol. 5, no. 1, 2015.

[60] J. Zhang, H. Chen, L. Liu et al., "Chemokine (C-C motif) receptor 2 mediates mast cell migration to abdominal aortic aneurysm lesions in mice," *Cardiovascular Research*, vol. 96, no. 3, pp. 543–551, 2012.

[61] R. Martinez-Pinna, J. Madrigal-Matute, C. Tarin et al., "Proteomic analysis of intraluminal thrombus highlights complement activation in human abdominal aortic aneurysms," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 33, no. 8, pp. 2013–2020, 2013.

[62] H. F. Zhou, H. Yan, C. M. Stover et al., "Antibody directs properdin-dependent activation of the complement alternative pathway in a mouse model of abdominal aortic aneurysm," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 7, pp. E415–E422, 2012.

[63] H. F. Zhou, H. Yan, P. Bertram et al., "Fibrinogen-specific antibody induces abdominal aortic aneurysm in mice through complement lectin pathway activation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 46, pp. E4335–E4344, 2013.

[64] C. E. Fernandez-García, E. Burillo, J. S. Lindholt et al., "Association of ficolin-3 with abdominal aortic aneurysm presence and progression," *Journal of Thrombosis and Haemostasis*, vol. 15, no. 3, pp. 575–585, 2017.

[65] E. Niinimaki, T. Paavonen, T. Valo, M. Tarkka, and A. Mennander, "Lack of C4d deposition may reveal susceptibility for ascending aortic dissection," *Scandinavian Cardiovascular Journal*, vol. 46, no. 3, pp. 177–182, 2012.

[66] K. D. Rizas, N. Ippagunta, and M. D. Tilson III, "Immune cells and molecular mediators in the pathogenesis of the abdominal aortic aneurysm," *Cardiology in Review*, vol. 17, no. 5, pp. 201–210, 2009.

[67] A. L. Blomkalns, D. Gavrila, M. Thomas et al., "CD14 directs adventitial macrophage precursor recruitment: role in early abdominal aortic aneurysm formation," *Journal of the American Heart Association*, vol. 2, no. 2, article e000065, 2013.

[68] K. Maiellaro and W. Taylor, "The role of the adventitia in vascular inflammation," *Cardiovascular Research*, vol. 75, no. 4, pp. 640–648, 2007.

[69] M. A. Dale, W. Xiong, J. S. Carson et al., "Elastin-derived peptides promote abdominal aortic aneurysm formation by modulating M1/M2 macrophage polarization," *The Journal of Immunology*, vol. 196, no. 11, pp. 4536–4543, 2016.

[70] N. H. Pope, M. Salmon, J. P. Davis et al., "D-series resolvins inhibit murine abdominal aortic aneurysm formation and increase M2 macrophage polarization," *The FASEB Journal*, vol. 30, no. 12, pp. 4192–4201, 2016.

[71] J. Rao, B. N. Brown, J. S. Weinbaum et al., "Distinct macrophage phenotype and collagen organization within the intraluminal thrombus of abdominal aortic aneurysm," *Journal of Vascular Surgery*, vol. 62, no. 3, pp. 585–593, 2015.

[72] Q. Cao, Y. Wang, D. Zheng et al., "IL-10/TGF-β-modified macrophages induce regulatory T cells and protect against adriamycin nephrosis," *Journal of the American Society of Nephrology*, vol. 21, no. 6, pp. 933–942, 2010.

[73] Y. Wang, H. Ait-Oufella, O. Herbin et al., "TGF-β activity protects against inflammatory aortic aneurysm progression and complications in angiotensin II-infused mice," *The Journal of Clinical Investigation*, vol. 120, no. 2, pp. 422–432, 2010.

[74] J. Khallou-Laschet, A. Varthaman, G. Fornasa et al., "Macrophage plasticity in experimental atherosclerosis," *PLoS One*, vol. 5, no. 1, article e8852, 2010.

[75] M. M. Tiemessen, A. L. Jagger, H. G. Evans, M. J. C. van Herwijnen, S. John, and L. S. Taams, "$CD4^+CD25^+Foxp3^+$ regulatory T cells induce alternative activation of human monocytes/macrophages," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 49, pp. 19446–19451, 2007.

[76] G. Liu, H. Ma, L. Qiu et al., "Phenotypic and functional switch of macrophages induced by regulatory $CD4^+CD25^+$ T cells in mice," *Immunology & Cell Biology*, vol. 89, no. 1, pp. 130–142, 2011.

[77] S. Katz, V. Zsiros, N. Dóczi, A. Szabó, Á. Biczó, and A. L. Kiss, "GM-CSF and GM-CSF receptor have regulatory role in transforming rat mesenteric mesothelial cells into macrophage-like cells," *Inflammation Research*, vol. 65, no. 10, pp. 827–836, 2016.

[78] E. Sho, M. Sho, K. Hoshina, H. Kimura, T. K. Nakahashi, and R. L. Dalman, "Hemodynamic forces regulate mural macrophage infiltration in experimental aortic aneurysms," *Experimental and Molecular Pathology*, vol. 76, no. 2, pp. 108–116, 2004.

[79] G. Weissen-Plenz, Ö. Sezer, C. Vahlhaus et al., "Aortic dissection associated with Cogan's syndrome: deleterious loss of vascular structural integrity is associated with GM-CSF overstimulation in macrophages and smooth muscle cells," *Journal of Cardiothoracic Surgery*, vol. 5, no. 1, p. 66, 2010.

[80] B. K. Son, D. Sawaki, S. Tomida et al., "Granulocyte macrophage colony-stimulating factor is required for aortic dissection/intramural haematoma," *Nature Communications*, vol. 6, no. 1, p. 6994, 2015.

[81] P. Ye, W. Chen, J. Wu et al., "GM-CSF contributes to aortic aneurysms resulting from *SMAD3* deficiency," *The Journal of Clinical Investigation*, vol. 123, no. 5, pp. 2317–2331, 2013.

[82] J. Lin, V. Kakkar, and X. Lu, "Impact of MCP-1 in atherosclerosis," *Current Pharmaceutical Design*, vol. 20, no. 28, pp. 4580–4588, 2014.

[83] D. Y. Li, Q. Zhang, Y. Che et al., "Effects of macrophage infiltration and related gene expression on the pathogenesis of early abdominal aortic aneurysm," *Zhonghua Yi Xue Za Zhi*, vol. 83, no. 18, pp. 1624–1627, 2003.

[84] R. K. Middleton, G. M. Lloyd, M. J. Bown, N. J. Cooper, N. J. London, and R. D. Sayers, "The pro-inflammatory and chemotactic cytokine microenvironment of the abdominal aortic aneurysm wall: a protein array study," *Journal of Vascular Surgery*, vol. 45, no. 3, pp. 574–580, 2007.

[85] K. G. Battiston, B. Ouyang, R. S. Labow, C. A. Simmons, and J. P. Santerre, "Monocyte/macrophage cytokine activity regulates vascular smooth muscle cell function within a degradable polyurethane scaffold," *Acta Biomaterialia*, vol. 10, no. 3, pp. 1146–1155, 2014.

[86] J. Fan, X. Li, L. Zhong et al., "MCP-1, ICAM-1 and VCAM-1 are present in early aneurysmal dilatation in experimental rats," *Folia Histochemica et Cytobiologica*, vol. 48, no. 3, pp. 455–461, 2010.

[87] C. Q. Yang, W. Li, S. Q. Li et al., "MCP-1 stimulates MMP-9 expression via ERK 1/2 and p38 MAPK signaling pathways in human aortic smooth muscle cells," *Cellular Physiology and Biochemistry*, vol. 34, no. 2, pp. 266–276, 2014.

[88] J. S. Colonnello, K. A. Hance, M. L. Shames et al., "Transient exposure to elastase induces mouse aortic wall smooth muscle cell production of MCP-1 and RANTES during development of experimental aortic aneurysm," *Journal of Vascular Surgery*, vol. 38, no. 1, pp. 138–146, 2003.

[89] Q. Wang, J. Ren, S. Morgan, Z. Liu, C. Dou, and B. Liu, "Monocyte chemoattractant protein-1 (MCP-1) regulates macrophage cytotoxicity in abdominal aortic aneurysm," *PLoS One*, vol. 9, no. 3, article e92053, 2014.

[90] Y. Zhou, W. Wu, J. S. Lindholt et al., "Regulatory T cells in human and angiotensin II-induced mouse abdominal aortic aneurysms," *Cardiovascular Research*, vol. 107, no. 1, pp. 98–107, 2015.

[91] G. Vallés, F. Bensiamar, L. Crespo, M. Arruebo, N. Vilaboa, and L. Saldaña, "Topographical cues regulate the crosstalk between MSCs and macrophages," *Biomaterials*, vol. 37, pp. 124–133, 2015.

[92] X. Ju, T. Ijaz, H. Sun et al., "IL-6 regulates extracellular matrix remodeling associated with aortic dilation in a fibrillin-1 hypomorphic mgR/mgR mouse model of severe Marfan syndrome," *Journal of the American Heart Association*, vol. 3, no. 1, article e000476, 2014.

[93] Q. Wang, C. Shu, J. Su, and X. Li, "A crosstalk triggered by hypoxia and maintained by MCP-1/miR-98/IL-6/p38 regulatory loop between human aortic smooth muscle cells and macrophages leads to aortic smooth muscle cells apoptosis via Stat1 activation," *International Journal of Clinical & Experimental Pathology*, vol. 8, no. 3, pp. 2670–2679, 2015.

[94] B. C. Tieu, C. Lee, H. Sun et al., "An adventitial IL-6/MCP1 amplification loop accelerates macrophage-mediated vascular inflammation leading to aortic dissection in mice," *The Journal of Clinical Investigation*, vol. 119, no. 12, pp. 3637–3651, 2009.

[95] A. K. Abbas, K. M. Murphy, and A. Sher, "Functional diversity of helper T lymphocytes," *Nature*, vol. 383, no. 6603, pp. 787–793, 1996.

[96] T. R. Mosmann, L. Li, H. Hengartner, D. Kagi, W. Fu, and S. Sad, "Differentiation and functions of T cell subsets," *Ciba Foundation Symposium*, vol. 204, pp. 148–154, 1997.

[97] D. Saadoun, M. Garrido, C. Comarmond et al., "Th1 and Th17 cytokines drive inflammation in Takayasu arteritis," *Arthritis & Rhematology*, vol. 67, no. 5, pp. 1353–1360, 2015.

[98] L. Wang, S. Gao, W. Xu et al., "Allergic asthma accelerates atherosclerosis dependent on Th2 and Th17 in apolipoprotein E deficient mice," *Journal of Molecular and Cellular Cardiology*, vol. 72, pp. 20–27, 2014.

[99] W. Yu, H. du, Q. Fu, N. Cui, and X. du, "The influence of Th1/Th2 and $CD4^+$ regulatory T cells of mesenteric lymph nodes on systemic lipopolysaccharide," *Polish Journal of Pathology*, vol. 65, no. 2, pp. 125–129, 2014.

[100] K. Shimizu, M. Shichiri, P. Libby, R. T. Lee, and R. N. Mitchell, "Th2-predominant inflammation and blockade of IFN-γ signaling induce aneurysms in allografted aortas," *The Journal of Clinical Investigation*, vol. 114, no. 2, pp. 300–308, 2004.

[101] U. Schönbeck, G. K. Sukhova, N. Gerdes, and P. Libby, "T_H2 predominant immune responses prevail in human abdominal aortic aneurysm," *The American Journal of Pathology*, vol. 161, no. 2, pp. 499–506, 2002.

[102] S. Ellison, K. Gabunia, S. E. Kelemen et al., "Attenuation of experimental atherosclerosis by interleukin-19," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 33, no. 10, pp. 2316–2324, 2013.

[103] V. L. King, A. Y. Lin, F. Kristo et al., "Interferon-γ and the interferon-inducible chemokine CXCL10 protect against aneurysm formation and rupture," *Circulation*, vol. 119, no. 3, pp. 426–435, 2009.

[104] C. Galle, L. Schandené, P. Stordeur et al., "Predominance of type 1 $CD4^+$ T cells in human abdominal aortic aneurysm," *Clinical & Experimental Immunology*, vol. 142, no. 3, pp. 519–527, 2005.

[105] W. Xiong, Y. Zhao, A. Prall, T. C. Greiner, and B. T. Baxter, "Key roles of $CD4^+$ T cells and IFN-γ in the development of abdominal aortic aneurysms in a murine model," *The Journal of Immunology*, vol. 172, no. 4, pp. 2607–2612, 2004.

[106] D. A. A. Vignali, L. W. Collison, and C. J. Workman, "How regulatory T cells work," *Nature Reviews Immunology*, vol. 8, no. 7, pp. 523–532, 2008.

[107] N. Ohkura, Y. Kitagawa, and S. Sakaguchi, "Development and maintenance of regulatory T cells," *Immunity*, vol. 38, no. 3, pp. 414–423, 2013.

[108] H. Ait-Oufella, Y. Wang, O. Herbin et al., "Natural regulatory T cells limit angiotensin II-induced aneurysm formation and rupture in mice," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 33, no. 10, pp. 2374–2379, 2013.

[109] M. Yin, J. Zhang, Y. Wang et al., "Deficient $CD4^+CD25^+$ T regulatory cell function in patients with abdominal aortic aneurysms," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 30, no. 9, pp. 1825–1831, 2010.

[110] K. Yodoi, T. Yamashita, N. Sasaki et al., "$Foxp3^+$ regulatory T cells play a protective role in angiotensin II-induced aortic aneurysm formation in mice," *Hypertension*, vol. 65, no. 4, pp. 889–895, 2015.

[111] S. Kasashima, A. Kawashima, Y. Zen et al., "Upregulated interleukins (IL-6, IL-10, and IL-13) in immunoglobulin G4-related aortic aneurysm patients," *Journal of Vascular Surgery*, vol. 67, no. 4, pp. 1248–1262, 2018.

[112] D. Vucevic, V. Maravic-Stojkovic, S. Vasilijic et al., "Inverse production of IL-6 and IL-10 by abdominal aortic aneurysm explant tissues in culture," *Cardiovascular Pathology*, vol. 21, no. 6, pp. 482–489, 2012.

[113] R. Sharir, J. Semo, A. Shaish et al., "Regulatory T cells influence blood flow recovery in experimental hindlimb ischaemia in an IL-10-dependent manner," *Cardiovascular Research*, vol. 103, no. 4, pp. 585–596, 2014.

[114] A. F. Baas, J. Medic, R. van't Slot et al., "Association of the TGF-β receptor genes with abdominal aortic aneurysm," *European Journal of Human Genetics*, vol. 18, no. 2, pp. 240–244, 2010.

[115] A. J. Doyle, J. J. Doyle, S. L. Bessling et al., "Mutations in the TGF-β repressor *SKI* cause Shprintzen-Goldberg syndrome with aortic aneurysm," *Nature Genetics*, vol. 44, no. 11, pp. 1249–1254, 2012.

[116] F. Gao, P. Chambon, S. Offermanns et al., "Disruption of TGF-β signaling in smooth muscle cell prevents elastase-induced abdominal aortic aneurysm," *Biochemical and Biophysical Research Communications*, vol. 454, no. 1, pp. 137–143, 2014.

[117] L. Q. Bian, L. Mao, Y. Bi et al., "Loss of regulatory characteristics in $CD4^+CD25^{+/hi}$ T cells induced by impaired

transforming growth factor beta secretion in pneumoconiosis," *APMIS*, vol. 125, no. 12, pp. 1108–1116, 2017.

[118] Y. Flores-García, J. L. Rosales-Encina, V. H. Rosales-García, A. R. Satoskar, and P. Talamás-Rohana, "$CD4^{+}CD25^{+-}$ $FOXP3^{+}$ Treg cells induced by rSSP4 derived from *T. cruzi* amastigotes increase parasitemia in an experimental Chagas disease model," *BioMed Research International*, vol. 2013, Article ID 632436, 10 pages, 2013.

[119] Y. Lu, F. Liu, C. Li, Y. Chen, D. Weng, and J. Chen, "IL-10-producing B cells suppress effector T cells activation and promote regulatory T cells in crystalline silica-induced inflammatory response in vitro," *Mediators of Inflammation*, vol. 2017, Article ID 8415094, 11 pages, 2017.

[120] P. Salamon, I. Shefler, I. Moshkovits et al., "IL-33 and IgE stimulate mast cell production of IL-2 and regulatory T cell expansion in allergic dermatitis," *Clinical & Experimental Allergy*, vol. 47, no. 11, pp. 1409–1416, 2017.

[121] Z. Zeng, K. Yu, L. Chen, W. Li, H. Xiao, and Z. Huang, "Interleukin-2/anti-interleukin-2 immune complex attenuates cardiac remodeling after myocardial infarction through expansion of regulatory T cells," *Journal of Immunology Research*, vol. 2016, Article ID 8493767, 13 pages, 2016.

[122] S. C. Balmert, C. Donahue, J. R. Vu, G. Erdos, L. D. Falo Jr., and S. R. Little, "In vivo induction of regulatory T cells promotes allergen tolerance and suppresses allergic contact dermatitis," *Journal of Controlled Release*, vol. 261, pp. 223–233, 2017.

[123] T. Kyaw, A. Winship, C. Tay et al., "Cytotoxic and proinflammatory $CD8^{+}$ T lymphocytes promote development of vulnerable atherosclerotic plaques in ApoE-deficient mice," *Circulation*, vol. 127, no. 9, pp. 1028–1039, 2013.

[124] H. F. Zhou, H. Yan, J. L. Cannon, L. E. Springer, J. M. Green, and C. T. N. Pham, "CD43-mediated IFN-γ production by $CD8^{+}$ T cells promotes abdominal aortic aneurysm in mice," *The Journal of Immunology*, vol. 190, no. 10, pp. 5078–5085, 2013.

[125] J. M. B. Prieto and M. J. B. Felippe, "Development, phenotype, and function of non-conventional B cells," *Comparative Immunology, Microbiology and Infectious Diseases*, vol. 54, pp. 38–44, 2017.

[126] G. Pasquinelli, P. Preda, M. Gargiulo et al., "An immunohistochemical study of inflammatory abdominal aortic aneurysms," *Journal of Submicroscopic Cytology and Pathology*, vol. 25, no. 1, pp. 103–112, 1993.

[127] E. Ocana, J. C. Bohórquez, J. Pérez-Requena, J. A. Brieva, and C. Rodríguez, "Characterisation of T and B lymphocytes infiltrating abdominal aortic aneurysms," *Atherosclerosis*, vol. 170, no. 1, pp. 39–48, 2003.

[128] B. Schaheen, E. A. Downs, V. Serbulea et al., "B-cell depletion promotes aortic infiltration of immunosuppressive cells and is protective of experimental aortic aneurysm," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 36, no. 11, pp. 2191–2202, 2016.

[129] S. Mellak, H. Ait-Oufella, B. Esposito et al., "Angiotensin II mobilizes spleen monocytes to promote the development of abdominal aortic aneurysm in *Apoe*$^{-/-}$ mice," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 35, no. 2, pp. 378–388, 2015.

[130] A. K. Meher, W. F. Johnston, G. Lu et al., "B2 cells suppress experimental abdominal aortic aneurysms," *The American Journal of Pathology*, vol. 184, no. 11, pp. 3130–3141, 2014.

[131] W. L. Chan, N. Pejnovic, T. V. Liew, and H. Hamilton, "Predominance of Th2 response in human abdominal aortic aneurysm: mistaken identity for IL-4-producing NK and NKT cells?," *Cellular Immunology*, vol. 233, no. 2, pp. 109–114, 2005.

[132] W. L. Chan, N. Pejnovic, H. Hamilton et al., "Atherosclerotic abdominal aortic aneurysm and the interaction between autologous human plaque-derived vascular smooth muscle cells, type 1 NKT, and helper T cells," *Circulation Research*, vol. 96, no. 6, pp. 675–683, 2005.

[133] J. Juvonen, H. M. Surcel, J. Satta et al., "Elevated circulating levels of inflammatory cytokines in patients with abdominal aortic aneurysm," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 17, no. 11, pp. 2843–2847, 1997.

[134] E. L. Henderson, Y. J. Geng, G. K. Sukhova, A. D. Whittemore, J. Knox, and P. Libby, "Death of smooth muscle cells and expression of mediators of apoptosis by T lymphocytes in human abdominal aortic aneurysms," *Circulation*, vol. 99, no. 1, pp. 96–104, 1999.

[135] I. Hinterseher, C. Schworer, J. Lillvis et al., "Immunohistochemical analysis of the natural killer cell cytotoxicity pathway in human abdominal aortic aneurysms," *International Journal of Molecular Sciences*, vol. 16, no. 12, pp. 11196–11212, 2015.

[136] H. Dai and R. J. Korthuis, "Mast cell proteases and inflammation," *Drug Discovery Today: Disease Models*, vol. 8, no. 1, pp. 47–55, 2011.

[137] J. Sun, J. Zhang, J. S. Lindholt et al., "Critical role of mast cell chymase in mouse abdominal aortic aneurysm formation," *Circulation*, vol. 120, no. 11, pp. 973–982, 2009.

[138] J. Zhang, J. Sun, J. S. Lindholt et al., "Mast cell tryptase deficiency attenuates mouse abdominal aortic aneurysm formation," *Circulation Research*, vol. 108, no. 11, pp. 1316–1327, 2011.

[139] H. Sillesen, N. Eldrup, R. Hultgren et al., "Randomized clinical trial of mast cell inhibition in patients with a medium-sized abdominal aortic aneurysm," *The British Journal of Surgery*, vol. 102, no. 8, pp. 894–901, 2015.

[140] W. R. W. Wilson, E. C. Schwalbe, J. L. Jones, P. R. F. Bell, and M. M. Thompson, "Matrix metalloproteinase 8 (neutrophil collagenase) in the pathogenesis of abdominal aortic aneurysm," *The British Journal of Surgery*, vol. 92, no. 7, pp. 828–833, 2005.

[141] H. Yan, H. F. Zhou, A. Akk et al., "Neutrophil proteases promote experimental abdominal aortic aneurysm via extracellular trap release and plasmacytoid dendritic cell activation," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 36, no. 8, pp. 1660–1669, 2016.

[142] P. Ramos-Mozo, J. Madrigal-Matute, M. Vega de Ceniga et al., "Increased plasma levels of NGAL, a marker of neutrophil activation, in patients with abdominal aortic aneurysm," *Atherosclerosis*, vol. 220, no. 2, pp. 552–556, 2012.

[143] A. Lacquaniti, M. Giardina, S. Lucisano et al., "Neutrophil gelatinase-associated lipocalin (NGAL) and endothelial progenitor cells (EPCs) evaluation in aortic aneurysm repair," *Current Vascular Pharmacology*, vol. 11, no. 6, pp. 1001–1010, 2013.

[144] V. Brinkmann, U. Reichard, C. Goosmann et al., "Neutrophil extracellular traps kill bacteria," *Science*, vol. 303, no. 5663, pp. 1532–1535, 2004.

[145] S. Delbosc, J. M. Alsac, C. Journe et al., "*Porphyromonas gingivalis* participates in pathogenesis of human abdominal aortic aneurysm by neutrophil activation. Proof of concept in rats," *PLoS One*, vol. 6, no. 4, article e18679, 2011.

[146] A. K. Meher, M. Spinosa, J. P. Davis et al., "Novel role of IL (interleukin)-1β in neutrophil extracellular trap formation and abdominal aortic aneurysms," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 38, no. 4, pp. 843–853, 2018.

[147] S. A. O'Leary, E. G. Kavanagh, P. A. Grace, T. M. McGloughlin, and B. J. Doyle, "The biaxial mechanical behaviour of abdominal aortic aneurysm intraluminal thrombus: classification of morphology and the determination of layer and region specific properties," *Journal of Biomechanics*, vol. 47, no. 6, pp. 1430–1437, 2014.

[148] R. Adolph, D. A. Vorp, D. L. Steed, M. W. Webster, M. V. Kameneva, and S. C. Watkins, "Cellular content and permeability of intraluminal thrombus in abdominal aortic aneurysm," *Journal of Vascular Surgery*, vol. 25, no. 5, pp. 916–926, 1997.

[149] M. Folkesson, M. Kazi, C. Zhu et al., "Presence of NGAL/MMP-9 complexes in human abdominal aortic aneurysms," *Thrombosis and Haemostasis*, vol. 98, no. 2, pp. 427–433, 2007.

[150] X. Houard, V. Ollivier, L. Louedec, J. B. Michel, and M. Bäck, "Differential inflammatory activity across human abdominal aortic aneurysms reveals neutrophil-derived leukotriene B_4 as a major chemotactic factor released from the intraluminal thrombus," *The FASEB Journal*, vol. 23, no. 5, pp. 1376–1383, 2009.

[151] Z. Touat, V. Ollivier, J. Dai et al., "Renewal of mural thrombus releases plasma markers and is involved in aortic abdominal aneurysm evolution," *The American Journal of Pathology*, vol. 168, no. 3, pp. 1022–1030, 2006.

[152] M. Kazi, J. Thyberg, P. Religa et al., "Influence of intraluminal thrombus on structural and cellular composition of abdominal aortic aneurysm wall," *Journal of Vascular Surgery*, vol. 38, no. 6, pp. 1283–1292, 2003.

[153] A. Parr, M. McCann, B. Bradshaw, A. Shahzad, P. Buttner, and J. Golledge, "Thrombus volume is associated with cardiovascular events and aneurysm growth in patients who have abdominal aortic aneurysms," *Journal of Vascular Surgery*, vol. 53, no. 1, pp. 28–35, 2011.

[154] C. S. Moran, S. W. Seto, S. M. Krishna et al., "Parenteral administration of factor Xa/IIa inhibitors limits experimental aortic aneurysm and atherosclerosis," *Scientific Reports*, vol. 7, article 43079, 2017.

[155] A. Daugherty, M. W. Manning, and L. A. Cassis, "Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice," *The Journal of Clinical Investigation*, vol. 105, no. 11, pp. 1605–1612, 2000.

[156] A. Nirula, S. M. Glaser, S. L. Kalled, and F. R. Taylora, "What is IgG4? A review of the biology of a unique immunoglobulin subtype," *Current Opinion in Rheumatology*, vol. 23, no. 1, pp. 119–124, 2011.

[157] R. Perez Alamino, C. Martinez, and L. R. Espinoza, "IgG4-associated vasculitis," *Current Rheumatology Reports*, vol. 15, no. 8, p. 348, 2013.

[158] C. A. Perugino, Z. S. Wallace, N. Meyersohn, G. Oliveira, J. R. Stone, and J. H. Stone, "Large vessel involvement by IgG4-related disease," *Medicine*, vol. 95, no. 28, article e3344, 2016.

[159] K. Raparia, C. P. Molina, G. Quiroga-Garza, D. Weilbaecher, A. G. Ayala, and J. Y. Ro, "Inflammatory aortic aneurysm: possible manifestation of IgG4-related sclerosing disease," *International Journal of Clinical & Experimental Pathology*, vol. 6, no. 3, pp. 469–475, 2013.

[160] R. Hourai, S. Kasashima, K. Sohmiya et al., "IgG4-positive cell infiltration in various cardiovascular disorders - results from histopathological analysis of surgical samples," *BMC Cardiovascular Disorders*, vol. 17, no. 1, p. 52, 2017.

[161] H. Kajander, T. Paavonen, T. Valo, M. Tarkka, and A. A. Mennander, "Immunoglobulin G4-positive ascending thoracic aortitis may be prone to dissection," *The Journal of Thoracic and Cardiovascular Surgery*, vol. 146, no. 6, pp. 1449–1455, 2013.

[162] M. Tajima, R. Nagai, and Y. Hiroi, "IgG4-related cardiovascular disorders," *International Heart Journal*, vol. 55, no. 4, pp. 287–295, 2014.

[163] H. Ito, Y. Kaizaki, Y. Noda, S. Fujii, and S. Yamamoto, "IgG4-related inflammatory abdominal aortic aneurysm associated with autoimmune pancreatitis," *Pathology International*, vol. 58, no. 7, pp. 421–426, 2008.

[164] N. Sakata, T. Tashiro, N. Uesugi et al., "IgG4-positive plasma cells in inflammatory abdominal aortic aneurysm: the possibility of an aortic manifestation of IgG4-related sclerosing disease," *The American Journal of Surgical Pathology*, vol. 32, no. 4, pp. 553–559, 2008.

[165] S. Kasashima, Y. Zen, A. Kawashima et al., "Inflammatory abdominal aortic aneurysm: close relationship to IgG4-related periaortitis," *The American Journal of Surgical Pathology*, vol. 32, no. 2, pp. 197–204, 2008.

[166] S. Kasashima, Y. Zen, A. Kawashima, M. Endo, Y. Matsumoto, and F. Kasashima, "A new clinicopathological entity of IgG4-related inflammatory abdominal aortic aneurysm," *Journal of Vascular Surgery*, vol. 49, no. 5, pp. 1264–1271, 2009.

[167] S. Kasashima and Y. Zen, "IgG4-related inflammatory abdominal aortic aneurysm," *Current Opinion in Rheumatology*, vol. 23, no. 1, pp. 18–23, 2011.

[168] I. Tuleta, D. Skowasch, F. Aurich et al., "Asthma is associated with atherosclerotic artery changes," *PLoS One*, vol. 12, no. 10, article e0186820, 2017.

[169] K. Z. Abd-Elmoniem, N. Ramos, S. K. Yazdani et al., "Coronary atherosclerosis and dilation in hyper IgE syndrome patients: depiction by magnetic resonance vessel wall imaging and pathological correlation," *Atherosclerosis*, vol. 258, pp. 20–25, 2017.

[170] D. Tsiantoulas, I. Bot, M. Ozsvar-Kozma et al., "Increased plasma IgE accelerate atherosclerosis in secreted IgM deficiency," *Circulation Research*, vol. 120, no. 1, pp. 78–84, 2017.

[171] A. F. Freeman, E. M. Avila, P. A. Shaw et al., "Coronary artery abnormalities in hyper-IgE syndrome," *Journal of Clinical Immunology*, vol. 31, no. 3, pp. 338–345, 2011.

[172] C. L. Liu, J. Y. Zhang, and G. P. Shi, "Interaction between allergic asthma and atherosclerosis," *Translational Research*, vol. 174, pp. 5–22, 2016.

[173] C. L. Liu, H. Wemmelund, Y. Wang et al., "Asthma associates with human abdominal aortic aneurysm and rupture," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 36, no. 3, pp. 570–578, 2016.

[174] C. L. Liu, Y. Wang, M. Liao et al., "Allergic lung inflammation aggravates angiotensin II-induced abdominal aortic

aneurysms in mice," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 36, no. 1, pp. 69–77, 2016.

[175] L. M. Villar, B. Casanova, N. Ouamara et al., "Immunoglobulin M oligoclonal bands: biomarker of targetable inflammation in primary progressive multiple sclerosis," *Annals of Neurology*, vol. 76, no. 2, pp. 231–240, 2014.

[176] G. M. Diepenhorst, C. L. Ciurana, N. Diaz Padilla et al., "IgM antibodies against apoptotic cells and phosphorylcholine in patients with acute myocardial infarction in relation to infarct size and inflammatory response," *Advances in Clinical and Experimental Medicine*, vol. 21, no. 4, pp. 455–467, 2012.

[177] C. A. Gleissner, C. Erbel, J. Haeussler et al., "Low levels of natural IgM antibodies against phosphorylcholine are independently associated with vascular remodeling in patients with coronary artery disease," *Clinical Research in Cardiology*, vol. 104, no. 1, pp. 13–22, 2015.

[178] T. Kyaw, P. Tipping, A. Bobik, and B. H. Toh, "Protective role of natural IgM-producing B1a cells in atherosclerosis," *Trends in Cardiovascular Medicine*, vol. 22, no. 2, pp. 48–53, 2012.

[179] X. Wang, Q. Wang, and Z. Sun, "Normal IgG downregulates the intracellular superoxide level and attenuates migration and permeability in human aortic endothelial cells isolated from a hypertensive patient," *Hypertension*, vol. 60, no. 3, pp. 818–826, 2012.

[180] S. V. Kaveri, M. Lecerf, C. Saha, M. D. Kazatchkine, S. Lacroix-Desmazes, and J. Bayry, "Intravenous immunoglobulin and immune response," *Clinical & Experimental Immunology*, vol. 178, no. S1, pp. 94–96, 2014.

[181] M. Ahmadi, S. Abdolmohammadi-vahid, M. Ghaebi et al., "Effect of intravenous immunoglobulin on Th1 and Th2 lymphocytes and improvement of pregnancy outcome in recurrent pregnancy loss (RPL)," *Biomedicine & Pharmacotherapy*, vol. 92, pp. 1095–1102, 2017.

[182] M. S. Maddur, S. V. Kaveri, and J. Bayry, "Circulating normal IgG as stimulator of regulatory T cells: lessons from intravenous immunoglobulin," *Trends in Immunology*, vol. 38, no. 11, pp. 789–792, 2017.

[183] J. Zschüntzsch, Y. Zhang, F. Klinker et al., "Treatment with human immunoglobulin G improves the early disease course in a mouse model of Duchenne muscular dystrophy," *Journal of Neurochemistry*, vol. 136, no. 2, pp. 351–362, 2016.

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Review Article

Microbiota Composition and the Integration of Exogenous and Endogenous Signals in Reactive Nasal Inflammation

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The prevalence of reactive nasal inflammatory conditions, for example, allergic rhinitis and chronic rhinosinusitis, is steadily increasing in parallel with significant environmental changes worldwide. Allergens and as yet undefined environmental agents may trigger these conditions via the involvement of host intrinsic factors, including the innate and adaptive immune system, the nasal epithelium, and the nasal nervous system. The critical role of the nasal microbiota in coordinating these components has emerged in recent studies documenting a significant association between microbial composition and the onset and progression of allergic or nonallergic inflammation. It is now clear that the local microbiota is a major player in the development of the mucosa-associated lymphoid tissue and in the regulation of such adaptive responses as IgA production and the function of effector and regulatory T cells. Microbial components also play a major role in the regulation of epithelial barrier functions, including mucus production and the control of paracellular transport across tight junctions. Bacterial components, including lipopolysaccharide, have also been shown to induce or amplify neuroinflammatory responses by engaging specific nociceptors. Finally, bacterial products may promote tissue remodeling processes, including nasal polyp formation, by interacting with formyl peptide receptors and inducing the expression of angiogenic factors and matrix-degrading enzymes.

1. Introduction

The nose, the uppermost portion of the respiratory tract, serves important physiologic functions, such as air filtration, warming, humidification, and olfaction. It consists of two cavities or *fossae* extending from the external nostrils (anterior nares) to the choanae and separated longitudinally by an osteocartilaginous septum. The lateral wall of each fossa provides insertion to three turbinates, or *conchae*, which divide the cavity in three passages, or *meatuses* [1]. These anatomical structures are essential to the air conditioning functions of the nose in that they expand the surface exposed to inhaled air. While the anterior nares and vestibule are lined with a skin-like stratified, keratinized epithelium, the nasal fossa proper is entirely coated with respiratory mucosa, consisting of a ciliated, highly vascularized, pseudostratified epithelium containing a sizeable number of mucus-producing goblet cells. The extensive vascularization of the nasal mucosa favors its air warming and humidifying functions, whereas the sticky seromucous secretions contribute to air filtering by effectively trapping inhaled particulate matter [2].

If the anatomy and physiology of the nasal cavities are complex, at least as complex are the pathophysiological processes that underlie the onset and progression of reactive nasal inflammatory conditions. These include a heterogeneous group of disorders, ranging from seasonal allergic rhinitis to nonallergic, persisting, refractory forms of chronic

rhinosinusitis (CRS). About 400 million people worldwide are affected by allergic rhinitis, and another 200 million are thought to be affected by nonallergic forms of nasal inflammation including CRS [3, 4]. The overall prevalence of these conditions has been on a steady rise for almost 25 years concomitant with gross environmental changes in developed and developing countries [5]. While the inflammatory responses underlying allergic rhinitis are triggered by exposure to molecules with intrinsic allergenic properties, which promote type 2 T helper cell- (Th2-) biased, IgE-dependent immune responses, triggers of nonallergic rhinitis or CRS are nonspecific and largely unknown [4, 6]. Regardless, a number of common factors variably contribute to favoring and worsening the inflammatory response in these reactive nasal conditions [7–9]. These include the innate and adaptive immune system, the epithelial barrier function, a neuroinflammatory component (i.e., neurogenic inflammation), tissue remodeling processes, and the nasal microbiota.

In spite of the growing level of interest by the scientific community, still very little is known on the relationship between the nasal mucosal microenvironment, nasal allergic or nonallergic inflammation, and the nasal microbiota. Conversely, for a number of reasons, including the availability of suitable animal models, the central role of the microbiota in the coordination of the host homeostasis and specific disease processes is amply documented in several studies of gut immunopathology. In this review, we will touch on some of these studies in parallel with discussing more recent acquisitions in allergic rhinitis and related reactive nasal inflammatory conditions.

2. Towards the Definition of a “Healthy” Nasal Microbiota

The human microbiota, that is, the population of symbiotic microbes in the human body, has gained growing attention in the past few years, accounting for over 30,000 articles indexed in PubMed, over 25,000 of which published in the last five years [10, 11]. In recent years, studies of tissue-associated microbial communities have increasingly exploited the striking advances in next-generation sequencing and quantitative PCR of microbial genomes, or metagenomes [12]. Sequencing strategies vary greatly in different studies, the most common involving amplicon analysis of the 16S ribosomal RNA (rRNA), whereby bacterial operational taxonomic units (OTU) are mostly defined at the phyla or genera level depending on the sequence similarity threshold [13, 14]. However, coverage of larger, more complete sets of genes, as in whole-genome shotgun sequencing, is required to more accurately define microbial taxa down to the species and strain level and provide specific information on their physiological state, including the acquisition of accessory genes involved in virulence or antibiotic resistance [15]. Regardless of the breadth of coverage, current metagenomics tools have allowed to fully appreciate the extreme diversity of microbial communities and document the relationship between their imbalance, or *dysbiosis*, and seemingly unrelated disease processes, for example, obesity, autoimmunity, cancer, and mental disorders [12, 16–19]. On the other hand, they have allowed extending and overcoming most basic assumptions from earlier studies relying on semiquantitative cultures of bacterial colonies from fecal samples or other sources [12, 15]. It is now clear that distinct microbial communities exist on almost all epithelial surfaces of the human body [12, 13, 20] and that these consist of a highly diverse repertoire of bacterial, archaeal, viral, and fungal species [21–23]. In fact, recent estimates challenge the long-standing notion that a healthy human microbiota mostly consists of bacteria and that their numbers vastly exceed the total number of human cells [24]. Viruses, especially bacteriophages, are currently thought to outnumber the bacterial community by a ratio of at least 10 : 1 and contribute at least as substantially to the host homeostasis by acting on the bacterial phenotype and function or by directly interacting with the host mucosa [22, 23, 25].

Regardless of the association with clinical disease, the microbiota composition varies greatly in different individuals or different anatomical sites [12, 13]. As inferred in recent metagenomics studies of gut and oral samples from large populations sharing relatively common environments, interindividual variability largely reflects the environmental biodiversity rather than the host genetic background [26, 27]. On the other hand, the basis for intraindividual variability is still a matter of conjecture. The gastrointestinal microbiota has been investigated in most studies to date and is possibly the most abundant and diverse in the human body [13, 28]. Comparable OTU numbers, a measure of species richness, as well as disease-specific perturbations, for instance in patients with rheumatoid arthritis or cirrhosis, have been observed in distinct niches within the upper or lower gastrointestinal tract [29–31]. However, profound differences have been reported in the relative stabilities and recovery rates of commensal bacteria within the oral and gut mucosa following the administration of several classes of antibiotics [32]. Studies of the Human Microbiome Project cohort, in which 16S rRNA sequence clusters were examined at 18 different sites, have provided a possible explanation to these apparent discrepancies [13, 14]. In these studies, a *core* microbiota, defined as OTU shared across at least 95% of all samples for a given site, is identified as a stable, relatively ubiquitous, well-adapted microbial community, whereas noncore, *satellite* communities are identified at the subgenus level which are more variable across individuals, anatomical niches, time, and response to treatment [13, 14].

The nasal mucosa, given its affinities and contiguity to the lower respiratory tract and the sinus mucosa, the heterogeneity of its cellular components, its air conditioning and olfactory functions, and its permanent exposure to the external environment, represents a quite unique, attractive model for studies of host-microbe relationships in health and disease. The complex anatomy of the nasal cavities offers a highly diversified habitat to microbial species in a relatively narrow space [33, 34]. Most culture- or sequence-based studies of the nasal microbiota have until recently been limited to sampling the anterior nares and the vestibule, which exhibit similar overall histology to the external skin. Not surprisingly then, the distribution of certain phyla within this niche, namely, *Actinobacteria* and *Firmicutes*, as well as the overall

richness in bacterial communities have been found to resemble those of the skin [13, 35]. A systematic study of 16S rRNA sequence clusters within two additional nasal sites besides the vestibule—the middle meatus and the sphenoethmoidal recess—identified in these mucosal sites a superimposable microbial colonization, consisting of about 50% *Actinobacteria*, 25% *Firmicutes*, and 20% *Proteobacteria* [33]. In contrast, the nasal vestibule was relatively enriched in *Firmicutes*, including *Staphylococcus aureus* [33]. A similar study identified almost 140 different taxa down to the species level by combining 16S rRNA sequencing and extensive culture of samples obtained during surgery from the anterior and posterior vestibule and the middle and inferior meatuses [34]. Core species identified at all sites by either approach included distinct *Staphylococcus* and *Corynebacterium* species, among others [34]. Similar core profiles were identified in a more recent study comparing the 16S rRNA amplicon sequence variants in samples from the anterior nasal cavity and the nasopharynx [36]. Aside from the relative abundance of species also detected in the lower respiratory tract, for example, *Streptococcus* and *Haemophilus* [37], nasopharynx samples from the majority of donors showed a more diverse "nasal" community, where *Corynebacterium*, *Staphylococcus*, and/or *Dolosigranulum* were the dominant core members [36].

3. Microbial Communities in Nasal Inflammation

Taken together, the findings outlined in the previous section are in line with the notion that, rather than being distributed in discrete niches, microbial communities throughout the nasal cavities and the upper respiratory tract may represent a continuum [36, 38]. This lends support to the idea, backing common clinical practice, that at least certain disease-specific associations can be recapitulated in swabs from a single site within the nasal cavities [39]. A number of studies have investigated the relationship between the nasal or nasopharynx microbiota and the frequency and severity of acute viral infections of the upper or lower respiratory tract (reviewed in [40]). A consistent association of 16S rRNA sequence profiles with disease severity was found in the anterior nares or nasopharyngeal swabs from over 800 infants hospitalized for bronchiolitis, whereby a *Haemophilus*- or *Moraxella*-dominated profile in either site appeared to be predictive of a higher or lower likelihood of intensive care use [39]. Along this line, a positive correlation between bacterial diversity, the relative abundance of *Haemophilus* and other species, and disease severity is documented in a metagenomics study of nasopharyngeal swabs from children hospitalized for influenza [41]. In contrast, a robust association was found between the frequency of symptomatic human rhinovirus (HRV) infections and a loss of microbial diversity in anterior nares swabs from an unselected cohort of 32 infants [42]. Evidence in support of a direct impact of viral pathogens on the nasal bacterial community is provided in a controlled study in which significant, long-lasting increases in *Staphylococcus* species relative abundance were seen in nasal swabs from healthy adult volunteers administered a live attenuated influenza virus vaccine [43]. Consistently, an up to 13-fold increase in *Staphylococcus* abundance was reported in the nasal lavage of volunteers subjected to experimental infection with HRV serotype 16 (HRV-16) [44].

Changes in microbiota composition subsequent to acute respiratory infections presumably reflect direct and diverse interactions of pathogenic viruses with the resident virome and bacteriome and/or the host immune system (reviewed in [45]). Studies in mouse models of influenza virus respiratory infection show that superinfection from *S. aureus* strains, including methicillin-resistant *S. aureus* (MRSA), is mediated by immune activation and the production of type I and III interferons (IFN) [43, 46]. Staphylococcal carriage within the nasal mucosa can be detected in about 30% of the general population and is a major risk factor for clinically significant, often severe infections of the lower respiratory tract, the skin, the bone, and other deep tissues [47–49]. An association between staphylococcal carriage, nasal dysbiosis, and nasal reactive inflammation was hypothesized by Salzano et al. in the early 1990s, who documented, using traditional culture methods and nasal challenges with bacterial antigens, the onset of more severe nasal symptoms concomitant with nasal colonization by *Chlamydia* and *Staphylococcus* species [50]. The relationship between nasal allergic inflammation and *S. aureus* carriage has been conclusively documented in a meta-analysis of ten studies conducted between 2000 and 2007: in nine out of ten studies, significantly higher numbers of adult or pediatric patients with allergic rhinitis were shown to test positive, at the local or systemic level, for *S. aureus* colonization [51]. A more recent 16S rRNA sequencing study documented increased microbial diversity in the middle nasal meatus of adult patients with seasonal allergic rhinitis and possible implications for airway inflammatory disease more in general [52]. Studies in patients with CRS, a more persisting form of nasal inflammation, presumably reflect the heterogeneity of this condition, as well as the protocols used for sample collection and processing, in that reduced or increased microbial diversity is detected from case to case; regardless, in most cases a relative enrichment is documented in staphylococcal species, especially *S. aureus* (reviewed in [53]).

Thus, studies of nasal inflammatory conditions of infectious or noninfectious etiology not necessarily fit the notion, inferred from studies of the gut or skin microbiota, that reduced microbial diversity, resulting from dietary changes, antibiotic overuse, and overall declining biodiversity, is most consistently associated with chronic disease, including allergic disease [54–56]. Conversely, clinical or subclinical infection with staphylococcal pathogenic strains emerges as a common denominator in the onset and progression of these conditions. Of note, superinfection by *S. aureus* strains in the context of a less diverse microbial community is a quite consistent finding in skin isolates from patients with atopic eczema (reviewed in [57]). Whether staphylococcal outgrowth in the atopic skin or nose is an initiating event that affects the relative abundance of symbiotic species or, rather, the result of changes in the microbial environment induced by other factors has yet to be determined. Such changes presumably reflect complex interactions between a genetically

biased, imbalanced host response and more or less identifiable environmental signals. Substantial changes in the respiratory microbiota, staphylococcal outgrowth, and the development of a Th2-driven inflammatory response are consistently seen in human and animal models of infection with HRV, influenza, and other respiratory viruses [41–44, 58]. Indeed, these viruses are well-known for contributing to the onset and exacerbations of rhinitis, CRS, and asthma (reviewed in [59]).

While a body of evidence from studies of the gut microbiota may be extrapolated to such other districts, as the oral, vaginal, and respiratory mucosa, clearly more studies are required, above and beyond association studies, to understand how changes in the nasal microbiota affect the local homeostasis in health and disease. On the other hand, intestinal dysbiosis can precede and be conducive to the development of respiratory allergy [56, 60]. A possible cause-effect relationship between enrichment in a clostridial gut symbiont, *Ruminococcus gnavus*, and allergic rhinitis was convincingly demonstrated in a recent study combining prospective findings in fecal samples from an infant twin cohort and a suitable mouse model of airway inflammation [60]. Moreover, interventions aimed at rebalancing enteric communities in gastrointestinal disorders, as the oral administration of probiotics or prebiotics, have proved beneficial in several studies of apparently unrelated conditions of the respiratory tract, including cystic fibrosis, allergic asthma, and rhinitis [61–64]. Thus, regardless of site-specific differences in the core microbiota lining the respiratory and gastrointestinal mucosa, connections must exist between these mucosal sites and the factors that regulate their homeostasis, which will be discussed in the following sections.

4. The Microbiota in the Development and Regulation of the Immune System

It was shown as early as in 1970 that Peyer's patches, the spleen, and the lymph nodes of mice hosted in a sterile environment are underdeveloped and do not contain germinal centers, resulting in reduced serum immunoglobulin levels, and that normal immune system development and function were restored following oral administration of *Salmonella paratyphi* A [65]. Such germ-free, or *gnotobiotic*, mice, lacking a microbial antigenic stimulus and presenting an immature immune system, are still a widely used *in vivo* model to dissect host-microbe and microbe-microbe interactions at the gut mucosa [66]. An alternative mouse model allows dissecting the contribution of at least certain bacterial commensals to the host immune response via the sustained administration of distinct classes of antibiotics [67]. Knowledge acquired in these overall study models represents the experimental basis for the dominant current paradigms on how these interactions regulate the immune response and other processes.

The gut microbiota contributes to shaping both the innate and adaptive components of the immune systems. These include the gut-associated lymphoid tissue (GALT), effector T cells, regulatory T cells (Treg), IgA-producing B cells and plasma cells, innate lymphoid cells (ILC), and resident macrophages and dendritic cells in the lamina propria [68, 69]. The development and function of Peyer's patches is a case in point, as it is the macroscopic epiphenomenon of a complex molecular process. A number of studies have focused on the mediators involved in the interaction between the gut microbiota and the production of IgA from B cells in Peyer's patches. It has been shown that the coadministration of retinoic acid and the Toll-like receptor- (TLR-) 4 ligand, lipopolysaccharide (LPS), a toxic by-product of Gram-negative bacteria also referred to as *endotoxin*, stimulates follicular dendritic cells to secrete B-cell activating factor (BAFF), the chemokine, C-X-C motif ligand 13, and transforming growth factor- (TGF-) β, which collectively act onto Peyer's patch B cells to promote class switch recombination and the production of IgA [70].

It is well known that dimeric IgA are a fundamental effector arm of mucosal immunity and that IgA dimers produced from B cells activated in Peyer's patches play a significant role in the mucosal firewall and the prevention of infections both locally and at distant sites [71, 72]. Several lines of evidence, including studies in patients with selective IgA deficiency, suggest that proper IgA induction within the GALT also confers protection against allergic and autoimmune inflammatory conditions at distant sites [73, 74]. Accordingly, IgA-inducing, viable strains of *Bifidobacterium* and *Lactobacillus* have been shown to alleviate symptoms of pollen-induced rhinitis when administered orally at the onset of pollen season [62]. In addition, recent work has identified significant associations between the development of influenza-specific nasal IgA responses and the presence of such microbial species in the nasal mucosa, as certain *Lactobacillus* and *Bacteroides* strains [75]. While the GALT is considered the primary induction site for body-wide IgA production, antigen-specific, mucosal IgA responses can in fact be promoted in subjects administered an intranasal vaccine [76, 77]. The demonstration of comparable levels of IgA class switch by-products in Peyer's patches and the nasal mucosa provide factual evidence that the nasopharynx-associated lymphoid tissue may represent a primary IgA induction site (Figure 1) [78, 79]. While it was thought that primary antibody responses could only develop in secondary lymphoid organs, for example, lymph nodes and Peyer's patches, it is now clear that more or less organized ectopic or *tertiary* lymphoid tissue may form in the respiratory mucosa, where naïve B cells undergo class-switch recombination and the production of high-affinity antibodies (reviewed in [80]). This phenomenon is more accentuated in inflammatory conditions and on instances has been associated with the development of autoimmunity [80]. Of note, increased numbers of B cells and plasma cells, elevated levels of BAFF, and the local production of antinuclear autoantibodies of the IgG and IgA isotypes have been observed in the nasal polyps of patients with CRS [81, 82]. Naïve or IgA$^+$ B cells in the nasal mucosa have also been shown to switch to IgE production in allergic rhinitis patients, which represents a conceptual basis in the appreciation of a subset of patients with local allergic rhinitis [8, 83].

Studies in gnotobiotic or antibiotic-treated mice have also provided evidence for a central role of the gut microbiota

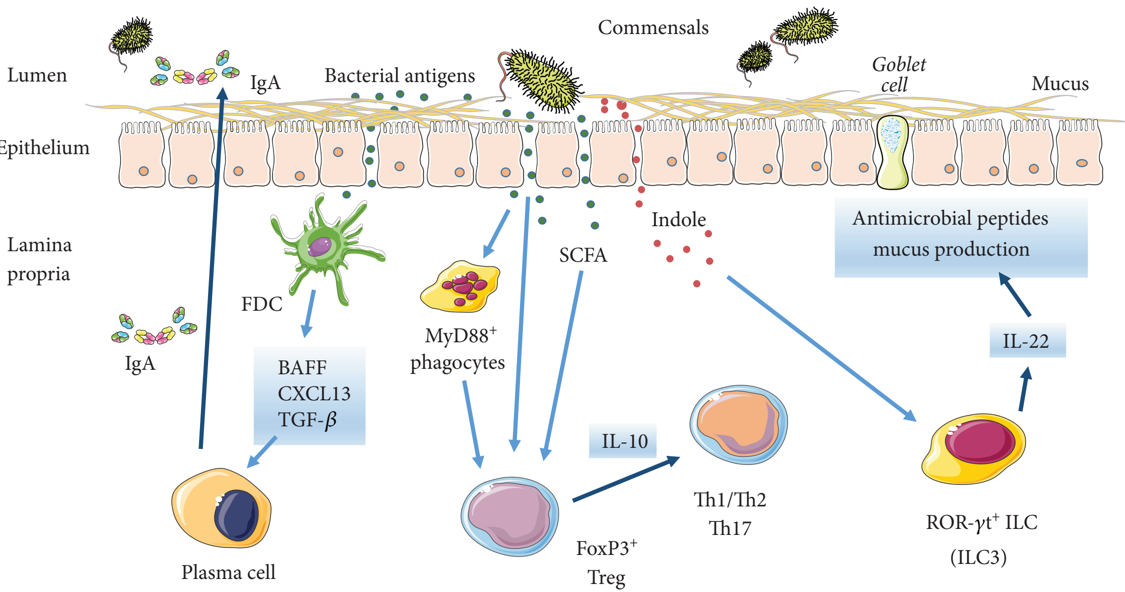

Figure 1: The regulation of immune responses by microbiota-associated factors. Follicular dendritic cells (FDC), myeloid differentiation primary response 88 $(MyD88)^+$ phagocytes, and ROR-γt^+ ILC are stimulated by commensal bacteria to produce cytokines and other proinflammatory mediators. These responses involve, among other outcomes, the secretion of dimeric IgA, mucus, and antimicrobial peptides and are critically regulated by $FoxP3^+$ Treg cells (see text).

in the regulation of effector T cell responses. Selective depletion of gut-associated Gram-positive communities in neomycin-treated mice was shown to be sufficient to impair the airway innate and adaptive response to influenza virus infection, which could be rescued following rectal or nasal inoculation of a mix of TLR ligands [67]. Allergic airway inflammation is typically enhanced in germ-free animals as a result of skewed activation of Th2 clones, suggesting a major role for gut colonization in the development of a balanced type 1 T helper (Th1)/Th2 response [84]. Consistently, reduced Th1 responses can be observed in early infancy, especially in infants delivered by cesarean section, in whom an increased predisposition to develop allergic disease is associated with delayed gut colonization of *Bacteroides* species and a less diverse microbial community [85]. These and similar studies provide factual evidence in support of the *hygiene* hypothesis, according to which exposure to a declining environmental biodiversity is a major contributing factor in the increasing prevalence of allergic and other chronic inflammatory diseases as it adversely affects the human microbiota and its central functions in the development and regulation of the immune system [55].

Several studies have focused on the interactions of $FoxP3^+$ Treg cells, a fundamental player in the immune regulatory network, with microbiota-delivered signals. A minimally diverse flora is required for activation and expansion of Treg cells and the production of interleukin- (IL-) 10, which involves the interaction of a host of bacterial components with distinct TLR or other pattern recognition receptors and depends on myeloid differentiation primary response 88-mediated signaling (Figure 1) [86, 87]. One such component is polysaccharide A from *Bacteroides fragilis*, which interacts with TLR-2 [88]. These effects, however, are apparently ligand-specific, as various TLR-2 ligands have been reported to either augment or decrease IL-10 production and Treg suppressive function [89]. Microbial metabolites, for example, butyrate and other short-chain fatty acids from *Bacteroides* and *Clostridium* species, can also direct the development and function of Treg cells and do so via the interaction with G protein-coupled receptors expressed by the intestinal epithelial cells and mucosal $CD103^+$ dendritic cells [90, 91].

Induction and maintenance of a tolerogenic, Treg-dominated immune profile in the gut mucosa has been invoked to explain the beneficial anti-inflammatory properties of *Lactobacillus* and *Bifidobacterium* probiotic mixtures and of high-fiber diet [90, 92–94]. It is inferred, then, that an imbalanced diet would be associated with a dysbiotic microbial community and an augmented predisposition for inflammatory disease. Studies in germ-free mice have identified distinct bacterial species, for example, segmented filamentous bacteria, which can sustain autoimmune inflammation in models of arthritis and multiple sclerosis via the activation of IL-17-producing T helper (Th17) cells [95–97]. A more or less direct connection between diet, the microbiota composition, and allergic inflammation is postulated in several studies [64, 98]. Studies in germ-free and mutant mice have shown that the microbiota can regulate Th2-driven immunity through the induction of Th17 cells and of a subset of Treg cells expressing the Th17 hormone receptor, retinoid-related orphan receptor-(ROR-) γt [99]. Symbiotic *Lactobacillus* strains and other bacterial species may directly activate these cells, as well as ROR-γt^+ type 3 ILC (ILC3), through the production of tryptophan-indole derivatives [100]. By engaging the aryl-hydrocarbon receptor, these metabolites induce in these cells the production of IL-22, a cytokine that promotes epithelial cell regeneration and the secretion of

antimicrobial peptides and mucus, thus contributing to intestinal homeostasis (Figure 1) [100].

The beneficial effects of a properly balanced diet and/or the supplementation with oral probiotics in respiratory allergy support the notion that signals from the gut microbiota can shape local immunity at distant sites, including the upper and lower airways [64, 98]. In fact, evidence for a bidirectional crosstalk between the respiratory and gastrointestinal mucosa is provided in several studies [101, 102]. It has long been known that airway inflammatory changes of some sort are detectable in about 50% of patients with inflammatory bowel disease [101]. Conversely, gastrointestinal symptoms, including such disorders as eosinophilic esophagitis or gastroenteritis, are frequent comorbidities in children with asthma or allergic rhinitis (reviewed in [103]). This might reflect recirculation of inflammatory cells, for example, eosinophils, redirection of gut- or lung-homing antigen-specific cells, antigen cross-sensitization between the two sites, and/or concomitant changes in site-specific microbial communities (reviewed in [101]). However, changes in the nose and lung microbiota are found in patients with airway inflammation which hardly reflect the microbial environment in the gut [51, 52, 104, 105]. It has been shown that substantial changes in the lung microbiota, which also take place in the first few weeks of life, are sufficient to drastically reduce Th2-driven eosinophilic responses to aeroallergens by promoting the emergence of a Helios^{-} Treg subset via engagement of programmed death ligand 1 [106]. Nasal administration of *Lactobacillus* strains is sufficient to accelerate the recovery of functional humoral immune responses against respiratory pathogens in malnourished mice [107]. A reduced load of *S. aureus*, a pathogen commonly hosted in the inflamed nasal mucosa, and lower goblet cell counts are seen following intranasal administration of *S. epidermidis* [108]. Preclinical evaluations of similar probiotics preparations, which are shown to induce IL-10 expression in human PBMC, support the notion that they promote tolerance via the activation of distinct Treg cell subsets [109]. A decreased ratio of Treg to effector T cells can be appreciated in the inflamed nasal mucosa, as shown in adults with seasonal allergic rhinitis and, regardless of the allergic status, in those with CRS with nasal polyps (CRSwNP) or without nasal polyps (CRSsNP) [110, 111]. Consistently, significantly increased numbers of FoxP3^{+} Treg cells are seen in the nasal mucosa of adult patients with allergic rhinitis patients who successfully underwent grass pollen immunotherapy [112]. It can be envisioned then, as implicated in studies in mice administered nasal *Lactobacillus* probiotics carrying a specific allergen, that direct manipulation of the nasal commensal flora may significantly contribute to shaping the local immune response to promote antigen-specific tolerance via the induction of Treg cells [113].

5. Regulation of Epithelial Cell Functions by the Microbiota

Epithelial cells and their functions are well-established direct targets of microbiota-delivered signals. Epithelial cells lining the mucosa contribute to immune regulation via the production of cytokines and chemokines and by providing a dynamical barrier to corpuscular and molecular antigens present in the environment. The mucin layer that coats epithelial surfaces physically excludes commensal microbes [114]. Direct microbial contact with epithelial cell surfaces can occur in the absence of mucin layers or when specific microbes can penetrate mucin (Figure 1) [114]. Muc2 is the predominant mucin secreted by goblet cells in the intestine [115]. Two mucus layers are organized in the colonic mucosa, the innermost of which is dense and impenetrable to bacteria [114]. A more penetrable mucus layer and reduced barrier function have been detected in C57BL/6 mice being fed an autoclaved chow relative to wild or experimental mice on a standard diet [116]. This was associated with pronounced differences in the composition of the colonic microbiota at the class and genus level and could be reproduced in germ-free mice transferred with caecal contents from either diet group [116]. While the mechanisms of microbial regulation of mucus formation and stability have not been elucidated, it is likely that these reflect the differential abilities of bacterial strains to process and degrade carbohydrates of dietary or host origin, including Muc2-associated glycans [117].

The human nasal mucosa contains a substantial number of mucus-producing goblet cells. These are evenly distributed within the pseudostratified ciliated epithelium and are mostly concentrated in the maxillary sinus, where they represent about 40–70% of the surface cells [118]. Muc5B and Muc5AC are the prevailing mucins produced by the human respiratory epithelium and are produced at similar levels in the upper and lower airways [119]. Expression of both mucin genes has been found upregulated in the nasal and sinus mucosa of patients with CRSwNP or CRSsNP [120]. However, the pattern of mucin glycosylation appears to be altered in CRS and especially so in patients in which the nasal bacterial community assembles a biofilm [121]. While the cause-effect relationship of biofilm development and a dysfunctional mucus barrier has yet to be elucidated, it is well known that biofilm development favors antibiotic resistance and is associated with persisting inflammatory changes in the nasal and sinus mucosa, worse sinus symptoms, and poor clinical improvement following polyp removal [122]. One possibility is that increased mucin production in the absence of sufficient mucociliary clearance might lead to the formation of thickened mucus patches providing a favorable milieu for bacterial outgrowth, as previously postulated for *Pseudomonas aeruginosa* biofilm development in cystic fibrosis lungs [123]. Notably, a sizeable proportion of patients with refractory forms of CRSwNP are carriers of mutated alleles of the cystic fibrosis transmembrane regulator gene [124]. Besides *P. aeruginosa*, *S. aureus* is the most common isolate in biofilms from CRSwNP patients with relapsing disease after functional endoscopic sinus surgery [125].

Factors produced by *S. aureus* can promote nasal inflammatory changes by directly acting on epithelial tight-junction (TJ) components to compromise mucosal barrier function [126, 127]. It has been documented that exposure of nasal epithelial cells grown at the air-liquid interface (ALI) to *S.*

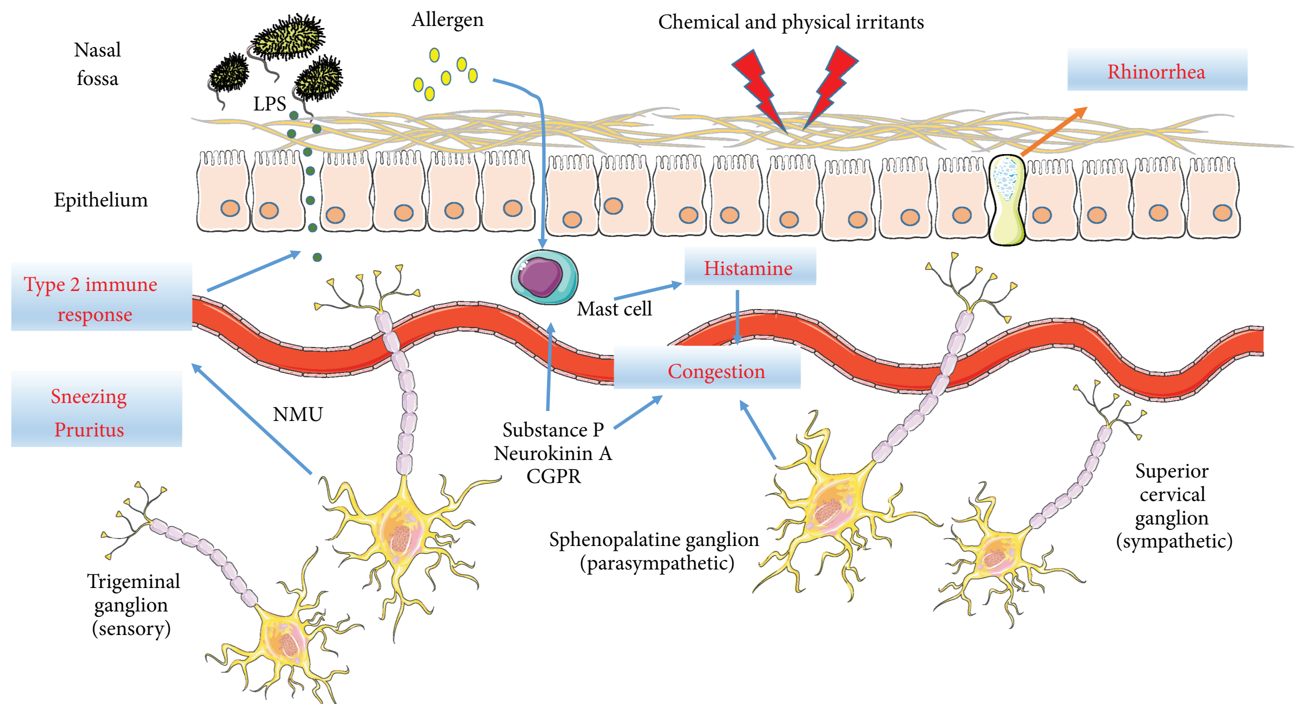

Figure 2: The relationship of allergen-induced, type 2 immune responses and the nasal nervous system. Trigeminal fibers are responsible for tactile sensitivity, including nociception, and release such neuropeptides as substance P, neurokinin A, CGPR, and possibly NMU (see text). These mediators induce vasodilation and directly activate cytotypes involved in the inflammatory response, for example, mast cells, eosinophils, lymphocytes, and macrophages. Parasympathetic postganglionic fibers release acetylcholine and induce vasodilation and mucus production, while norepinephrine from sympathetic fibers may induce vasoconstriction by interacting with α-adrenergic receptors, which typically prevails over vasodilation induced by concomitant ligation of β-receptors.

aureus-conditioned media determines a reduction of transepithelial electric resistance (TEER), a measure of barrier integrity, which is paralleled by the detection of a distinct separation between adjacent cells in the apical region, where TJ proteins are harbored [126]. These effects could be recapitulated upon exposure of these ALI cultures to staphylococcal V8 protease, which appeared to act on the assembly and expression of the TJ integral components, claudin-1 and *zonula occludens* protein- (ZO-) 1 [127]. As shown by Steelant et al. in two separate studies, the nasal epithelium from subjects allergic to dust mite or other inhalants expresses, both ex vivo and when cultured *in vitro*, reduced levels of occludin and ZO-1 and exhibits reduced TEER and increased permeability to fluorescein isothiocyanate-labelled 4 kDa dextran [128, 129]. Allergenic proteases, including dust mite major allergen, *Der* p 1, can at least in part account for these findings (Figure 2) [130]. TEER was further reduced in cells cultured in IL-4-supplemented media, suggesting that Th2-dependent immunity can affect the barrier function of the nasal mucosa [128]. In line with this notion, Saatian et al. demonstrated that the addition of IL-4 or IL-13 increases the permeability of airway epithelial cell monolayers *in vitro*, which is accompanied by the appearance of intercellular gaps and the accumulation of claudin-4 in cytoplasmic vesicles [131]. Besides Th2 cytokines, histamine, tumor necrosis factor-α, and the Th1 signature cytokine, IFN-γ, can also promote similar changes, including a decreased TEER and reduced claudin-1 and claudin-4 expression [129, 132]. Indeed, dysfunctional TJ and barrier function are not an exclusive feature of allergic inflammation but of chronic inflammatory processes in general [132].

The mechanisms of epithelial barrier disruption by pathogenic bacteria are not completely understood. It has long been known that certain bacterial toxins, for example, *C. perfringens* enterotoxin and *Vibrio cholerae zonula occludens* toxin (ZOT), may affect TJ integrity either by targeting its specific components or by binding to receptors shared with host-expressed homeostatic factors [133, 134]. One such factor, zonulin, shares with ZOT the ability to reversibly disassemble TJ complexes in the gut epithelium by interacting with protease-activated receptor-2 [135]. Zonulin may be induced upon exposure of small intestine monolayers to pathogenic enterobacteria or molecular patterns in certain nutrients, for example, gluten [136, 137]. Excess zonulin production, as a result of gluten intolerance or intestinal dysbiosis, and the ensuing loss of gut barrier integrity have been demonstrated in such chronic inflammatory conditions, as celiac disease and type I diabetes [135]. Less clear is zonulin involvement in the regulation of airway barrier function by the associated microbiota or aeroallergens. A molecule related to zonulin, presumably a serine protease, has been found to mediate albumin leak and complement activation in a mouse model of acute lunge injury [138]. An 8-mer peptide recapitulating zonulin and ZOT's effects on TJ complexes has the ability to increase the

permeability of the nasal mucosa and help deliver antigen to the submucosa, thereby acting as an effective adjuvant for mucosal vaccines [139]. Thus, it is feasible that zonulin, or a closely related molecule, may play a critical role in the regulation of the nasal epithelial barrier and the nasal response to allergens, irritants, and toxins.

6. The Integration of Microbial and Neuroimmune Signals

Neurogenic inflammation owes to the dense sensory innervation of the nasal mucosa. The nasal mucosa is densely innervated with trigeminal fibers. Trigeminal nociceptors in the nasal mucosa consist of myelinated and unmyelinated fibers. The former, named Aδ, transmit impulses faster than the slowly conducting, unmyelinated C fibers. C fibers mostly conduct nociceptive signals but also function as chemoreceptors in response to signals from transient receptor potential cation channel, subfamily A, member 1 (TRPA1), and transient receptor potential cation channel, subfamily V, member 1 (TRPV1), among others [140]. These are ionic channels that can be activated upon engagement by such specific ligands, as bradykinin to TRPA1 and lipid peroxidation or reactive oxygen species (ROS) to TRPV1. This implies that, besides sensory stimulation, also mediators of allergic inflammation can activate these receptors. The activation of TRPA1 and TRPV1 induces the inflow of cations and the antidromic depolarization of afferent fibers, also known as *axon reflex*. This in turn triggers the secretion, by the chemoreceptor itself, of such vasoactive mediators, as substance P, neurokinin A, neuropeptide Y, gastrin-releasing peptide (GRP), and calcitonin gene-related peptide (CGRP) (Figure 2) [141]. These neuropeptides contribute to vasodilation, glandular secretion, and lymphocyte and eosinophil effector function, resulting in such clinical symptoms, as congestion, rhinorrhea, sneezing, and headache, typical of nasal allergic inflammation (Figure 2) [142].

While nociceptive trigeminal fibers contribute to the development of allergic inflammation, they also directly affect nasal reactivity. In fact, the inhalation of irritating chemicals (gases, diesel particulate matter, etc.) induces more severe symptoms in allergic patients than in nonallergic controls, as shown in a 1998 study in which patients and controls were exposed to chlorine vapors [143]. Trigeminal nociceptors of allergic patients also have an increased activation threshold to tactile stimuli. We have detected a reduced tactile sensitivity of the nasal mucosa in elderly subjects following stimulation of the inferior turbinates with Semmes-Weinstein monofilaments [144]. Using a similar approach, we detected a reduction in the nasal tactile sensitivity of comparable grade in patients with allergic rhinitis compared to nonallergic controls [145]. Recent studies support a specific role for TRPA1 channels in the detection and response to microbial products. It has been shown that TRPA1 in vagal and somatic nociceptors can be activated by LPS, leading to the local release of such neuropeptides as CGRP, pain, neurogenic inflammation, and vasodilation (Figure 2) [146]. These effects of bacterial endotoxin are very fast, occurring within seconds of exposure and independently of TLR-4 ligation, and presumably involve specific structural features in lipid A, the LPS biologically active lipid moiety [146]. Similar results were obtained in a study in which distinct bacterial products, for example, N-formylated peptides, could mediate *S. aureus*-induced mechanical and thermal hyperalgesia in mice by directly activating nociceptors [147].

Taken together, these findings have led to the appreciation that bacterial products can induce a neuroinflammatory response independently of their interactions with the innate or adaptive immune system. The role of nociceptors in amplifying pathological immune responses to adaptive stimuli is in turn stressed in another study, in which ablation or pharmacological inhibition of Nav1.8-lineage neurons, which include the subpopulation of TRPA1$^+$ nociceptors, decreased eosinophilia and macrophage accumulation in bronchoalveolar lavage fluids of mice subjected to allergen challenge [148]. One possible mechanism by which neurotransmitters can enhance the mucosal immune response to pathogens and allergens is suggested in recent studies showing that the neuropeptide neuromedin U (NMU), expressed in cholinergic neurons localized in the mouse gastrointestinal tract, can directly activate type 2 ILC (ILC2) through the interaction with the specific receptor, NMUR1 (Figure 2) [149]. NMU release occurred subsequent to direct sensing of parasite products and alarmins, and NMUR1-dependent induction of the cytokines IL-5, IL-9, and IL-13 in this model was found to promote accelerated parasite expulsion [149, 150]. Moreover, coadministration of NMU and the ILC2-activating epithelial alarmin, IL-25, strongly amplified airway inflammation in mice who underwent allergen challenge [151]. A similar crosstalk of neuroinflammatory signals in the nasal mucosa is suggested in studies showing that ligands of the GRP and neuromedin B receptors can interact with related or unrelated G protein-coupled receptors (GPCR), such as N-formyl peptide receptors (FPR), to promote mucus secretion, neutrophil recruitment, and the production of ROS [152, 153]. Thus, it can be envisioned that signals emanating from the microbial community may engage a complex interaction with immune and nervous system components within the nasal ecosystem as recently appreciated in the enteric mucosa [154].

7. Microbial Regulation of Tissue Remodeling in Nasal Inflammation

Morphologic alterations of the nasal mucosa are variably observed in patients with chronic inflammatory disorders of the nose and sinuses. These range from simple hypertrophy, mesenchymal transition, collagen deposition and fibrosis, polypoid degeneration, to polyps of various sizes and extension, resulting from different grades of tissue remodeling processes [9]. Tissue remodeling is defined by transient or permanent changes in tissue architecture, which involves breakdown of tissue structures, for example, basement membranes and interstitial stroma, as well as repair [155]. A pseudostratified, respiratory epithelium comprising ciliated and secretory cells and supported by basal cells lines the nasal and paranasal sinuses. In reactive nasal inflammation, its morphology is subverted and characterized by squamous

metaplasia, ciliary destruction, increase of microfold cells, and mucous gland and goblet cell hyperplasia [9]. Although angiogenesis appears to be an important event in these processes, little is known about the mechanisms of vascular remodeling in the nasal mucosa. Numerous factors are dysregulated in the CRS mucosa which are involved in vessel remodeling, including TGF-β, platelet-derived growth factor, periostin, and vascular-endothelial growth factor (VEGF) [156–159]. Among these, VEGF could play a key role in polyp formation in CRS, thanks not only to its proedematous and angiogenic properties but also to its ability to promote nasal epithelial cell growth and resistance to apoptosis [156].

Several studies indicate that such innate immunity receptors, as FPR-1, FPR-2, and FPR-3, and matrix metalloproteases (MMP) may mediate the effects of microbial components on the tissue remodeling processes taking place in these conditions. The FPR are GPCR for the N-formylated peptides present in bacterial cell walls or in mitochondria and are expressed on the membrane of mononuclear and polymorphonuclear leukocytes. FPR ligation activates recruitment and activation of these cells via the engagement of phosphorylation cascades involving Akt, protein kinase C, and the Ras-mitogen-activated protein kinase pathway [160]. We hypothesized that engagement of FPR by bacterial ligands might be one possible mechanism linking nasal inflammation and dysbiosis to tissue remodeling processes leading to polyp formation. As shown by Prevete et al., the FPR agonists, f-Met-Leu-Phe and $uPAR_{84-95}$, induce the migration of nasal epithelial cells *in vitro* and the production of VEGF and TGF-β, two factors involved in tissue remodeling [161]. A significant increase in VEGF expression, both at the mRNA and protein levels, and of MMP species involved in tissue remodeling, including MMP-2 and MMP-9, has been detected in fibroblasts from nasal polyps following *in vitro* infection with HRV-16 [162]. Consistently, in the nasal mucosa of patients with CRSwNP, we have detected a marked increase in the expression of MMP-2, MMP-7, and MMP-9, which was paralleled by reduced expression of tissue inhibitor of MMP-(TIMP-) 1 and TIMP-2 [163].

That tissue remodeling and polyp formation may be favored in a dysbiotic microenvironment is inferred in a number of studies showing increased *S. aureus* colonization in the nasal cavities of patients with polyposis [164]. Recent work has shown that α-toxin, one of the major *S. aureus* toxins, can substantially contribute to airway remodeling via a combined effect on the epithelial cell cytoskeleton and endothelial TJ integrity, leading, respectively, to altered morphology and edema [165, 166]. However, the main mechanism this far ascertained by which *S. aureus* may induce or favor nasal polyp formation is the production of such superantigens as staphylococcal enterotoxins (SAE) [7, 167]. SAE can bind to invariant domains of the T-cell receptor and of the major histocompatibility complex-II of antigen-presenting cells (APC) and induce the production of T cell and APC cytokines and other factors. More than 20 SAE have been described to date, the most studied being type A (SEA) and type B (SEB) staphylococcal enterotoxins [168]. It has been documented that exposure to SEB can induce the secretion of both Th1- and Th2-restricted cytokines, for example, IFN-γ, IL-4, and IL-13, from the healthy nasal mucosa. However, a polypoid nasal mucosa would release increased amounts of these cytokines when exposed to a comparable load of SEB, possibly reflecting priming by co-colonizing microbial species [167]. Among these, fungal species, for example, *Malassezia* and *Aspergillus*, are present at higher abundance in at least certain CRS phenotypes and may contribute to immune activation in the nasal mucosa via the interaction with lectin and antigen receptors (discussed in [169]). Consistently, it has been shown that the polypoid tissue contains increased amounts of the eosinophil-specific chemokine, C-C motif ligand-11, eosinophil cationic protein, IL-5, total IgE, and SEA- and SEB-specific IgE, relative to controls with CRSsNP [7].

Thus, while the mechanism of *S. aureus*-induced polyp formation has not been elucidated to date, it is current belief that this process is mediated by chronic Th2-dependent, eosinophilic inflammation in a *S. aureus*-colonized mucosa. In contrast, CRSsNP is mostly characterized by a predominantly neutrophilic infiltrate and a diverse Th1, Th2, and Th17 cytokine profile [170]. Remodeling in this condition is characterized by excessive collagen production and thickening of the extracellular matrix [157]. This process is mediated by TGF-β, which is distinctly upregulated in the CRSsNP mucosa relative to CRSwNP [157]. These findings present important therapeutic implications, in that, while Th2-driven eosinophilic inflammation and polyp formation are relatively well controlled with, and at least partly reversed by, inhaled corticosteroids, Th17-dependent inflammation and TGF-β-mediated remodeling are not [171–173]. However, numerous exceptions challenge these paradigms [170]. As shown in Figure 3, patients with apparently similar clinical and histological pictures may respond differently to medical and surgical treatment. The identification of discrete endotypes within either CRS phenotype is a growing need given the enormous potential of newly available targeted biotechnological therapies, for example, anti-IgE and anticytokine monoclonal antibodies (reviewed in [174]). An initial definition of up to 10 CRS inflammatory endotypes and their clinical correlates is provided in recent studies of expression clusters of cytokines and other biomarkers in the nasal cavities [175, 176]. One of these studies confirmed the strong association of SAE-specific IgE, a proxy for *S. aureus* carriage within the nasal mucosa, with nasal polyposis, measures of Th2-driven inflammation, and asthma comorbidity [175].

These results imply that distinct microbial signatures might be recognized across the expanding repertoire of CRS clinical and inflammatory endotypes. In a recent study comparing 16S rRNA sequences in paired swabs from the middle and inferior meatuses of adults with distinct nasal reactive inflammatory phenotypes, samples from patients with CRSsNP exhibited significantly lower overall microbial diversity relative to patients with CRSwNP, allergic rhinitis, and healthy controls [177]. Taxa enriched in CRSsNP relative to CRSwNP included *Streptococcus* and *Haemophilus* among others, whereas *Staphylococcus* and *Alloiococcus* were enriched in CRSwNP [177]. A separate 16S rRNA sequencing study of sinus brushings from a heterogeneous group of

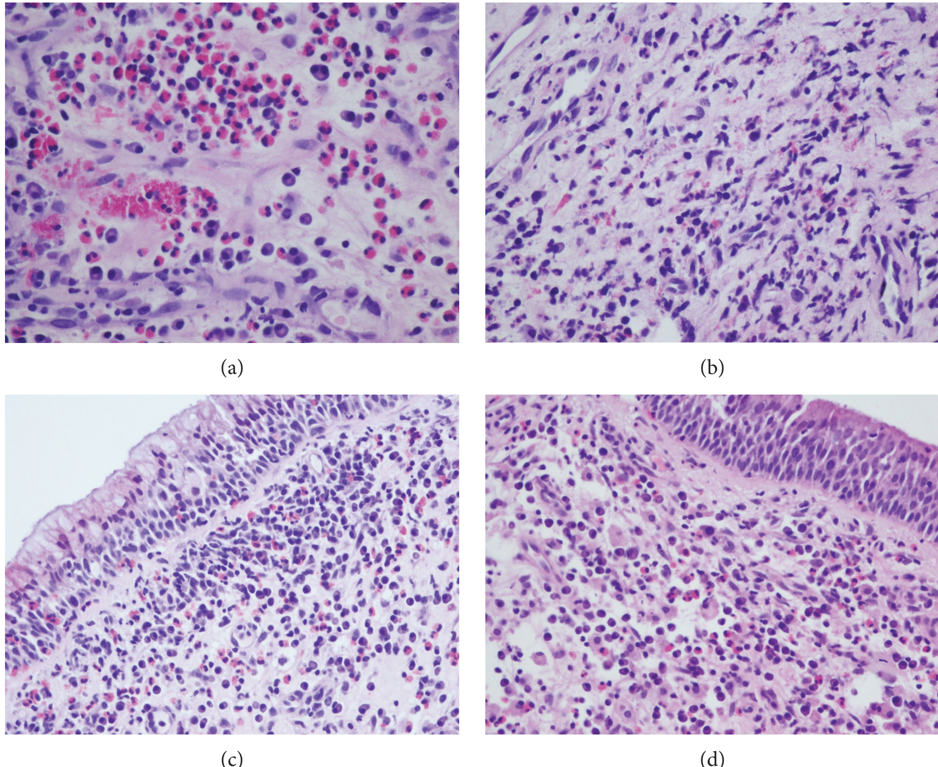

Figure 3: The effect of corticosteroid treatment on eosinophilic inflammation in CRSwNP. Hematoxylin and eosin stainings of polyp sections from two patients with CRSwNP before (a and c) and after (b and d) a short course of oral prednisone (0.4 mg/kg/day for 7 days) prior to polyp resection by functional endoscopic sinus surgery. Both patients did not have any allergies and were aspirin-intolerant. Polyp size and eosinophilic infiltration in the first patient (a and b), a 50-year-old female, were promptly reduced following prednisone administration, whereas polyp size and histology in the second patient (c and d), a 69-year-old male, remained substantially unchanged (F.A. Salzano and C. Stellato, unpublished observations).

adults with CRS identified three main groups of patients based on bacterial community composition [178]. The largest group of patients, mostly with CRSwNP and a Th2-biased immune phenotype, exhibited a sinus microbiota reciprocally dominated by *Corynebacteriaceae* or *Staphylococcaceae* [178]. This confirmed that competing interactions may exist between these microbial families, whereby an increased colonization of a corynebacterial species, *C. pseudodiphtheriticum*, is associated with a reduced colonization of *S. aureus* and vice versa [33]. This led to hypothesizing that a *C. pseudodiphtheriticum*-colonized mucosa provides a less favorable microenvironment to *S. aureus* growth [33]. Besides competition for nutrients, *S. aureus* outgrowth in the nasal mucosa might be limited by the antimicrobial molecules produced by certain microbial species, as documented for species colonizing the intestinal mucosa [179]. One such species, the coagulase-negative staphylococcal strain *S. lugdunensis*, a common dweller in the human nose, produces a natural antibiotic, named lugdunin, which exerts a distinctive antimicrobial activity against *S. aureus* strains including MRSA [180]. Taken together, these findings emphasize the potential impact of interventions aimed at manipulating the nasal microbial community in the setup of a less favorable milieu for colonization from pathogenic species and ultimately the predisposition to develop nasal inflammation with different grades of such associated morphologic alterations, as hyperplasia, thickening, fibrosis, polypoid degeneration, or polyps of various sizes and extensions.

8. Concluding Remarks

The relationship between nasal dysbiosis and reactive, allergic or nonallergic, nasal inflammation involves a complex network of processes regulating mucosal permeability and TJ function, neurogenic signals, innate immunity cells and receptors, vascular and mucosal remodeling factors, effector T cells and related cytokines, and the production of specific IgE or IgA antibodies. The literature to date has not clarified the timing and reciprocity of these connections, and whether, for instance, intrinsic alterations in the mechanisms governing barrier function would typically precede or follow immune activation and inflammation. Moreover, the precise mechanisms that lead to distinct clinical phenotypes and endotypes and the inherent specific inflammatory processes are still largely unknown. Regardless, it can be concluded that the barrier function of the nasal mucosa, or *mucosal firewall*, represents the key element linking nasal dysbiosis to the cellular and molecular processes that lead to and sustain inflammation. An increased mucosal permeability may in turn favor bacterial translocation to the submucosa, where antigen presentation and recognition take place, as well as the

interaction of bacterial components with innate immune and nociceptive receptors.

In light of these considerations, given the complex interactions between the microbial microenvironment, the nasal epithelium, the innate and adaptive immune system, and the nasal nervous system, it would be quite reductionist to classify nasal inflammatory processes based on the prevailing inflammatory cytotypes, for example, neutrophils, eosinophils, or mast cells, but should include at a minimum a definition of the immune phenotype or endotype to allow for a more targeted and effective line of intervention in the clinical management of patients with these conditions [174, 181]. In this light, even the resection of largely hyperplastic or extended, frankly polypoid lesions of the chronically inflamed nasal and sinus mucosa by minimally invasive, functional endoscopic surgery would not be seen as just the last resort whereby all other treatments have failed, but as the integral part of an organic strategy including conventional and biotechnological anti-inflammatory agents, antibiotics, and probiotics [182]. The possible effectiveness and appropriateness of probiotics in the management of at least certain CRS endotypes, and of reactive nasal inflammatory disorders in general, cannot be stressed enough. As confirmed in a study comparing the 16S rRNA sequence profiles before and after surgery of patients with refractory, relapsing forms of CRSwNP, conventional management of these patients with antibiotics and intranasal steroids is insufficient to prevent the rapid repopulation of the nasal mucosa with the baseline bacterial communities [183].

As discussed in this review, the rationale for probiotics administration in allergic rhinitis, CRS, and related nasal reactive disorders comes from studies documenting the antagonistic interactions of symbiotic and pathogenic species within the nasal mucosa or other niches [33, 108, 179, 180] and the ability of certain symbiotic species to regulate the fine balance between host immunity and tolerance [61, 75, 90–94, 100, 107, 113]. However, studies looking at the effects of oral probiotics on several clinical measures in pediatric patients with allergic rhinitis do not show consistent results [63, 184], and the findings from sparse reports in adults with CRS are discouraging [185]. On the other hand, the clinical use of nasal probiotics is supported in a number of preclinical studies and has shown promise in early-stage clinical studies in children with recurrent upper respiratory infections [107, 108, 186]. More studies are needed to fully understand the potential of this approach as a support treatment in nasal inflammatory conditions, for example, in allergic rhinitis patients undergoing specific immunotherapy and in surgical patients with resistant and relapsing forms of CRS.

In this review, we have focused on the possible mechanisms mediating disruption of the basic homeostatic functions of the human nasal mucosa concomitant to alterations in the local microbiota, which have been documented in nasal inflammatory conditions. Much of our knowledge comes from studies of gut bacterial communities, which provide a solid basis to understand the complex interactions between the host mucosa and the microbial milieu. Future studies will hopefully reveal how unique changes in the nasal microbiota, including viral and fungal components, result in distinct clinical phenotypes and how its manipulation may contribute to their current and prospective treatments.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

References

[1] C. Bodino, R. Jankowski, B. Grignon, A. Jimenez-Chobillon, and M. Braun, “Surgical anatomy of the turbinal wall of the ethmoidal labyrinth,” *Rhinology*, vol. 42, no. 2, pp. 73–80, 2004.

[2] J. R. Harkema, S. A. Carey, and J. G. Wagner, “The nose revisited: a brief review of the comparative structure, function, and toxicologic pathology of the nasal epithelium,” *Toxicologic Pathology*, vol. 34, no. 3, pp. 252–269, 2006.

[3] R. Pawankar, G. W. Canonica, S. T. Holgate, and R. F. Lockey, “Allergic diseases and asthma: a major global health concern,” *Current Opinion in Allergy and Clinical Immunology*, vol. 12, no. 1, pp. 39–41, 2012.

[4] P. W. Hellings, L. Klimek, C. Cingi et al., “Non-allergic rhinitis: position paper of the European Academy of Allergy and Clinical Immunology,” *Allergy*, vol. 72, no. 11, pp. 1657–1665, 2017.

[5] G. D’Amato, S. T. Holgate, R. Pawankar et al., “Meteorological conditions, climate change, new emerging factors, and asthma and related allergic disorders. A statement of the World Allergy Organization,” *World Allergy Organization Journal*, vol. 8, no. 1, pp. 1–52, 2015.

[6] Y.-G. Min, “The pathophysiology, diagnosis and treatment of allergic rhinitis,” *Allergy, Asthma & Immunology Research*, vol. 2, no. 2, pp. 65–76, 2010.

[7] C. Bachert, P. Gevaert, G. Holtappels, S. G. O. Johansson, and P. Van Cauwenberge, “Total and specific IgE in nasal polyps is related to local eosinophilic inflammation,” *Journal of Allergy and Clinical Immunology*, vol. 107, no. 4, pp. 607–614, 2001.

[8] C. Rondón, J. J. Romero, S. López et al., “Local IgE production and positive nasal provocation test in patients with persistent nonallergic rhinitis,” *Journal of Allergy and Clinical Immunology*, vol. 119, no. 4, pp. 899–905, 2007.

[9] R. Pawankar and M. Nonaka, “Inflammatory mechanisms and remodeling in chronic rhinosinusitis and nasal polyps,” *Current Allergy and Asthma Reports*, vol. 7, no. 3, pp. 202–208, 2007.

[10] M. J. Blaser, “The microbiome revolution,” *Journal of Clinical Investigation*, vol. 124, no. 10, pp. 4162–4165, 2014.

[11] M. Rescigno, “The microbiota revolution: excitement and caution,” *European Journal of Immunology*, vol. 47, no. 9, pp. 1406–1413, 2017.

[12] I. Cho and M. J. Blaser, “The human microbiome: at the interface of health and disease,” *Nature Reviews Genetics*, vol. 13, no. 4, pp. 260–270, 2012.

[13] S. M. Huse, Y. Ye, Y. Zhou, and A. A. Fodor, “A core human microbiome as viewed through 16S rRNA sequence clusters,” *PLoS One*, vol. 7, no. 6, article e34242, 2012.

[14] K. Li, M. Bihan, and B. A. Methé, “Analyses of the stability and core taxonomic memberships of the human microbiome,” *PLoS One*, vol. 8, no. 5, article e63139, 2013.

[15] J. Jovel, J. Patterson, W. Wang et al., "Characterization of the gut microbiome using 16S or shotgun metagenomics," *Frontiers in Microbiology*, vol. 7, 2016.

[16] T. C. Fung, C. A. Olson, and E. Y. Hsiao, "Interactions between the microbiota, immune and nervous systems in health and disease," *Nature Neuroscience*, vol. 20, no. 2, pp. 145–155, 2017.

[17] K. F. Budden, S. L. Gellatly, D. L. A. Wood et al., "Emerging pathogenic links between microbiota and the gut-lung axis," *Nature Reviews Microbiology*, vol. 15, no. 1, pp. 55–63, 2017.

[18] B. O. Schroeder and F. Bäckhed, "Signals from the gut microbiota to distant organs in physiology and disease," *Nature Medicine*, vol. 22, no. 10, pp. 1079–1089, 2016.

[19] S. Roy and G. Trinchieri, "Microbiota: a key orchestrator of cancer therapy," *Nature Reviews Cancer*, vol. 17, no. 5, pp. 271–285, 2017.

[20] L. N. Segal and M. J. Blaser, "A brave new world: the lung microbiota in an era of change," *Annals of the American Thoracic Society*, vol. 11, Supplement 1, pp. S21–S27, 2014.

[21] G. B. Huffnagle and M. C. Noverr, "The emerging world of the fungal microbiome," *Trends in Microbiology*, vol. 21, no. 7, pp. 334–341, 2013.

[22] N. Rascovan, R. Duraisamy, and C. Desnues, "Metagenomics and the human virome in asymptomatic individuals," *Annual Review of Microbiology*, vol. 70, no. 1, pp. 125–141, 2016.

[23] N. Yutin, K. S. Makarova, A. B. Gussow et al., "Discovery of an expansive bacteriophage family that includes the most abundant viruses from the human gut," *Nature Microbiology*, vol. 3, no. 1, pp. 38–46, 2018.

[24] R. Sender, S. Fuchs, and R. Milo, "Are we really vastly outnumbered? Revisiting the ratio of bacterial to host cells in humans," *Cell*, vol. 164, no. 3, pp. 337–340, 2016.

[25] S. Nguyen, K. Baker, B. S. Padman et al., "Bacteriophage transcytosis provides a mechanism to cross epithelial cell layers," *MBio*, vol. 8, no. 6, pp. e01874–e01817, 2017.

[26] D. Rothschild, O. Weissbrod, E. Barkan et al., "Environment dominates over host genetics in shaping human gut microbiota," *Nature*, vol. 555, no. 7695, pp. 210–215, 2018.

[27] L. Shaw, A. L. R. Ribeiro, A. P. Levine et al., "The human salivary microbiome is shaped by shared environment rather than genetics: evidence from a large family of closely related individuals," *MBio*, vol. 8, no. 5, pp. e01237–e01217, 2017.

[28] K. Kodukula, D. V. Faller, D. N. Harpp et al., "Gut microbiota and salivary diagnostics: the mouth is salivating to tell us something," *BioResearch Open Access*, vol. 6, no. 1, pp. 123–132, 2017.

[29] J. S. Bajaj, N. S. Betrapally, P. B. Hylemon et al., "Salivary microbiota reflects changes in gut microbiota in cirrhosis with hepatic encephalopathy," *Hepatology*, vol. 62, no. 4, pp. 1260–1271, 2015.

[30] X. Zhang, D. Zhang, H. Jia et al., "The oral and gut microbiomes are perturbed in rheumatoid arthritis and partly normalized after treatment," *Nature Medicine*, vol. 21, no. 8, pp. 895–905, 2015.

[31] A. Tsuda, W. Suda, H. Morita et al., "Influence of proton-pump inhibitors on the luminal microbiota in the gastrointestinal tract," *Clinical and Translational Gastroenterology*, vol. 6, no. 6, pp. e89–e89, 2015.

[32] E. Zaura, B. W. Brandt, M. J. Teixeira de Mattos et al., "Same exposure but two radically different responses to antibiotics: resilience of the salivary microbiome versus long-term microbial shifts in feces," *MBio*, vol. 6, no. 6, pp. e01693–e01615, 2015.

[33] M. Yan, S. J. Pamp, J. Fukuyama et al., "Nasal microenvironments and interspecific interactions influence nasal microbiota complexity and S. aureus carriage," *Cell Host & Microbe*, vol. 14, no. 6, pp. 631–640, 2013.

[34] U. Kaspar, A. Kriegeskorte, T. Schubert et al., "The culturome of the human nose habitats reveals individual bacterial fingerprint patterns," *Environmental Microbiology*, vol. 18, no. 7, pp. 2130–2142, 2016.

[35] K. P. Lemon, V. Klepac-Ceraj, H. K. Schiffer, E. L. Brodie, S. V. Lynch, and R. Kolter, "Comparative analyses of the bacterial microbiota of the human nostril and oropharynx," *MBio*, vol. 1, no. 3, article e00129-10, 2010.

[36] I. De Boeck, S. Wittouck, S. Wuyts et al., "Comparing the healthy nose and nasopharynx microbiota reveals continuity as well as niche-specificity," *Frontiers in Microbiology*, vol. 8, 2017.

[37] G. G. Einarsson, D. M. Comer, L. McIlreavey et al., "Community dynamics and the lower airway microbiota in stable chronic obstructive pulmonary disease, smokers and healthy non-smokers," *Thorax*, vol. 71, no. 9, pp. 795–803, 2016.

[38] E. Allen, A. F. Koeppel, J. Hendley, S. D. Turner, B. Winther, and M. M. Sale, "Characterization of the nasopharyngeal microbiota in health and during rhinovirus challenge," *Microbiome*, vol. 2, no. 1, p. 22, 2014.

[39] P. N. Luna, K. Hasegawa, N. J. Ajami et al., "The association between anterior nares and nasopharyngeal microbiota in infants hospitalized for bronchiolitis," *Microbiome*, vol. 6, no. 1, p. 2, 2018.

[40] M. Pichon, B. Lina, and L. Josset, "Impact of the respiratory microbiome on host responses to respiratory viral infection," *Vaccines*, vol. 5, no. 4, 2017.

[41] S. Langevin, M. Pichon, E. Smith et al., "Early nasopharyngeal microbial signature associated with severe influenza in children: a retrospective pilot study," *Journal of General Virology*, vol. 98, no. 10, pp. 2425–2437, 2017.

[42] I. Korten, M. Mika, S. Klenja et al., "Interactions of respiratory viruses and the nasal microbiota during the first year of life in healthy infants," *mSphere*, vol. 1, no. 6, pp. e00312–e00316, 2016.

[43] Y. Tarabichi, K. Li, S. Hu et al., "The administration of intranasal live attenuated influenza vaccine induces changes in the nasal microbiota and nasal epithelium gene expression profiles," *Microbiome*, vol. 3, no. 1, p. 74, 2015.

[44] J. J. Hofstra, S. Matamoros, M. A. van de Pol et al., "Changes in microbiota during experimental human Rhinovirus infection," *BMC Infectious Diseases*, vol. 15, no. 1, p. 336, 2015.

[45] B. A. Duerkop and L. V. Hooper, "Resident viruses and their interactions with the immune system," *Nature Immunology*, vol. 14, no. 7, pp. 654–659, 2013.

[46] P. J. Planet, D. Parker, T. S. Cohen et al., "Lambda interferon restructures the nasal microbiome and increases susceptibility to Staphylococcus aureus superinfection," *MBio*, vol. 7, no. 1, pp. e01939–e01915, 2016.

[47] D. N. Frank, L. M. Feazel, M. T. Bessesen, C. S. Price, E. N. Janoff, and N. R. Pace, "The human nasal microbiota and Staphylococcus aureus carriage," *PLoS One*, vol. 5, no. 5, article e10598, 2010.

[48] B. Tilahun, A. C. Faust, P. McCorstin, and A. Ortegon, "Nasal colonization and lower respiratory tract infections with

methicillin-resistant Staphylococcus aureus," *American Journal of Critical Care*, vol. 24, no. 1, pp. 8–12, 2015.

[49] M. E. Mulcahy and R. M. McLoughlin, "Staphylococcus aureus and influenza a virus: partners in coinfection," *MBio*, vol. 7, no. 6, pp. e02068–e02016, 2016.

[50] F. A. Salzano, L. d'Angelo, S. Motta, A. del Prete, M. Gentile, and G. Motta Jr, "Allergic rhinoconjunctivitis: diagnostic and clinical assessment," *Rhinology*, vol. 30, no. 4, pp. 265–275, 1992.

[51] C. Pastacaldi, P. Lewis, and P. Howarth, "Staphylococci and staphylococcal superantigens in asthma and rhinitis: a systematic review and meta-analysis," *Allergy*, vol. 66, no. 4, pp. 549–555, 2011.

[52] C. H. Choi, V. Poroyko, S. Watanabe et al., "Seasonal allergic rhinitis affects sinonasal microbiota," *American Journal of Rhinology and Allergy*, vol. 28, no. 4, pp. 281–286, 2014.

[53] M. Mahdavinia, A. Keshavarzian, M. C. Tobin, A. L. Landay, and R. P. Schleimer, "A comprehensive review of the nasal microbiome in chronic rhinosinusitis (CRS)," *Clinical & Experimental Allergy*, vol. 46, no. 1, pp. 21–41, 2016.

[54] Y. Belkaid and T. W. Hand, "Role of the microbiota in immunity and inflammation," *Cell*, vol. 157, no. 1, pp. 121–141, 2014.

[55] I. Hanski, L. von Hertzen, N. Fyhrquist et al., "Environmental biodiversity, human microbiota, and allergy are interrelated," *Proceedings of the National Academy of Sciences*, vol. 109, no. 21, pp. 8334–8339, 2012.

[56] T. R. Abrahamsson, H. E. Jakobsson, A. F. Andersson, B. Björkstén, L. Engstrand, and M. C. Jenmalm, "Low gut microbiota diversity in early infancy precedes asthma at school age," *Clinical & Experimental Allergy*, vol. 44, no. 6, pp. 842–850, 2014.

[57] J. A. Geoghegan, A. D. Irvine, and T. J. Foster, "Staphylococcus aureus and atopic dermatitis: a complex and evolving relationship," *Trends in Microbiology*, vol. 26, no. 6, pp. 484–497, 2018.

[58] T. T. Hansel, T. Tunstall, M. B. Trujillo-Torralbo et al., "A comprehensive evaluation of nasal and bronchial cytokines and chemokines following experimental rhinovirus infection in allergic asthma: increased interferons (IFN-γ and IFN-λ) and type 2 inflammation (IL-5 and IL-13)," *eBioMedicine*, vol. 19, pp. 128–138, 2017.

[59] F. Lan, N. Zhang, E. Gevaert, L. Zhang, and C. Bachert, "Viruses and bacteria in Th2-biased allergic airway disease," *Allergy*, vol. 71, no. 10, pp. 1381–1392, 2016.

[60] H. H. Chua, H. C. Chou, Y. L. Tung et al., "Intestinal dysbiosis featuring abundance of Ruminococcus gnavus associates with allergic diseases in infants," *Gastroenterology*, vol. 154, no. 1, pp. 154–167, 2018.

[61] E. Bruzzese, V. Raia, M. I. Spagnuolo et al., "Effect of Lactobacillus GG supplementation on pulmonary exacerbations in patients with cystic fibrosis: a pilot study," *Clinical Nutrition*, vol. 26, no. 3, pp. 322–328, 2007.

[62] A. C. Ouwehand, M. Nermes, M. C. Collado, N. Rautonen, S. Salminen, and E. Isolauri, "Specific probiotics alleviate allergic rhinitis during the birch pollen season," *World Journal of Gastroenterology*, vol. 15, no. 26, pp. 3261–3268, 2009.

[63] R. R. Das, M. Singh, and N. Shafiq, "Probiotics in treatment of allergic rhinitis," *World Allergy Organization Journal*, vol. 3, no. 9, pp. 239–244, 2010.

[64] Y. J. Huang, B. J. Marsland, S. Bunyavanich et al., "The microbiome in allergic disease: current understanding and future opportunities—2017 PRACTALL document of the American Academy of Allergy, Asthma & Immunology and the European Academy of Allergy and Clinical Immunology," *Journal of Allergy and Clinical Immunology*, vol. 139, no. 4, pp. 1099–1110, 2017.

[65] M. Pollard and N. Sharon, "Responses of the Peyer's patches in germ-free mice to antigenic stimulation," *Infection and Immunity*, vol. 2, no. 1, pp. 96–100, 1970.

[66] R. Martín, L. G. Bermúdez-Humarán, and P. Langella, "Gnotobiotic rodents: an in vivo model for the study of microbe-microbe interactions," *Frontiers in Microbiology*, vol. 7, 2016.

[67] T. Ichinohe, I. K. Pang, Y. Kumamoto et al., "Microbiota regulates immune defense against respiratory tract influenza A virus infection," *Proceedings of the National Academy of Sciences*, vol. 108, no. 13, pp. 5354–5359, 2011.

[68] K. Honda and D. R. Littman, "The microbiota in adaptive immune homeostasis and disease," *Nature*, vol. 535, no. 7610, pp. 75–84, 2016.

[69] C. A. Thaiss, N. Zmora, M. Levy, and E. Elinav, "The microbiome and innate immunity," *Nature*, vol. 535, no. 7610, pp. 65–74, 2016.

[70] K. Suzuki, M. Maruya, S. Kawamoto et al., "The sensing of environmental stimuli by follicular dendritic cells promotes immunoglobulin a generation in the gut," *Immunity*, vol. 33, no. 1, pp. 71–83, 2010.

[71] A. J. Macpherson, M. B. Geuking, and K. D. McCoy, "Homeland security: IgA immunity at the frontiers of the body," *Trends in Immunology*, vol. 33, no. 4, pp. 160–167, 2012.

[72] N. Lycke, "Recent progress in mucosal vaccine development: potential and limitations," *Nature Reviews Immunology*, vol. 12, no. 8, pp. 592–605, 2012.

[73] R. Yazdani, A. H. Latif, F. Tabassomi et al., "Clinical phenotype classification for selective immunoglobulin A deficiency," *Expert Review of Clinical Immunology*, vol. 11, no. 11, pp. 1245–1254, 2015.

[74] A. Bonnegarde-Bernard, J. Jee, M. J. Fial et al., "IKKβ in intestinal epithelial cells regulates allergen-specific IgA and allergic inflammation at distant mucosal sites," *Mucosal Immunology*, vol. 7, no. 2, pp. 257–267, 2014.

[75] H. M. Salk, W. L. Simon, N. D. Lambert et al., "Taxa of the nasal microbiome are associated with influenza-specific IgA response to live attenuated influenza vaccine," *PLoS One*, vol. 11, no. 9, article e0162803, 2016.

[76] N. J. Carter and M. P. Curran, "Live attenuated influenza vaccine (FluMist®; Fluenz™): a review of its use in the prevention of seasonal influenza in children and adults," *Drugs*, vol. 71, no. 12, pp. 1591–1622, 2011.

[77] A. Rigter, I. Widjaja, H. Versantvoort et al., "A protective and safe intranasal RSV vaccine based on a recombinant prefusion-like form of the F protein bound to bacterium-like particles," *PLoS One*, vol. 8, no. 8, article e71072, 2013.

[78] H. Kiyono and S. Fukuyama, "NALT- versus Peyer's-patch-mediated mucosal immunity," *Nature Reviews Immunology*, vol. 4, no. 9, pp. 699–710, 2004.

[79] M. Lin, L. Du, P. Brandtzaeg, and Q. Pan-Hammarström, "IgA subclass switch recombination in human mucosal and systemic immune compartments," *Mucosal Immunology*, vol. 7, no. 3, pp. 511–520, 2014.

[80] A. Kato, K. E. Hulse, B. K. Tan, and R. P. Schleimer, "B-lymphocyte lineage cells and the respiratory system," *Journal of Allergy and Clinical Immunology*, vol. 131, no. 4, pp. 933–957, 2013.

[81] A. Kato, A. Peters, L. Suh et al., "Evidence of a role for B cell-activating factor of the TNF family in the pathogenesis of chronic rhinosinusitis with nasal polyps," *Journal of Allergy and Clinical Immunology*, vol. 121, no. 6, pp. 1385–1392.e2, 2008.

[82] B. K. Tan, Q. Z. Li, L. Suh et al., "Evidence for intranasal antinuclear autoantibodies in patients with chronic rhinosinusitis with nasal polyps," *Journal of Allergy and Clinical Immunology*, vol. 128, no. 6, pp. 1198–1206.e1, 2011.

[83] H. A. Coker, S. R. Durham, and H. J. Gould, "Local somatic hypermutation and class switch recombination in the nasal mucosa of allergic rhinitis patients," *The Journal of Immunology*, vol. 171, no. 10, pp. 5602–5610, 2003.

[84] T. Herbst, A. Sichelstiel, C. Schär et al., "Dysregulation of allergic airway inflammation in the absence of microbial colonization," *American Journal of Respiratory and Critical Care Medicine*, vol. 184, no. 2, pp. 198–205, 2011.

[85] H. E. Jakobsson, T. R. Abrahamsson, M. C. Jenmalm et al., "Decreased gut microbiota diversity, delayed Bacteroidetes colonisation and reduced Th1 responses in infants delivered by Caesarean section," *Gut*, vol. 63, no. 4, pp. 559–566, 2014.

[86] M. B. Geuking, J. Cahenzli, M. A. E. Lawson et al., "Intestinal bacterial colonization induces mutualistic regulatory T cell responses," *Immunity*, vol. 34, no. 5, pp. 794–806, 2011.

[87] S. Wang, L. M. Charbonnier, M. Noval Rivas et al., "MyD88 adaptor-dependent microbial sensing by regulatory T cells promotes mucosal tolerance and enforces commensalism," *Immunity*, vol. 43, no. 2, pp. 289–303, 2015.

[88] J. L. Round and S. K. Mazmanian, "Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota," *Proceedings of the National Academy of Sciences*, vol. 107, no. 27, pp. 12204–12209, 2010.

[89] M. H. Nyirenda, L. Sanvito, P. J. Darlington et al., "TLR2 stimulation drives human naive and effector regulatory T cells into a Th17-like phenotype with reduced suppressive function," *Journal of Immunology*, vol. 187, no. 5, pp. 2278–2290, 2011.

[90] J. Tan, C. McKenzie, P. J. Vuillermin et al., "Dietary fiber and bacterial SCFA enhance oral tolerance and protect against food allergy through diverse cellular pathways," *Cell Reports*, vol. 15, no. 12, pp. 2809–2824, 2016.

[91] N. Arpaia, C. Campbell, X. Fan et al., "Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation," *Nature*, vol. 504, no. 7480, pp. 451–455, 2013.

[92] M. Roselli, A. Finamore, S. Nuccitelli et al., "Prevention of TNBS-induced colitis by different Lactobacillus and Bifidobacterium strains is associated with an expansion of $\gamma\delta$T and regulatory T cells of intestinal intraepithelial lymphocytes," *Inflammatory Bowel Diseases*, vol. 15, no. 10, pp. 1526–1536, 2009.

[93] T. Poutahidis, M. Kleinewietfeld, C. Smillie et al., "Microbial reprogramming inhibits western diet-associated obesity," *PLoS One*, vol. 8, no. 7, article e68596, 2013.

[94] H. H. Smits, A. Engering, D. van der Kleij et al., "Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin," *Journal of Allergy and Clinical Immunology*, vol. 115, no. 6, pp. 1260–1267, 2005.

[95] A. Haghikia, S. Jörg, A. Duscha et al., "Dietary fatty acids directly impact central nervous system autoimmunity via the small intestine," *Immunity*, vol. 43, no. 4, pp. 817–829, 2015.

[96] I. I. Ivanov, K. Atarashi, N. Manel et al., "Induction of intestinal Th17 cells by segmented filamentous bacteria," *Cell*, vol. 139, no. 3, pp. 485–498, 2009.

[97] H.-J. Wu, I. I. Ivanov, J. Darce et al., "Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells," *Immunity*, vol. 32, no. 6, pp. 815–827, 2010.

[98] A. Trompette, E. S. Gollwitzer, K. Yadava et al., "Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis," *Nature Medicine*, vol. 20, no. 2, pp. 159–166, 2014.

[99] C. Ohnmacht, J.-H. Park, S. Cording et al., "The microbiota regulates type 2 immunity through ROR t+ T cells," *Science*, vol. 349, no. 6251, pp. 989–993, 2015.

[100] T. Zelante, R.. G. Iannitti, C. Cunha et al., "Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22," *Immunity*, vol. 39, no. 2, pp. 372–385, 2013.

[101] M. K. Tulic, T. Piche, and V. Verhasselt, "Lung-gut cross-talk: evidence, mechanisms and implications for the mucosal inflammatory diseases," *Clinical & Experimental Allergy*, vol. 46, no. 4, pp. 519–528, 2016.

[102] J. C. Madan, D. C. Koestler, B. A. Stanton et al., "Serial analysis of the gut and respiratory microbiome in cystic fibrosis in infancy: interaction between intestinal and respiratory tracts and impact of nutritional exposures," *MBio*, vol. 3, no. 4, pp. e00251–e00212, 2012.

[103] A. Cianferoni and J. M. Spergel, "Eosinophilic esophagitis and gastroenteritis," *Current Allergy and Asthma Reports*, vol. 15, no. 9, p. 58, 2015.

[104] K. F. Chung, "Airway microbial dysbiosis in asthmatic patients: a target for prevention and treatment?," *Journal of Allergy and Clinical Immunology*, vol. 139, no. 4, pp. 1071–1081, 2017.

[105] N. Li, R. Qiu, Z. Yang et al., "Sputum microbiota in severe asthma patients: relationship to eosinophilic inflammation," *Respiratory Medicine*, vol. 131, pp. 192–198, 2017.

[106] E. S. Gollwitzer, S. Saglani, A. Trompette et al., "Lung microbiota promotes tolerance to allergens in neonates via PD-L1," *Nature Medicine*, vol. 20, no. 6, pp. 642–647, 2014.

[107] N. Barbieri, J. Villena, M. Herrera, S. Salva, and S. Alvarez, "Nasally administered lactobacillus rhamnosus accelerate the recovery of humoral immunity in B lymphocyte-deficient malnourished mice," *The Journal of Nutrition*, vol. 143, no. 2, pp. 227–235, 2013.

[108] E. J. Cleland, A. Drilling, A. Bassiouni, C. James, S. Vreugde, and P. J. Wormald, "Probiotic manipulation of the chronic rhinosinusitis microbiome," *International Forum of Allergy & Rhinology*, vol. 4, no. 4, pp. 309–314, 2014.

[109] J. S. Schwartz, A. G. Peres, L. Mfuna Endam, B. Cousineau, J. Madrenas, and M. Desrosiers, "Topical probiotics as a therapeutic alternative for chronic rhinosinusitis: a preclinical proof of concept," *American Journal of Rhinology and Allergy*, vol. 30, no. 6, pp. 202–205, 2016.

[110] H. Pant, A. Hughes, M. Schembri, D. Miljkovic, and D. Krumbiegel, "CD4(+) and CD8(+) regulatory T cells in

chronic rhinosinusitis mucosa," *American Journal of Rhinology & Allergy*, vol. 28, no. 2, pp. e83–e89, 2014.

[111] A. Sogut, O. Yilmaz, C. Kirmaz et al., "Regulatory-T, T-helper 1, and T-helper 2 cell differentiation in nasal mucosa of allergic rhinitis with olive pollen sensitivity," *International Archives of Allergy and Immunology*, vol. 157, no. 4, pp. 349–353, 2012.

[112] S. Radulovic, M. R. Jacobson, S. R. Durham, and K. T. Nouri-Aria, "Grass pollen immunotherapy induces Foxp3-expressing CD4+CD25+ cells in the nasal mucosa," *Journal of Allergy and Clinical Immunology*, vol. 121, no. 6, pp. 1467–1472.e1, 2008.

[113] C. Daniel, A. Repa, C. Wild et al., "Modulation of allergic immune responses by mucosal application of recombinant lactic acid bacteria producing the major birch pollen allergen Bet v 1," *Allergy*, vol. 61, no. 7, pp. 812–819, 2006.

[114] M. E. V. Johansson, J. M. H. Larsson, and G. C. Hansson, "The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions," *Proceedings of the National Academy of Sciences*, vol. 108, Supplement_1, pp. 4659–4665, 2011.

[115] K. Godl, M. E. V. Johansson, M. E. Lidell et al., "The N terminus of the MUC2 mucin forms trimers that are held together within a trypsin-resistant core fragment," *Journal of Biological Chemistry*, vol. 277, no. 49, pp. 47248–47256, 2002.

[116] H. E. Jakobsson, A. M. Rodriguez-Pineiro, A. Schutte et al., "The composition of the gut microbiota shapes the colon mucus barrier," *EMBO Reports*, vol. 16, no. 2, pp. 164–177, 2015.

[117] D. W. Cockburn and N. M. Koropatkin, "Polysaccharide degradation by the intestinal microbiota and its influence on human health and disease," *Journal of Molecular Biology*, vol. 428, no. 16, pp. 3230–3252, 2016.

[118] M. S. Ali and J. P. Pearson, "Upper airway mucin gene expression: a review," *The Laryngoscope*, vol. 117, no. 5, pp. 932–938, 2007.

[119] D. A. Groneberg, C. Peiser, Q. T. Dinh et al., "Distribution of respiratory mucin proteins in human nasal mucosa," *The Laryngoscope*, vol. 113, no. 3, pp. 520–524, 2003.

[120] G. Q. Ding and C. Q. Zheng, "The expression of MUC5AC and MUC5B mucin genes in the mucosa of chronic rhinosinusitis and nasal polyposis," *American Journal of Rhinology*, vol. 21, no. 3, pp. 359–366, 2007.

[121] L. Tan, A. Psaltis, L. M. Baker, M. McGuckin, K. Rousseau, and P. J. Wormald, "Aberrant mucin glycoprotein patterns of chronic rhinosinusitis patients with bacterial biofilms," *American Journal of Rhinology and Allergy*, vol. 24, no. 5, pp. 319–324, 2010.

[122] J. H. Fastenberg, W. D. Hsueh, A. Mustafa, N. A. Akbar, and W. M. Abuzeid, "Biofilms in chronic rhinosinusitis: pathophysiology and therapeutic strategies," *World Journal of Otorhinolaryngology - Head and Neck Surgery*, vol. 2, no. 4, pp. 219–229, 2016.

[123] R. C. Boucher, "New concepts of the pathogenesis of cystic fibrosis lung disease," *European Respiratory Journal*, vol. 23, no. 1, pp. 146–158, 2004.

[124] X. Wang, "Mutation in the gene responsible for cystic fibrosis and predisposition to chronic rhinosinusitis in the general population," *JAMA*, vol. 284, no. 14, pp. 1814–1819, 2000.

[125] Z. Bendouah, J. Barbeau, W. A. Hamad, and M. Desrosiers, "Biofilm formation by Staphylococcus aureus and Pseudomonas aeruginosais associated with an unfavorable evolution after surgery for chronic sinusitis and nasal polyposis," *Otolaryngology-Head and Neck Surgery*, vol. 134, no. 6, pp. 991–996, 2006.

[126] Z. Malik, E. Roscioli, J. Murphy et al., "Staphylococcus aureus impairs the airway epithelial barrier in vitro," *International Forum of Allergy & Rhinology*, vol. 5, no. 6, pp. 551–556, 2015.

[127] J. Murphy, M. Ramezanpour, N. Stach et al., "Staphylococcus aureus V8 protease disrupts the integrity of the airway epithelial barrier and impairs IL-6 production in vitro," *The Laryngoscope*, vol. 128, no. 1, pp. E8–E15, 2018.

[128] B. Steelant, R. Farré, P. Wawrzyniak et al., "Impaired barrier function in patients with house dust mite-induced allergic rhinitis is accompanied by decreased occludin and zonula occludens-1 expression," *Journal of Allergy and Clinical Immunology*, vol. 137, no. 4, pp. 1043–1053.e5, 2016.

[129] B. Steelant, S. F. Seys, L. Van Gerven et al., "Histamine and T helper cytokine-driven epithelial barrier dysfunction in allergic rhinitis," *Journal of Allergy and Clinical Immunology*, vol. 141, no. 3, pp. 951–963.e8, 2018.

[130] H. Wan, H. L. Winton, C. Soeller et al., "Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions," *Journal of Clinical Investigation*, vol. 104, no. 1, pp. 123–133, 1999.

[131] B. Saatian, F. Rezaee, S. Desando et al., "Interleukin-4 and interleukin-13 cause barrier dysfunction in human airway epithelial cells," *Tissue Barriers*, vol. 1, no. 2, article e24333, 2013.

[132] M. B. Soyka, P. Wawrzyniak, T. Eiwegger et al., "Defective epithelial barrier in chronic rhinosinusitis: the regulation of tight junctions by IFN-γ and IL-4," *Journal of Allergy and Clinical Immunology*, vol. 130, no. 5, pp. 1087–1096.e10, 2012.

[133] M. Di Pierro, R. Lu, S. Uzzau et al., "Zonula occludens toxin structure-function analysis: identification of the fragment biologically active on tight junctions and of the zonulin receptor binding domain," *Journal of Biological Chemistry*, vol. 276, no. 22, pp. 19160–19165, 2001.

[134] N. Sonoda, M. Furuse, H. Sasaki et al., "Clostridium perfringens enterotoxin fragment removes specific claudins from tight junction strands: evidence for direct involvement of claudins in tight junction barrier," *The Journal of Cell Biology*, vol. 147, no. 1, pp. 195–204, 1999.

[135] C. Sturgeon and A. Fasano, "Zonulin, a regulator of epithelial and endothelial barrier functions, and Its involvement in chronic inflammatory diseases," *Tissue Barriers*, vol. 4, no. 4, article e1251384, 2016.

[136] R. E. Asmar, P. Panigrahi, P. Bamford et al., "Host-dependent zonulin secretion causes the impairment of the small intestine barrier function after bacterial exposure," *Gastroenterology*, vol. 123, no. 5, pp. 1607–1615, 2002.

[137] K. M. Lammers, R. Lu, J. Brownley et al., "Gliadin induces an increase in intestinal permeability and zonulin release by binding to the chemokine receptor CXCR3," *Gastroenterology*, vol. 135, no. 1, pp. 194–204.e3, 2008.

[138] D. Rittirsch, M. A. Flierl, B. A. Nadeau et al., "Zonulin as prehaptoglobin2 regulates lung permeability and activates the complement system," *American Journal of Physiology-Lung Cellular and Molecular Physiology*, vol. 304, no. 12, pp. L863–L872, 2013.

[139] M. Marinaro, A. Fasano, and M. T. De Magistris, "Zonula occludens toxin acts as an adjuvant through different mucosal routes and induces protective immune responses," *Infection and Immunity*, vol. 71, no. 4, pp. 1897–1902, 2003.

[140] F. Viana, "Chemosensory properties of the trigeminal system," *ACS Chemical Neuroscience*, vol. 2, no. 1, pp. 38–50, 2011.

[141] J. N. Baraniuk and S. J. Merck, "Neuroregulation of human nasal mucosa," *Annals of the New York Academy of Sciences*, vol. 1170, no. 1, pp. 604–609, 2009.

[142] S. Sarin, B. Undem, A. Sanico, and A. Togias, "The role of the nervous system in rhinitis," *Journal of Allergy and Clinical Immunology*, vol. 118, no. 5, pp. 999–1014, 2006.

[143] D. J. Shusterman, M. A. Murphy, and J. R. Balmes, "Subjects with seasonal allergic rhinitis and nonrhinitic subjects react differentially to nasal provocation with chlorine gas," *Journal of Allergy and Clinical Immunology*, vol. 101, no. 6, pp. 732–740, 1998.

[144] F. A. Salzano, L. Guastini, R. Mora et al., "Nasal tactile sensitivity in elderly," *Acta Oto-Laryngologica*, vol. 130, no. 12, pp. 1389–1393, 2010.

[145] F. A. Salzano, R. Mora, S. Penco et al., "Nasal tactile sensitivity in allergic rhinitis," *Acta Oto-Laryngologica*, vol. 131, no. 6, pp. 640–644, 2011.

[146] V. Meseguer, Y. A. Alpizar, E. Luis et al., "TRPA1 channels mediate acute neurogenic inflammation and pain produced by bacterial endotoxins," *Nature Communications*, vol. 5, 2014.

[147] I. M. Chiu, B. A. Heesters, N. Ghasemlou et al., "Bacteria activate sensory neurons that modulate pain and inflammation," *Nature*, vol. 501, no. 7465, pp. 52–57, 2013.

[148] S. Talbot, R. E. E. Abdulnour, P. R. Burkett et al., "Silencing nociceptor neurons reduces allergic airway inflammation," *Neuron*, vol. 87, no. 2, pp. 341–354, 2015.

[149] C. S. N. Klose, T. Mahlakõiv, J. B. Moeller et al., "The neuropeptide neuromedin U stimulates innate lymphoid cells and type 2 inflammation," *Nature*, vol. 549, no. 7671, pp. 282–286, 2017.

[150] V. Cardoso, J. Chesné, H. Ribeiro et al., "Neuronal regulation of type 2 innate lymphoid cells via neuromedin U," *Nature*, vol. 549, no. 7671, pp. 277–281, 2017.

[151] A. Wallrapp, S. J. Riesenfeld, P. R. Burkett et al., "The neuropeptide NMU amplifies ILC2-driven allergic lung inflammation," *Nature*, vol. 549, no. 7672, pp. 351–356, 2017.

[152] I. A. Schepetkin, L. N. Kirpotina, A. I. Khlebnikov, M. A. Jutila, and M. T. Quinn, "Gastrin-releasing peptide/neuromedin B receptor antagonists PD176252, PD168368, and related analogs are potent agonists of human formyl-peptide receptors," *Molecular Pharmacology*, vol. 79, no. 1, pp. 77–90, 2011.

[153] M. Ali, A. Z. Gawin, and J. N. Baraniuk, "Effects of bombesin family peptides and antagonists on guinea pig nasal mucosal secretion," *The Journal of Pharmacology and Experimental Therapeutics*, vol. 276, no. 3, pp. 1266–1271, 1996.

[154] B. B. Yoo and S. K. Mazmanian, "The enteric network: interactions between the immune and nervous systems of the gut," *Immunity*, vol. 46, no. 6, pp. 910–926, 2017.

[155] J. Bousquet, W. Jacquot, A. M. Vignola, C. Bachert, and P. Van Cauwenberge, "Allergic rhinitis: a disease remodeling the upper airways?," *Journal of Allergy and Clinical Immunology*, vol. 113, no. 1, pp. 43–49, 2004.

[156] H. S. Lee, A. Myers, and J. Kim, "Vascular endothelial growth factor drives autocrine epithelial cell proliferation and survival in chronic rhinosinusitis with nasal polyposis," *American Journal of Respiratory and Critical Care Medicine*, vol. 180, no. 11, pp. 1056–1067, 2009.

[157] N. Van Bruaene, L. Derycke, C. A. Perez-Novo et al., "TGF-β signaling and collagen deposition in chronic rhinosinusitis," *Journal of Allergy and Clinical Immunology*, vol. 124, no. 2, pp. 253–259.e2, 2009.

[158] H. Kouzaki, S. Seno, J. Fukui, S. Owaki, and T. Shimizu, "Role of platelet-derived growth factor in airway remodeling in rhinosinusitis," *American Journal of Rhinology & Allergy*, vol. 23, no. 3, pp. 273–280, 2009.

[159] J. A. Ebenezer, J. M. Christensen, B. G. Oliver et al., "Periostin as a marker of mucosal remodelling in chronic rhinosinusitis," *Rhinology*, vol. 55, no. 3, pp. 234–241, 2017.

[160] D. A. Dorward, C. D. Lucas, G. B. Chapman, C. Haslett, K. Dhaliwal, and A. G. Rossi, "The role of formylated peptides and formyl peptide receptor 1 in governing neutrophil function during acute inflammation," *The American Journal of Pathology*, vol. 185, no. 5, pp. 1172–1184, 2015.

[161] N. Prevete, F. A. Salzano, F. W. Rossi et al., "Role(s) of formyl-peptide receptors expressed in nasal epithelial cells," *Journal of Biological Regulators and Homeostatic Agents*, vol. 25, no. 4, pp. 553–564, 2011.

[162] J. H. Wang, H. J. Kwon, and Y. J. Jang, "Rhinovirus upregulates matrix metalloproteinase-2, matrix metalloproteinase-9, and vascular endothelial growth factor expression in nasal polyp fibroblasts," *The Laryngoscope*, vol. 119, no. 9, pp. 1834–1838, 2009.

[163] F. A. Salzano and D. Testa, "Etiopathogenesis of chronic rhinosinusitis with nasal polyposis," in *Translational Research on Otorhinolaryngology*, pp. 56–58, Grafica de las Nieves S.L., Malaga, 2012.

[164] T. Van Zele, P. Gevaert, J. B. Watelet et al., "Staphylococcus aureus colonization and IgE antibody formation to enterotoxins is increased in nasal polyposis," *Journal of Allergy and Clinical Immunology*, vol. 114, no. 4, pp. 981–983, 2004.

[165] K. A. Becker, B. Fahsel, H. Kemper et al., "Staphylococcus aureus alpha-toxin disrupts endothelial-cell tight junctions via acid sphingomyelinase and ceramide," *Infection and Immunity*, vol. 86, no. 1, pp. e00606–e00617, 2017.

[166] S. Ziesemer, I. Eiffler, A. Schönberg et al., "Staphylococcus aureus α-toxin induces actin filament remodeling in human airway epithelial model cells," *American Journal of Respiratory Cell and Molecular Biology*, vol. 58, no. 4, pp. 482–491, 2018.

[167] J. Patou, P. Gevaert, T. Van Zele, G. Holtappels, P. van Cauwenberge, and C. Bachert, "Staphylococcus aureus enterotoxin B, protein A, and lipoteichoic acid stimulations in nasal polyps," *Journal of Allergy and Clinical Immunology*, vol. 121, no. 1, pp. 110–115, 2008.

[168] I. V. Pinchuk, E. J. Beswick, and V. E. Reyes, "Staphylococcal enterotoxins," *Toxins*, vol. 2, no. 8, pp. 2177–2197, 2010.

[169] I. Zhang, S. D. Pletcher, A. N. Goldberg, B. M. Barker, and E. K. Cope, "Fungal microbiota in chronic airway inflammatory disease and emerging relationships with the host immune response," *Frontiers in Microbiology*, vol. 8, p. 2477, 2017.

[170] X. Wang, N. Zhang, M. Bo et al., “Diversity of T H cytokine profiles in patients with chronic rhinosinusitis: a multicenter study in Europe, Asia, and Oceania,” *Journal of Allergy and Clinical Immunology*, vol. 138, no. 5, pp. 1344–1353, 2016.

[171] J. Bousquet, J. Reid, C. van Weel et al., “Allergic rhinitis management pocket reference 2008,” *Allergy*, vol. 63, no. 8, pp. 990–996, 2008.

[172] J. Banuelos, S. Shin, Y. Cao et al., “BCL-2 protects human and mouse Th17 cells from glucocorticoid-induced apoptosis,” *Allergy*, vol. 71, no. 5, pp. 640–650, 2016.

[173] C. Wang, H. Lou, X. Wang et al., “Effect of budesonide transnasal nebulization in patients with eosinophilic chronic rhinosinusitis with nasal polyps,” *Journal of Allergy and Clinical Immunology*, vol. 135, no. 4, pp. 922–929.e6, 2015.

[174] J. Gurrola II and L. Borish, “Chronic rhinosinusitis: endotypes, biomarkers, and treatment response,” *Journal of Allergy and Clinical Immunology*, vol. 140, no. 6, pp. 1499–1508, 2017.

[175] P. Tomassen, G. Vandeplas, T. Van Zele et al., “Inflammatory endotypes of chronic rhinosinusitis based on cluster analysis of biomarkers,” *Journal of Allergy and Clinical Immunology*, vol. 137, no. 5, pp. 1449–1456.e4, 2016.

[176] J. H. Turner, R. K. Chandra, P. Li, K. Bonnet, and D. G. Schlundt, “Identification of clinically relevant chronic rhinosinusitis endotypes using cluster analysis of mucus cytokines,” *Journal of Allergy and Clinical Immunology*, vol. 141, no. 5, pp. 1895–1897.e7, 2018.

[177] D. Lal, P. Keim, J. Delisle et al., “Mapping and comparing bacterial microbiota in the sinonasal cavity of healthy, allergic rhinitis, and chronic rhinosinusitis subjects,” *International Forum of Allergy & Rhinology*, vol. 7, no. 6, pp. 561–569, 2017.

[178] E. K. Cope, A. N. Goldberg, S. D. Pletcher, and S. V. Lynch, “Compositionally and functionally distinct sinus microbiota in chronic rhinosinusitis patients have immunological and clinically divergent consequences,” *Microbiome*, vol. 5, no. 1, p. 53, 2017.

[179] G. Mamo, “Anaerobes as sources of bioactive compounds and health promoting tools,” *Advances in Biochemical Engineering/Biotechnology*, vol. 156, pp. 433–464, 2016.

[180] A. Zipperer, M. C. Konnerth, C. Laux et al., “Human commensals producing a novel antibiotic impair pathogen colonization,” *Nature*, vol. 535, no. 7613, pp. 511–516, 2016.

[181] M. Gelardi, M. Landi, and G. Ciprandi, “Nasal cytology: a precision medicine tool in clinical practice,” *Clinical & Experimental Allergy*, vol. 48, no. 1, pp. 96-97, 2018.

[182] M. A. Kohanski, E. Toskala, and D. W. Kennedy, “Evolution in the surgical management of chronic rhinosinusitis: current indications and pitfalls,” *Journal of Allergy and Clinical Immunology*, vol. 141, no. 5, pp. 1561–1569, 2018.

[183] L. J. Hauser, D. Ir, T. T. Kingdom, C. E. Robertson, D. N. Frank, and V. R. Ramakrishnan, “Investigation of bacterial repopulation after sinus surgery and perioperative antibiotics,” *International Forum of Allergy & Rhinology*, vol. 6, no. 1, pp. 34–40, 2016.

[184] G. Zuccotti, F. Meneghin, A. Aceti et al., “Probiotics for prevention of atopic diseases in infants: systematic review and meta-analysis,” *Allergy*, vol. 70, no. 11, pp. 1356–1371, 2015.

[185] S. S. Mukerji, M. A. Pynnonen, H. M. Kim, A. Singer, M. Tabor, and J. E. Terrell, “Probiotics as adjunctive treatment for chronic rhinosinusitis: a randomized controlled trial,” *Otolaryngology-Head and Neck Surgery*, vol. 140, no. 2, pp. 202–208, 2009.

[186] P. Marchisio, S. Esposito, and N. Principi, “The evidence for applying Streptococcus salivarius 24SMB by nasal spray for preventing recurrent acute otitis media,” *European Journal of Clinical Microbiology & Infectious Diseases*, vol. 35, no. 11, pp. 1889-1890, 2016.

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Research Article

NMO-IgG and AQP4 Peptide Can Induce Aggravation of EAMG and Immune-Mediated Muscle Weakness

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Neuromyelitis optica (NMO) and myasthenia gravis (MG) are autoimmune diseases mediated by autoantibodies against either aquaporin 4 (AQP4) or acetylcholine receptor (AChR), respectively. Recently, we and others have reported an increased prevalence of NMO in patients with MG. To verify whether coexisting autoimmune disease may exacerbate experimental autoimmune MG, we tested whether active immunization with AQP4 peptides or passive transfer of NMO-Ig can affect the severity of EAMG. Injection of either AQP4 peptide or NMO-Ig to EAMG or to naive mice caused increased fatigability and aggravation of EAMG symptoms as expressed by augmented muscle weakness (but not paralysis), decremental response to repetitive nerve stimulation, increased neuromuscular jitter, and aberration of immune responses. Thus, our study shows increased disease severity in EAMG mice following immunization with the NMO autoantigen AQP4 or by NMO-Ig, mediated by augmented inflammatory response. This can explain exacerbation or increased susceptibility of patients with one autoimmune disease to develop additional autoimmune syndrome.

1. Introduction

Neuromyelitis optica (NMO), also known as Devic's disease, is a central nervous system (CNS) autoimmune disease that preferentially affects the spinal cord and optic nerve [1]. The disease is mediated by autoantibodies against aquaporin 4 (AQP4) [2]. These antibodies have been proven pathogenic in NMO by several methods including complement-dependent cytotoxicity, antibody-dependent cell-mediated cytotoxicity, and induction of inflammation with a prominent granulocyte and macrophage response, which lead to secondary oligodendrocyte injury, demyelination, and neuronal injury [3].

Myasthenia gravis (MG) is a well-recognized antibody-mediated disease affecting the neuromuscular junction, caused by immunoglobulin G (IgG)1- and IgG3-complement, activating antibodies against the nicotinic acetylcholine receptor (AChR, AChR-Ab) in around 85% of patients [4].

Both AQP4-Ab-positive NMO and AChR-Ab-positive MG are associated with other organ-specific and systemic autoimmune diseases [5–7]. Despite the rarity of MG and of NMO, in recent years, there is considerable evidence for increased susceptibility of NMO in patients with MG. Our and others' studies have linked NMO to patients previously diagnosed with MG and pointed common immunological abnormalities between the two diseases [8–15].

Although AQP4 is expressed also outside the CNS, in muscles, lungs, and kidneys, until recently, no disease was described in those organs. There are currently indications that there might be mild muscle pathology in patients with NMO [16–18].

Currently, there is no satisfactory animal model of NMO. In order to study the pathogenesis of NMO and to test candidate therapies, it is important to have an animal model of the disease [19]. Several animal studies have shown that AQP4 antibodies are not pathogenic via simple transfer of AQP4

antibodies into the circulation of naive animals. In order to cause NMO pathology, NMO-IgG should reach the CNS parenchyma by penetrating through the blood-brain barrier (BBB). This was established using preexisting CNS inflammation in the experimental autoimmune encephalitis (EAE) model, to cross the BBB, or via direct intracerebral injection of recombinant NMO-IgG [20–23]. Direct administration of NMO-IgG into the CNS tissue, without coinjection of complement, produced NMO-like lesions with astrocyte and AQP4 loss [24]. By injection of NMO-IgG into mice lacking complement inhibitor, Zhang et al. induced long extensive myelitis comparable to the myelitis in humans with NMO [25]. Recently, several studies showed that induction of NMO-like syndrome can be induced by the transfer of AQP4-reactive T-cells directed to the second extracellular loop of AQP4. These T-cells were derived from AQP4 null mice and injected to wild type or to B cell-deficient mice [23, 26, 27].

The present study was aimed at establishing an animal model for NMO together with MG, based on previous observation of increased NMO susceptibility in patients with MG. We used experimental autoimmune MG (EAMG) mice immunized with Torpedo AChR and subjected the animals to passive transfer of NMO-IgG or to immunization with AQP4-derived peptide for inducing NMO and MG models.

Our study shows that injection of either AQP4 peptide or NMO-Ig to naive mice caused increased fatigability and that the same agents' administered to EAMG mice significantly increased disease severity mediated by muscle weakness.

2. Materials and Methods

2.1. EAMG and NMO Induction and Clinical Evaluation. Induction of EAMG C57BL/6JOlaHsd mice were purchased from Harlan Laboratories (Rehovot, Israel) and were housed under specific pathogen-free conditions in the animal facility of the Hebrew University Medical School, in accordance with NIH guidelines for the care and use of laboratory animals. Torpedo AChR was purified from *Torpedo californica* as previously described [28].

Purified Torpedo AChR (25 μg and 5 mg/ml of M. tuberculosis H37Ra, (Difco, Detroit MI)), emulsified in complete Freund's adjuvant (CFA), was subcutaneously (s.c.) injected into the hind footpads and into the flank of 6-7-week-old C57BL female mice as previously described [29]. The clinical status was graded as the following: (0) no weakness or fatigue; (1) mildly decreased activity, weak grip with fatigue, weight loss > 3% body weight in one week; (2) moderate weakness accompanied by weak grip, 5–10% weight loss; (3) moderate-severe weakness, hunched back posture at rest, head down, and forelimb digit flexed, tremulous ambulation, 10% weight loss; and (4) severe general weakness, weak grip, weight loss > 10% [29].

2.1.1. Preparation of NMO-Ig. Plasma exchange fluid was collected during the procedure from clinically definite, seropositive NMO patient according to the guidelines and with the approval of the Hebrew University's Bioethics Committee. The diagnosis of NMO was based on positive titer of anti-AQP4 antibodies and typical clinical symptoms, including optic neuritis and myelitis [30]. Immunoglobulin (Ig) was purified by saturated ammonium sulfate precipitation followed by dialysis.

2.1.2. Injection of NMO-Ig or AQP4 Peptide. Seven groups of mice were used in 3 different sets of experiments:

(1) Twenty-one control EAMG

(2) Thirteen naive mice injected intraperitoneally (i.p.) with Ig isolated from NMO patient, 4 weeks after the induction of EAMG (the amount of Ig was 17.5 mg protein; Ig injection was repeated 3 times with 10-day intervals between each injection)

(3) Six EAMG mice immunized with 40 μg AQP4 peptide p249-323 (Alomone Labs, Israel), AQP4 peptide emulsified in complete Freund's adjuvant (CFA)

(4) Thirteen naive mice that were injected i.p. with 17.5 mg Ig protein (Ig injection was repeated 3 times with 10-day intervals between each injection)

(5) Fifteen naive mice immunized with 40 μg AQP4 peptide p249-323 (Alomone Labs, Israel), AQP4 peptide emulsified with CFA

(6) Six naive mice injected with CFA alone

(7) Eight naive untreated mice

All the animals were weighed and inspected once a week during the first month and daily after AQP4 peptide and NMO-Ig injection to evaluate muscle weakness.

In addition, 10 naive and EAMG mice were injected with scrambled AQP4 peptides (amino acids 207-221, 217-231, 222-239, and 299-213, chosen from loop E- and C-terminus regions) in CFA and inspected as above. Those animals did not develop any measured clinical weakness (data not shown).

2.1.3. Anti-AChR Ab Determination. Sera from EAMG animals were assayed by direct radioimmunoassay for anti-T-AChR, or mouse M-AChR. All the EAMG mice displayed high anti-T-AChR and/or anti-mouse AChR levels, with the serum mean ± SE values of 82.1 ± 16.0 nM for anti-T-AChR Abs and 19.9 ± 1.8 nM for anti-M-AChR.

2.1.4. Repetitive Nerve Conduction Studies (RNS). Mice were anesthetized by i.p. injection of 157 mg/kg ketamine + 3.5 mg/kg xylazine and immobilized on silver foil over an AccuBlock™ at 41°C to maintain rectal temperature between 36-37°C. The left hind limb was abducted slightly and immobilized with the knee and ankle in slight flexion and shaved. An electromyography (EMG) machine was used to assess motor nerve conduction, RNS, and single fiber electromyography (SFEMG). Baseline motor conduction filter settings were 0.01–10 kHz, amplifier gain 2 mV/division, sweep duration 2 ms/division, and stimulus duration 0.1 ms (rectangular pulses). Disposable sensory needle electrodes (SNEs) (28G, Alpine Biomed ApS) were used for both stimulating

and recording motor conduction and RNS studies. The left sciatic-tibial nerve was stimulated as described by Osuchowski et al. [31]. The stimulator anode SNE was advanced subdermally lateral and parallel to the spine 11 mm from the tail-base, and cathode SNE was inserted subdermally 3 mm lateral and parallel to the anode. For recording, an active pick-up SNE was inserted into the gastrocnemius muscle and reference SNE was placed subdermally over the Achilles tendon. Baseline supramaximal sciatic-tibial nerve compound muscle action potential (CMAP) amplitudes were recorded from gastrocnemius, followed by a 5 Hz train of RNS at supramaximal intensity. A decrement was calculated as percent amplitude drops between the first and fifth CMAPs evoked by a train of 10 impulses, which were determined in two sets of repetitive stimulation for each animal. A reduction of 10% or more indicated neuromuscular transmission dysfunction [32]. Normal muscle showed no decrement in action potential amplitude.

2.1.5. Concentric Needle Stimulation SFEMG. Anesthetized mice were kept immobilized, and temperatures maintained as above. Stimulation SFEMG was performed by direct extramuscular nerve stimulation and was picked up by concentric electromyography needle (CN). SFEMG filter settings were 0.5–10 kHz, amplifier gain 1 mV/division, and sweep duration 1 ms/division. Left sciatic-tibial nerve stimulation [31] was initially of low rate and intensity (3 Hz, 0.1–0.3 mA), stimulus duration 0.1 ms (rectangular pulses) to obtain correct CN positioning and spike wave forms. A disposable CN electrode (30G, Alpine Biomed ApS) was inserted into the midpoint of the twitching gastrocnemius, and reference (anode) via a surface cup electrode (Alpine Biomed ApS, 10 mm diameter, gold-plated) was affixed with gel onto the skin 1.5 cm from the CN insertion site. The motor unit potential recorded with a CN electrode represents the sum of single fiber action potentials (SFAPs) from all muscle fibers from a single motor unit that lays within the uptake range of the electrode [33]. The "apparent single fiber action potentials" (ASFAPs) spikes were identified with amplitudes > 200 IV, rise time < 200 ìs, [33]. Thereafter, stimulation intensity was increased to abolish false positive blocking due to insufficient stimulus strength, to 10 Hz, with muscle movement minimized. Five to fourteen apparent single muscle fiber potentials per animal were used for the analysis. The jitter of each ASFAP was computed as the mean consecutive difference (MCD) of 100 consecutive intervals between stimulus artifact and muscle fiber potential. A jitter (MCD) value ≤ 5 was rejected as being due to muscle fiber splitting or technical factors [32, 34]. Neuromuscular blocking was calculated as the percent of all fibers in which any blocking was seen among the 5–14 ASFAPs studied per animal.

2.1.6. Grip Strength Analysis. Starting from week 10 and for 8 consecutive weeks, forelimbs' muscle strength was determined using the electronic grip strength meter (Columbus Instruments). Forelimb muscle strength was assessed once a week according Tanase et al. [35]. Five measurements were performed on the animal's forelimbs. The three highest measurements in each mouse group were averaged to give the mean strength score. The results are given as grams force divided by body weight (gr).

2.1.7. Cell Culture and Cytokine and Chemokine Production. Pooled lymphocytes from spleens were harvested from naive mice or from mice immunized 7 weeks earlier with T-AChR in CFA or with NMO-Ig or AQP4 peptide. 1×10^6 cells were cultured in 24-well plates, in a total volume of 0.5 ml. stimulated with purified anti-CD3 antibodies and incubated for 24 h to 96 h. The conditioned media were collected, and the following cytokines interferon γ (IFNγ), interleukin 6 (IL-6), and interleukin 10 (IL-10) were tested using enzyme-linked immunosorbent assay (ELISA) kits (Biolegend) according to the manufacturer's instructions, as previously reported [36].

2.1.8. RNA Purification and cDNA Synthesis. Total RNA was prepared using a 5 Prime RNA kit (GmBh Hamburg, Germany). cDNA was prepared from 1 mg of total RNA, using a qScript cDNA synthesis kit (Qunta).

2.1.9. Cytokines and Chemokines Expressed in Muscles of EAMG- and NMO-Ig- and AQP4 Peptide-Injected Animals. The following cytokines and chemokines were tested on muscle specimens using RT-PCR. The reaction mixture contained 40 ng cDNA and 4 μl buffer containing 300 nM of the appropriate forward and reverse primers and PerfeCTa SYBR Green FastMix ROX (Quanta) in a total volume of 7 μl. Gene amplification was carried out using the GeneAmp 7000 Sequence Detection System (Applied Biosystems). The results are expressed as relative quantification (RQ), and the fold increase of gene expression in samples from concanavalin A (Con A) stimulated cells above those from control cells. The results for gene expression were normalized to the HPRT gene; the expression of which was not changed under stimulation conditions. The primers used were HPRT: fwd: 5′TCCTCCTCAGACCGCTTTT3′ and rev: 5′CCTGGTTCATCATCGCTAATC3′; TGF: fwd: 5′TCAGACATTCGGGAAGCAGT3′ and rev: 5′ACGCCAGGAATTGTTGCTAT3′; IP-10: fwd: 5′GGATGGCTGTCCTAGCTCTG3′ and rev: 5′ATAACCCCTTGGGAAGATGG3′; CXCR3: fwd: 5′TGGGGCGGTGGCTGCTGTGCT3′ and rev: 5′TGCAGAGGCCAGGGCCGAAAACCC3′; atrogin: fwd: 5′AAGAAGAGAGCAGTATGGGGTCA3′ and rev: 5′ACGGATGGTCAGTGCCCTT3′; MCP-1: fwd: 5′ACCTGTAAATGCCATGCAAGT3′ and rev: 5′TGTCTTCCATTTCCTTTGATTT3′; and IL-6: fwd: 5′AGTTGCCTTCTTGGGACTGA3′ and rev: 5′TCCACGATTTCCCAGAGAAC3′.

2.1.10. Pathology. On the day of sacrifice, the optic nerve, spinal cords, and brain were harvested and fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Longitudinal sections were cut to include the majority of the length of the spinal cord, containing both gray and white matter, and stained with hematoxylin-eosin.

2.1.11. Statistical Analysis. The data were analyzed using Student's t-test and one-way ANOVA. $P < 0.05$ was considered statistically significant.

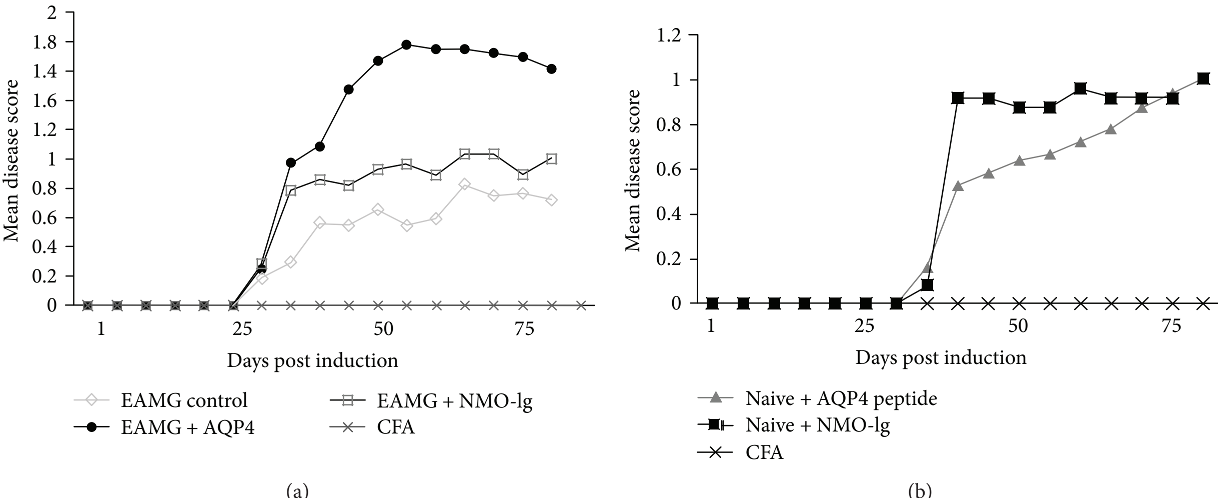

FIGURE 1: Aggravation of muscle weakness symptoms in naive and EAMG mice by exposure to NMO-Ig or AQP4 peptide. (a) Injection of AQP4 peptide or NMO-IgG in addition to EAMG induction resulted in aggravation of EAMG weakness symptoms. (b) Mice injected with AQP4 peptide or NMO-IgG had significant muscle weakness as compared to CFA-injected mice. Results are the mean of 3 separated experiments. EAMG control $n = 16$, EAMG + AQP4 peptide $n = 6$, EAMG + NMO-Ig $n = 7$, AQP4 peptide $n = 9$, NMO-Ig $n = 6$, and CFA $n = 6$.

TABLE 1: Aggravation of clinical severity and muscle strength in EAMG and naive mice by immunization with AQP4 peptide and NMO-IgG.

	EAMG control	EAMG + NMO-IgG	EAMG + AQP4 peptide	Naive + NMO-IgG	Naive + AQP4 peptide	Naive
Mean severity	0.624 ± 0.01	0.82 ± 0.01	$1.5 \pm 0.02^{*}$	0.72 ± 0.01	0.63 ± 0.02	0
Gram force	242.8 ± 4.5	$214.3 \pm 4.5^{*}$	$219.4 \pm 5.6^{*}$	$227.7 \pm 8.9^{**}$	$219.6 \pm 5.4^{**}$	307.9 ± 7.4
Decrease in force (versus naive)	21.13%	30.40%	28.73%	26.03%	28.73%	0

$*$ versus EAMG control. $**$ versus naive.

3. Results

3.1. NMO-Ig and AQP4 Peptide Aggravate EAMG and Induce Immune-Mediated Muscle Weakness. Noting that NMO clinical symptoms and the presence of the pathogenic anti-AQP4 antibodies have recently been reported in patients with MG [9, 12] and that anti-AChR antibodies were found in 11% of NMO patients [10], we examined whether and how one disease affects the severity of the autoimmune course of the other disease. EAMG mice were injected with AQP4 antibodies or peptide, and we verified the disease pattern and determined the effect of AQP4 antibodies or peptide on the immune pathophysiology in the CNS and in the muscle.

A total of 82 mice (21 with EAMG alone, 13 with EAMG + NMO-Ig, 6 with EAMG + AQP4 peptide, 13 injected with NMO-Ig alone, 15 injected with AQP4 peptide alone, 6 with CFA, and 8 naive mice) were included in this study in 3 different sets of experiments. EAMG mice that received AQP4 antibodies or peptide developed increased EAMG scale severity of 0.82 ± 0.01 and 1.5 ± 0.02, respectively, with increased muscle weakness and fatigability, compared with $0.62 + 0.01$ in mice with EAMG alone (Figure 1(a) and Table 1). Thus, the additional immunological challenge to AQP4 components significantly aggravated EAMG. Moreover, exposure to AQP4 antibodies or peptide alone also induced measurable impairment in muscle force. Naive mice that were subjected to AQP4 antibodies or AQP4 peptide developed mild fatigability that was quantified as 0.72 ± 0.01 and 0.63 ± 0.02, respectively, in disease scores (Figure 1(b)). The naive mice injected with NMO-Ig or AQP4 peptide displayed weakness, which was expressed by mildly decreased activity (relative to CFA, scrambled peptides, and Ig-injected control mice).

Aside from the clinical severity score, several measurements of muscle strength were performed, including weight changes and grip strength, and a fraction of the mice were subjected to RNS and SFEMG.

3.2. Forelimb Grip Strength Test. In order to quantify muscle weakness symptoms in the different mice groups, we performed a longitudinal assessment with 8 weekly measurements of the forelimb grip strength test. Grip strength was determined and divided by the mouse weight. As shown in Figure 2, and Table 1, all 5 experimental groups EAMG, NMO-Ig, AQP4 peptide, EAMG plus NMO-Ig, and EAMG plus AQP4 peptide (except the control CFA) showed significant reduction in the force values of 21.1–30.4% and increased fatigability compared to CFA ($P < 0.05$).

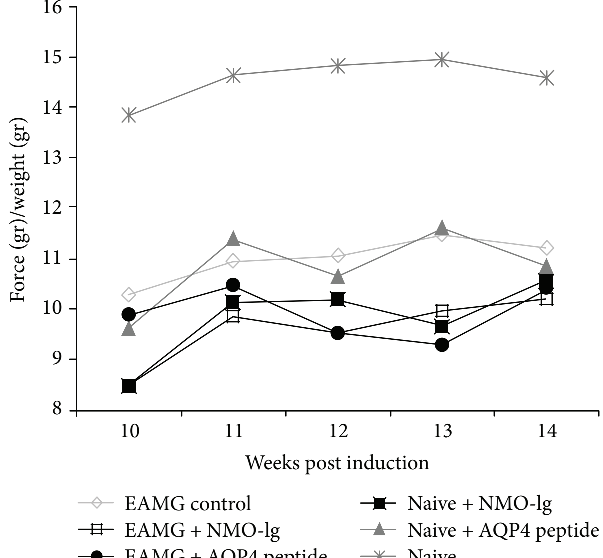

Figure 2: Forelimb muscle strength in AQP4 peptide- or NMO-IgG-injected mice. Using an electronic grip strength meter, the forelimb force was determined once a week. All the groups showed a significant reduction in forelimb strength in comparison to the CFA control mice. Results are the mean of 2 separated experiments with 4 to 7 mice in each group.

3.3. Measurements of CNS and Optic Nerve Histopathology. Since NMO disease in humans affects mainly the spinal cord (SC) and the optic nerves (ON), we studied the brain, SC, and ON obtained from 3 mice in each group (control naive mice, EAMG alone, and EAMG injected with NMO-Ig or with AQP4 peptide). To our surprise, although there was increased disease severity in mice with EAMG injected with either AQP4 peptide or NMO-Ig, no CNS pathological abnormalities were observed in these mice (data not shown).

3.4. Evidence of Neuromuscular Junction Pathology on Repetitive Nerve Stimulation and Single Fiber Electromyography. Since there was no evidence of CNS pathology, to verify the possibility that the fatigability of mice injected with AQP4 peptide or NMO-Ig originated from neuromuscular junction pathology using a nonbiased test, the mice were tested for their response to RNS and SFEMG. Compound muscle action potentials (CMAPs) from the gastrocnemius muscle were recorded in naive mice injected with NMO-Ig or AQP4 peptide alone and compared to EAMG- and CFA-injected mice. EAMG mice displayed a decrement in CMAP amplitudes. The baseline pathological decrement ranged from $11 \pm 0.66\%$ to $20 \pm 3\%$, as shown in Figure 3(a) (with decrement above 10% considered abnormal).

Concentric needle stimulation SFEMG was performed on the same mice groups described above; the mean jitter values (mean MCD) of CFA-injected mice, EAMG mice, and mice injected with NMO-Ig or AQP4 peptide alone were calculated. The CFA-injected mice showed a baseline jitter (mean MCD) of 13.5 ± 0.1 ìs. EAMG control and mice injected with NMO-Ig or AQP4 peptide showed significantly increased neuromuscular jitter as shown in Figure 3(b) (22.03 ± 1.8 ìs, 35.9 ± 2.2 ìs, and 32.2 ± 6.5 ìs, resp., $P < 0.01$). We also observed neuromuscular blocking in 50% of the EAMG controls, in 100% of the mice injected with NMO-Ig, in 50% of mice injected with AQP4 peptide, and none in the naive CFA-injected mice. All of these electrophysiological findings support neuromuscular junction pathology.

3.5. Immune Responses in AQP4 Peptide or NMO-Ig Injected to Both Naive and EAMG Mice. To investigate whether the systemic immune response differs in EAMG and EAMG/NMO mice, we studied the spleen and lymph node cell repertoire using flow cytometry and the secretion of cytokines from spleen cells stimulated by anti-CD3 using ELISA assay.

Lymphocyte cultures derived from CFA-injected mice were compared with lymphocytes from EAMG injected with AQP4 peptide or with NMO-Ig and cultures derived from naive mice that were injected with this peptide or antibodies. As shown in Figure 4(a), we found significantly increased IFNγ secretion when the cells were stimulated with anti-CD3, in both EAMG control mice and naive mice injected with AQP4 peptide ($18{,}469 \pm 830$, $18{,}086 \pm 1121$, resp., and $13{,}230 \pm 848$ in the CFA injected mice, $P < 0.05$). Significantly increased IL-6 secretion (compared to CFA-injected mice) was detected (Figure 4(b)), in EAMG control mice, in EAMG mice injected with NMO-Ig, and in naive mice injected with AQP4 peptide (1521 ± 59, 1004 ± 17.4 1303.7 ± 54, resp., and 560 ± 106.5 in the CFA-injected mice, $P < 0.05$). IL-10 secretion did not change significantly (Figure 4(c)).

Flow cytometry analysis of lymphocytes derived from the various mouse groups measuring CD4+, CD8+, CD19+,CD19+/CD27+, CD11b+,CD11c, and MHC-class II cells did not show any statistical difference between the treated groups as compared with the CFA-injected group. Although, EAMG + NMO mice had a significantly more severe disease score and aggravated impairment in force compared with the EAMG mice alone. No significant differences were found in these immune cell markers in these groups (data not shown).

3.6. Expression of Inflammatory Markers in Muscle. We were interested to verify whether the increased severity of muscle fatigability in the EAMG + NMO mice was mediated by the expression of muscle proinflammatory markers. Using RT-PCR, the expression levels of cytokines (IL-6, transforming growth factor β (TGF β)), chemokines, and chemokine receptors C-X-C motif chemokine receptor 3 (CXCR3), interferon gamma inducible protein 10 (IP-10), and monocyte chemoattractant protein 1 (MCP-1), and the atrophy-associated gene atrogin-1 were analyzed. As shown in Figure 5, our study confirmed the findings of increased MCP-1, CXCR3, and IP-10 in EAMG mice as compared to CFA control mice ($P = 0.05$, and 0.06, resp.) [37]. Interestingly, expression of the atrogin-1 muscle atrophy-associated gene was increased in mice that received NMO-Ig and in those injected with AQP4 peptide, while atrogin-1 expression did not change in the EAMG

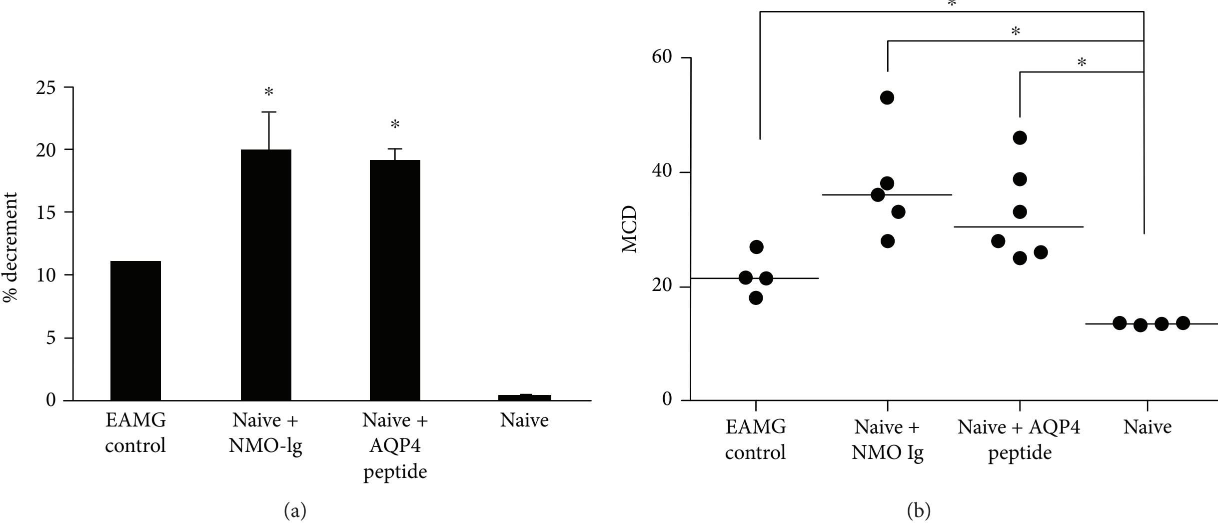

Figure 3: The injection of AQP4 peptide or NMO-Ig induces pathological repetitive nerve stimulation and jitter. (a) Changes in the 5th/1st muscle action potential amplitude ratio calculated as percent decrement and expressed as the mean ± SE in EAMG mice and NMO-Ig- and AQP4 peptide-injected mice. Naive mice had no decrement. (b) NMO-Ig or AQP4 peptide increased neuromuscular mean consecutive difference (MCD). The baseline MCD value of the control CFA-injected mice was $13.5 \pm 0.1\ \mu$s. The MCD values of EAMG mice as well as mice injected with AQP4 peptide or NMO antibodies were significantly elevated and pathologic. $^*P < 0.05$.

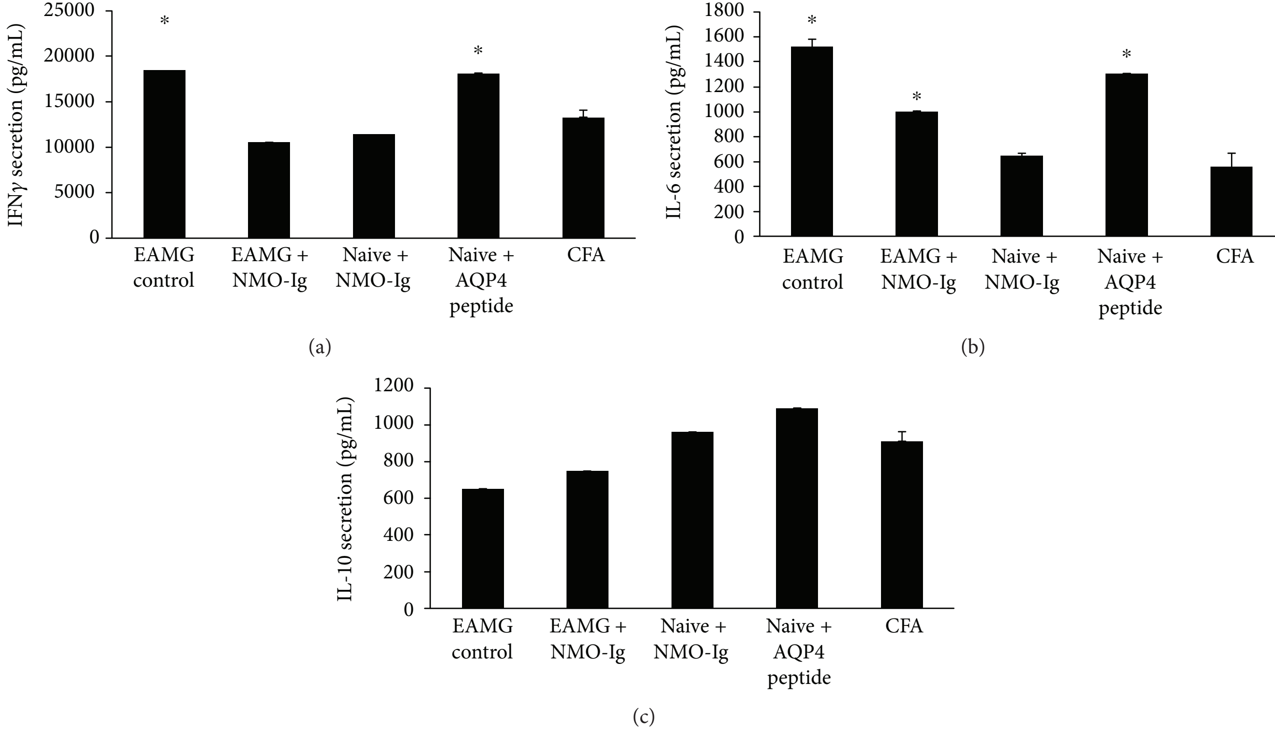

Figure 4: Increased proinflammatory cytokine secretion. Splenocytes derived from EAMG control, EAMG with NMO-Ig, and from naive mice injected with NMO-Ig or AQP4 peptide, or CFA, was activated by anti-CD3. Cell-free supernatants were tested for the cytokine content by specific ELISA. IFNγ was tested after 24 h of stimulation, IL-6 (48 h of stimulation), and IL-10 (after 72 h). Results are the mean of 2 different sets of experiments of 3–5 mice in each group and are expressed as the mean ± SE. $^*P < 0.05$ compared to CFA-injected mice.

mice (Figure 5(d)). Proinflammatory markers (MCP-1, CXCR3, and IP-10, Figure 5(a)–5(c)) were increased in EAMG mice, while expression of IL-6 and TGFβ did not change (data not shown).

4. Discussion

In the present study, we observed that coexisting autoimmune disease—EAMG—can be exacerbated by the NMO

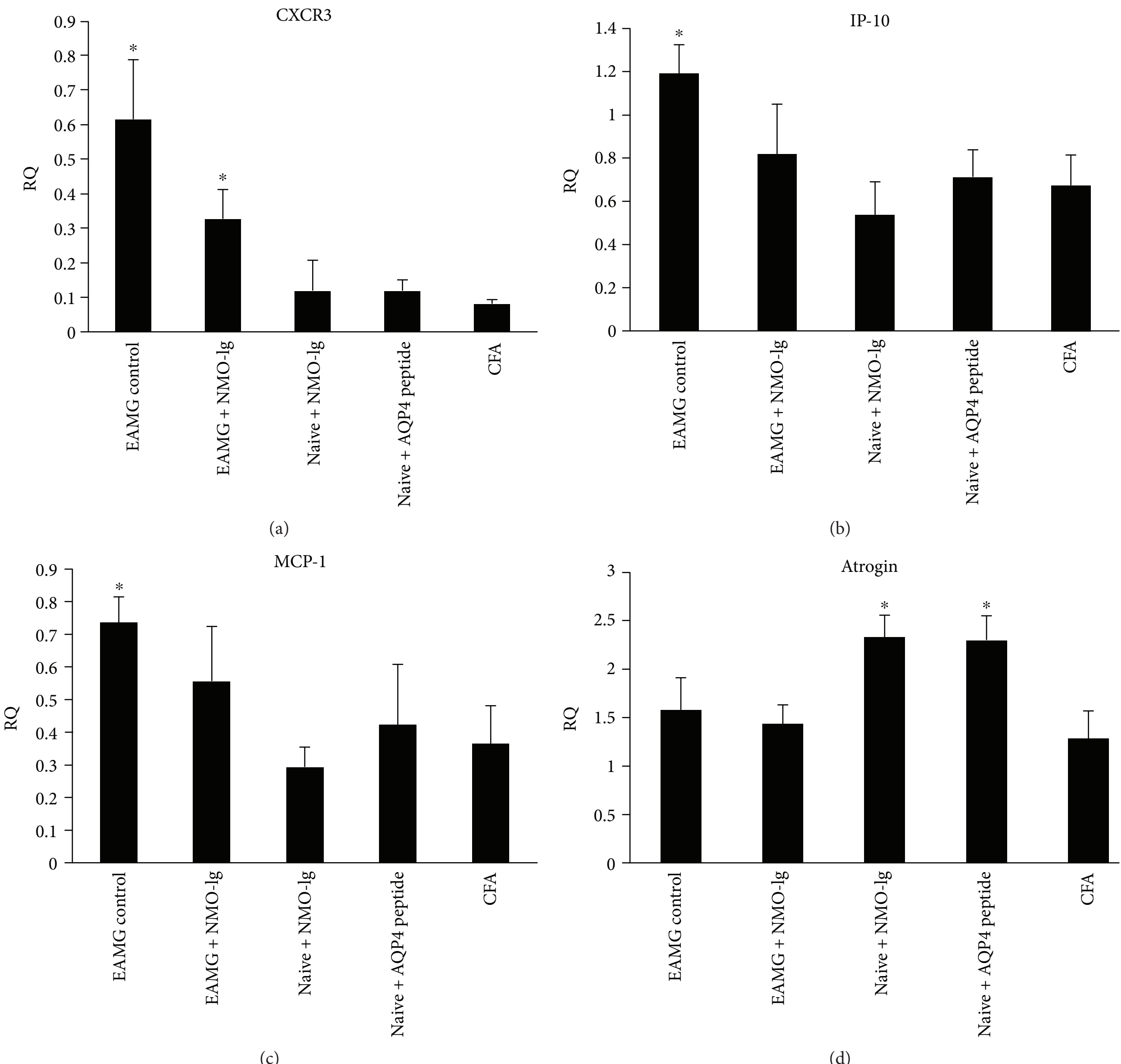

Figure 5: Changes in the expression of muscle markers. The expression of proinflammatory chemokines CXCR3 (a), IP-10 (b), and MCP-1(c) as well as the atrophy marker atrogin (d) were measured by RT-PCR. Results are the mean of 3 different sets of experiments of 3–5 mice in each group and are expressed as the mean ± SE. $^{*}P < 0.05$.

antigen or antibody. The injection of AQP4 peptide or NMO-Ig increased disease severity in these mice and induced mild fatigability in naive mice.

Muscle weakness was expressed by decreased grip force in AQP4 peptide- or NMO-Ig naive-injected mice. This weakness could be attributed to CNS and/or neuromuscular inflammation. However, we did not find evidence for CNS inflammation by brain, spinal cord, and optic nerve histopathology, arguing against major CNS involvement in these mice. On the other hand, we found clear neuromuscular pathology including abnormal neuromuscular transmission with decrement response on RNS and prolonged jitter and blocking. The neuromuscular pathology can be either inflammatory or degenerative. The abnormal neuromuscular transmission was well correlated with an increase in the expression of the atrophy-associated gene, atrogin-1. This change may indicate muscle wasting rather than direct inflammatory process-associated damage. In the EAMG mice, there was a trend toward significant increase in local muscle inflammation as expressed by increased expression of MCP-1, IP-10, and CXCR3. This support a major role for peripheral immune response in the pathogenesis of the observed weakness in the EAMG mice but not in those injected with AQP4 peptide or antibodies. Additional possible explanation for the exacerbated muscle weakness and mild EAMG phenotype in the naive-injected mice could be

due to the use of fatigability—MG-like examinations. We observed distinct fatigability and decreased grip force in both AQP4 peptide- and NMO-Ig-injected mice (Figure 2).

The exacerbated disease phenotype in EAMG mice injected with either AQP4 peptide or NMO-Ig may be explained by two pathogenic mechanisms: first, systemic and/or local immune response. EAMG mice as well as naive mice injected with NMO-Ig and EAMG with NMO-IgG revealed a significant increase in IL-6 secretion compared with CFA-injected mice. This is in accordance with the known IL-6 contribution to both NMO and MG [38–41] and the ample evidence for the role of the systemic immune response in MG and EAMG and disease severity [42]. Second, augmented activity of antigen-presenting cells. Both IL-10 and IL-6 are known to play a major role in NMO and MG [43, 44]. Increased secretion of IL-6 and lack of change in IL-10 were found in the cell cultures in our study supporting a divergence of the cytokine pattern and a possible role for antigen-presenting cells in mediating the pathological findings. The proinflammatory milieu can modify the immune balance; for example, increased IL-6 levels can alter T regulatory suppression activity and increase the resistance of effector T-cell suppression [42].

With regard to local immune response, the NMO autoantigen, AQP4, is also expressed in plasma membranes of cells outside the CNS, including skeletal muscle fibers. Several NMO patients have been reported with hyperCKemia. Recently, the histopathology of muscle involvement in NMO patients and hyperCKemia revealed mild endomysial and perivascular inflammation. Like the classic brain pathology found in patients with NMO, muscle lesions show lymphocyte and eosinophil infiltration with loss of immunoreactivity to AQP4 [16]. In our study, we found several indications that the AQP4 peptide- and NMO-Ig-injected mice had local muscle deviations. These included significantly decreased grip ability and signs of abnormal neuromuscular transmission with increased jitter and blocking on SFEMG. A possible mechanism by which NMO-Ig can exacerbate MG can be by interacting with the muscle AQP4, clustering more FC receptors and increasing the local neuromuscular junction pathology.

Additional possible scenario was raised by Guo et al., describing patients with NMO and hyperCKemia. The authors suggested that AQP4 may affect bioenergetic pathways and could be involved in the intracellular calcium dynamics of muscles [16]. Such mechanism can also explain the increased fatigability in EAMG and naive mice in our study. Calcium homeostasis is particularly perturbed in fast-twitch muscle fibers, which express AQP4 most abundantly and can therefore be affected by either NMO-Ig or by the specific immune response to AQP4 peptide. Furthermore, the report of mild myositis in EAE rats that received adoptively transferred AQP4-specific T-cells [45] is also in agreement to our findings.

Several animal studies have shown that the AQP4 antibodies are not pathogenic via simple transfer of AQP4 antibodies into naive animals. The NMO/EAE model clearly demonstrates that T-cells are required to facilitate the permeabilization of the BBB for the entry of antibodies and complement [21, 46–48]. We and the others have found increased AQP4-specific T-cell proliferation in patients with NMO [49, 50]. However, in the present study, T-cell proliferation was not affected by the stimulation with AQP4 peptide or NMO-Ig transfer.

Our approach of inducing a second autoimmune disease in mice that already developed one autoimmune syndrome can have clinical and pathophysiological relevance. Clinically, it is known that patients with one autoimmune disease tend to develop additional autoimmune disease [51, 52]. Immunologically, it is known that intermolecular epitope spreading occurs in animals and humans. For example, immunization of mice with peptides of the EBV, EBNA-1 protein, leads to the development of antibodies to SmB, SmD, nRNPs, and La/SSB [53]. Thus, immunization with the AQP4 peptide may increase immune reactivity to muscle epitopes, which can aggravate fatigability.

5. Conclusions

Our study shows exacerbation of disease severity in EAMG following immunization with the NMO autoantigen AQP4, or by NMO-Ig, that is accompanied by augmented inflammatory response. This finding could explain some of the clinical observations of increased susceptibility of patients with one autoimmune disease to develop a second autoimmune syndrome.

Abbreviations

NMO: Neuromyelitis optica
EAMG: Experimental autoimmune myasthenia gravis
AQP4: Aquaporin 4
MG: Myasthenia gravis
AChR: Acetylcholine receptor
CNS: Central nervous system
IgG: Immunoglobulin G
EMG: Electromyography
SFEMG: Single fiber electromyography
BBB: Blood-brain barrier
EAE: Experimental autoimmune encephalitis
BgT: Bungarotoxin
SNEs: Sensory needle electrodes
CMAP: Compound muscle action potential
CN: Concentric electromyography needle
SFAPs: Single fiber action potentials
RNS: Repetitive nerve stimulation
TGFβ: Transforming growth factor β
MCP-1: Monocyte chemoattractant protein
CXCR3: C-X-C motif chemokine receptor 3
IP-10: Interferon γ inducible protein 10.

Ethical Approval

The study is approved by the ethic committee of the Hebrew University. Ethic committee Research no. HD-15-1-14280-4.

Disclosure

The authors confirm that they have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Talma Brenner and Adi Vaknin-Dembinsky contributed equally to this work.

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References

[1] D. M. Wingerchuk, V. A. Lennon, C. F. Lucchinetti, S. J. Pittock, and B. G. Weinshenker, "The spectrum of neuromyelitis optica," *The Lancet Neurology*, vol. 6, no. 9, pp. 805–815, 2007.

[2] V. A. Lennon, D. M. Wingerchuk, T. J. Kryzer et al., "A serum autoantibody marker of neuromyelitis optica: distinction from multiple sclerosis," *The Lancet*, vol. 364, no. 9451, pp. 2106–2112, 2004.

[3] P. W. Phuan, J. Ratelade, A. Rossi, L. Tradtrantip, and A. S. Verkman, "Complement-dependent cytotoxicity in neuromyelitis optica requires aquaporin-4 protein assembly in orthogonal arrays," *Journal of Biological Chemistry*, vol. 287, no. 17, pp. 13829–13839, 2012.

[4] A. C. Hoedemaekers, P. J. van Breda Vriesman, and M. H. De Baets, "Myasthenia gravis as a prototype autoimmune receptor disease," *Immunologic Research*, vol. 16, no. 4, pp. 341–354, 1997.

[5] S. J. Pittock, V. A. Lennon, J. de Seze et al., "Neuromyelitis optica and non–organ-specific autoimmunity," *Archives of Neurology*, vol. 65, no. 1, pp. 78–83, 2008.

[6] A. Iyer, L. Elsone, R. Appleton, and A. Jacob, "A review of the current literature and a guide to the early diagnosis of autoimmune disorders associated with neuromyelitis optica," *Autoimmunity*, vol. 47, no. 3, pp. 154–161, 2014.

[7] P. B. Christensen, T. S. Jensen, I. Tsiropoulos et al., "Associated autoimmune diseases in myasthenia gravis A population-based study," *Acta Neurologica Scandinavica*, vol. 91, no. 3, pp. 192–195, 1995.

[8] A. Vaknin-Dembinsky, O. Abramsky, P. Petrou et al., "Myasthenia gravis–associated neuromyelitis optica–like disease: an immunological link between the central nervous system and muscle?," *Archives of Neurology*, vol. 68, no. 12, pp. 1557–1561, 2011.

[9] M. I. Leite, E. Coutinho, M. Lana-Peixoto et al., "Myasthenia gravis and neuromyelitis optica spectrum disorder: a multicenter study of 16 patients," *Neurology*, vol. 78, no. 20, pp. 1601–1607, 2012.

[10] A. McKeon, V. A. Lennon, A. Jacob et al., "Coexistence of myasthenia gravis and serological markers of neurological autoimmunity in neuromyelitis optica," *Muscle & Nerve*, vol. 39, no. 1, pp. 87–90, 2009.

[11] A. Uzawa, M. Mori, Y. Iwai et al., "Association of anti-aquaporin-4 antibody-positive neuromyelitis optica with myasthenia gravis," *Journal of the Neurological Sciences*, vol. 287, no. 1-2, pp. 105–107, 2009.

[12] S. Jarius, F. Paul, D. Franciotta et al., "Neuromyelitis optica spectrum disorders in patients with myasthenia gravis: ten new aquaporin-4 antibody positive cases and a review of the literature," *Multiple Sclerosis Journal*, vol. 18, no. 8, pp. 1135–1143, 2012.

[13] R. Ikeguchi, Y. Shimizu, S. Suzuki et al., "Japanese cases of neuromyelitis optica spectrum disorder associated with myasthenia gravis and a review of the literature," *Clinical Neurology and Neurosurgery*, vol. 125, pp. 217–221, 2014.

[14] J. Spillane, G. Christofi, K. C. Sidle, D. M. Kullmann, and R. S. Howard, "Myasthenia gravis and neuromyelitis opica: a causal link," *Multiple Sclerosis and Related Disorders*, vol. 2, no. 3, pp. 233–237, 2013.

[15] Z. Wang and Y. Yan, "Immunopathogenesis in myasthenia gravis and neuromyelitis optica," *Frontiers in Immunology*, vol. 8, article 1785, 2017.

[16] Y. Guo, V. A. Lennon, B. F. Popescu et al., "Autoimmune aquaporin-4 myopathy in neuromyelitis optica spectrum," *JAMA Neurology*, vol. 71, no. 8, pp. 1025–1029, 2014.

[17] J. Cosgrove, S. Alli, H. Ramadan, and H. L. Ford, "Myocarditis and diffuse skeletal muscle oedema: new features of neuromyelitis optica spectrum disorder? A case report," *Multiple Sclerosis Journal*, vol. 20, no. 1, pp. 120–122, 2014.

[18] M. Di Filippo, D. Franciotta, R. Massa et al., "Recurrent hyperCKemia with normal muscle biopsy in a pediatric patient with neuromyelitis optica," *Neurology*, vol. 79, no. 11, pp. 1182–1184, 2012.

[19] M. Bradl and H. Lassmann, "Experimental models of neuromyelitis optica," *Brain Pathology*, vol. 24, no. 1, pp. 74–82, 2014.

[20] S. Saadoun, P. Waters, B. A. Bell, A. Vincent, A. S. Verkman, and M. C. Papadopoulos, "Intra-cerebral injection of neuromyelitis optica immunoglobulin G and human complement produces neuromyelitis optica lesions in mice," *Brain*, vol. 133, no. 2, pp. 349–361, 2010.

[21] J. L. Bennett, C. Lam, S. R. Kalluri et al., "Intrathecal pathogenic anti–aquaporin-4 antibodies in early neuromyelitis optica," *Annals of Neurology*, vol. 66, no. 5, pp. 617–629, 2009.

[22] K. Kurosawa, T. Misu, Y. Takai et al., "Severely exacerbated neuromyelitis optica rat model with extensive astrocytopathy by high affinity anti-aquaporin-4 monoclonal antibody," *Acta Neuropathologica Communications*, vol. 3, no. 1, p. 82, 2015.

[23] M. V. Jones, H. Huang, P. A. Calabresi, and M. Levy, "Pathogenic aquaporin-4 reactive T cells are sufficient to induce mouse model of neuromyelitis optica," *Acta Neuropathologica Communications*, vol. 3, no. 1, p. 28, 2015.

[24] R. Marignier, A. Ruiz, S. Cavagna et al., "Neuromyelitis optica study model based on chronic infusion of autoantibodies in rat cerebrospinal fluid," *Journal of Neuroinflammation*, vol. 13, no. 1, p. 111, 2016.

[25] H. Zhang and A. S. Verkman, "Longitudinally extensive NMO spinal cord pathology produced by passive transfer of NMO-IgG in mice lacking complement inhibitor CD59," *Journal of Autoimmunity*, vol. 53, pp. 67–77, 2014.

[26] S. A. Sagan, R. C. Winger, A. Cruz-Herranz et al., "Tolerance checkpoint bypass permits emergence of pathogenic T cells to neuromyelitis optica autoantigen aquaporin-4," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 113, no. 51, pp. 14781–14786, 2016.

[27] A. L. Vogel, B. Knier, K. Lammens et al., "Deletional tolerance prevents AQP4-directed autoimmunity in mice," *European Journal of Immunology*, vol. 47, no. 3, pp. 458–469, 2017.

[28] A. Aharonov, R. Tarrab-Hazdai, I. Silman, and S. Fuchs, "Immunochemical studies on acetylcholine receptor from Torpedo californica," *Immunochemistry*, vol. 14, no. 2, pp. 129–137, 1977.

[29] D. Gur-Wahnon, T. Mizrachi, S. Wald-Altman, A. Al-Roof Higazi, and T. Brenner, "Tissue plasminogen activator involvement in experimental autoimmune myasthenia gravis: aggravation and therapeutic potential," *Journal of Autoimmunity*, vol. 52, pp. 36–43, 2014.

[30] D. M. Wingerchuk, V. A. Lennon, S. J. Pittock, C. F. Lucchinetti, and B. G. Weinshenker, "Revised diagnostic criteria for neuromyelitis optica," *Neurology*, vol. 66, no. 10, pp. 1485–1489, 2006.

[31] M. F. Osuchowski, J. Teener, and D. Remick, "Noninvasive model of sciatic nerve conduction in healthy and septic mice: reliability and normative data," *Muscle & Nerve*, vol. 40, no. 4, pp. 610–616, 2009.

[32] D. Dumitru, A. A. Amato, and M. J. Zwarts, "Neuromuscular junction disorders," in *Electrodiagnostic Medicine*, pp. 1148–1177, Hanley & Belfus Inc., Philadelphia, PA, USA, 2nd edition, 2002.

[33] E. V. Stålberg and D. B. Sanders, "Jitter recordings with concentric needle electrodes," *Muscle & Nerve*, vol. 40, no. 3, pp. 331–339, 2009.

[34] E. Stålberg, "Jitter and impulse blocking," in *Single Fiber Electromyography, Studies in Healthy and Diseased Muscle*, pp. 45–82, Raven Press, New York, NY, USA, 1994.

[35] K. Tanase, Q. Teng, A. A. Krishnaney, J. K. Liu, M. E. Garrity-Moses, and N. M. Boulis, "Cervical spinal cord delivery of a rabies G protein pseudotyped lentiviral vector in the SOD-1 transgenic mouse. Invited submission from the joint section meeting on disorders of the spine and peripheral nerves, March 2004," *Journal of Neurosurgery: Spine*, vol. 1, no. 1, pp. 128–136, 2004.

[36] E. Nizri and T. Brenner, "Modulation of inflammatory pathways by the immune cholinergic system," *Amino Acids*, vol. 45, no. 1, pp. 73–85, 2013.

[37] T. Feferman, P. K. Maiti, S. Berrih-Aknin et al., "Overexpression of IFN-induced protein 10 and its receptor CXCR3 in myasthenia gravis," *The Journal of Immunology*, vol. 174, no. 9, pp. 5324–5331, 2005.

[38] M. Maurer, S. Bougoin, T. Feferman et al., "IL-6 and Akt are involved in muscular pathogenesis in myasthenia gravis," *Acta Neuropathologica Communications*, vol. 3, no. 1, p. 1, 2015.

[39] R. Aricha, K. Mizrachi, S. Fuchs, and M. C. Souroujon, "Blocking of IL-6 suppresses experimental autoimmune myasthenia gravis," *Journal of Autoimmunity*, vol. 36, no. 2, pp. 135–141, 2011.

[40] S. İİçöz, E. Tüzün, M. Kürtüncü et al., "Enhanced IL-6 production in aquaporin-4 antibody positive neuromyelitis optica patients," *International Journal of Neuroscience*, vol. 120, no. 1, pp. 71–75, 2010.

[41] A. Uzawa, M. Mori, S. Sawai et al., "Cerebrospinal fluid interleukin-6 and glial fibrillary acidic protein levels are increased during initial neuromyelitis optica attacks," *Clinica Chimica Acta*, vol. 421, pp. 181–183, 2013.

[42] S. Berrih-Aknin, "Myasthenia gravis: paradox versus paradigm in autoimmunity," *Journal of Autoimmunity*, vol. 52, pp. 1–28, 2014.

[43] S. Cohen-Kaminsky, O. Devergne, R. M. Delattre et al., "Interleukin-6 overproduction by cultured thymic epithelial cells from patients with myasthenia gravis is potentially involved in thymic hyperplasia," *European Cytokine Network*, vol. 4, no. 2, pp. 121–132, 1993.

[44] N. Chihara, T. Aranami, W. Sato et al., "Interleukin 6 signaling promotes anti-aquaporin 4 autoantibody production from plasmablasts in neuromyelitis optica," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 9, pp. 3701–3706, 2011.

[45] M. Pohl, M. T. Fischer, S. Mader et al., "Pathogenic T cell responses against aquaporin 4," *Acta Neuropathologica*, vol. 122, no. 1, pp. 21–34, 2011.

[46] M. Bradl, T. Misu, T. Takahashi et al., "Neuromyelitis optica: pathogenicity of patient immunoglobulin in vivo," *Annals of Neurology*, vol. 66, no. 5, pp. 630–643, 2009.

[47] M. Kinoshita, Y. Nakatsuji, T. Kimura et al., "Neuromyelitis optica: passive transfer to rats by human immunoglobulin," *Biochemical and Biophysical Research Communications*, vol. 386, no. 4, pp. 623–627, 2009.

[48] N. Asavapanumas, J. Ratelade, and A. S. Verkman, "Unique neuromyelitis optica pathology produced in naïve rats by intracerebral administration of NMO-IgG," *Acta Neuropathologica*, vol. 127, no. 4, pp. 539–551, 2014.

[49] A. Vaknin-Dembinsky, L. Brill, I. Kassis et al., "T-cell reactivity against AQP4 in neuromyelitis optica," *Neurology*, vol. 79, no. 9, pp. 945-946, 2012.

[50] M. Varrin-Doyer, C. M. Spencer, U. Schulze-Topphoff et al., "Aquaporin 4-specific T cells in neuromyelitis optica exhibit a Th17 bias and recognize *Clostridium* ABC transporter," *Annals of Neurology*, vol. 72, no. 1, pp. 53–64, 2012.

[51] M. Hudson, A. Rojas-Villarraga, P. Coral-Alvarado et al., "Polyautoimmunity and familial autoimmunity in systemic sclerosis," *Journal of Autoimmunity*, vol. 31, no. 2, pp. 156–159, 2008.

[52] J. Y. Maeda, A. K. Moura, C. W. Maruta, C. G. Santi, P. S. Prisayanh, and V. Aoki, "Changes in the autoimmune blistering response: a clinical and immunopathological shift from pemphigus foliaceus to bullous pemphigoid," *Clinical and Experimental Dermatology*, vol. 31, no. 5, pp. 653–655, 2006.

[53] C. Cornaby, L. Gibbons, V. Mayhew, C. S. Sloan, A. Welling, and B. D. Poole, "B cell epitope spreading: mechanisms and contribution to autoimmune diseases," *Immunology Letters*, vol. 163, no. 1, pp. 56–68, 2015.

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Research Article

Downregulation of iNOS, IL-1β, and P2X7 Expression in Mast Cells via Activation of PAR4 Contributes to the Inhibition of Visceral Hyperalgesia in Rats

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Protease-activated receptor 4 (PAR4) is implicated in the inhibition of visceral hyperalgesia. In the present study, the effects of PAR4 activation on visceral hypersensitivity and expression of inflammatory mediators, including interleukin-1β (IL-1β), P2RX7 purinergic receptor (P2X7), inducible nitric oxide synthase (iNOS), and tryptase, in mast cells (MCs) were investigated via in vivo and in vitro studies. The numbers of tryptase-positive MCs with extensive PAR4, P2X7, and iNOS expression were increased in the colons of visceral hyperalgesia rats compared with controls. Intracolonic administration of PAR4-activating peptide (PAR4-AP) significantly attenuated the visceral hypersensitivity to colorectal distention and reduced the iNOS, IL-1β, P2X7, and tryptase protein and mRNA levels in the colonic mucosa. Treatment of rat bone marrow MCs (BMMCs) with PAR4-AP also reduced the iNOS, IL-1β, P2X7, and tryptase protein and mRNA levels. ERK1/2 and p38 activators (t-butylhydroquinone, tBHQ, and U-46619) reversed the suppressive effect of PAR4 activation on IL-1β and iNOS expression, whereas ERK1/2 and p38 inhibitors (PD98059 and SB203580) reversed the suppressive effect of PAR4 activation on P2X7 and tryptase expression. Our results indicate that the downregulation of inflammatory mediators, including iNOS, IL-1β, P2X7, and tryptase, in MCs that are mediated by PAR4 activation could inhibit visceral hyperalgesia via the mitogen-activated protein kinase (MAPK) signal pathway.

1. Introduction

Accumulating evidence suggests that mast cells (MCs), especially mucosal MCs, have crucial roles in the regulation of intestinal motility, visceral sensitivity, mucosal inflammation, the permeability of the epithelial barrier, and the immune system in irritable bowel syndrome (IBS) [1, 2]. Increased numbers of activated MCs and increases in MC products have been observed throughout the gastrointestinal mucosa of IBS patients [3, 4]. The soluble mediators released by activated MCs, particularly proinflammatory mediators and cytokines such as tryptase and interleukin-1β (IL-1β), contribute to visceral hyperalgesia [5, 6].

Protease-activated receptor 4 (PAR4) is a member of the G-protein coupled receptor family and may mediate an antinociceptive effect, which would indicate new roles in the modulation of visceral hyperalgesia and hypersensitivity [7, 8]. Previous research has demonstrated that the activation of PAR4 inhibits colonic hypersensitivity through the suppression of the excitability of colonic sensory neurons and their primary afferent responses to pronociceptive mediators [8, 9]. PAR4 is highly expressed in MCs in the colons of IBS patients [10, 11]. MCs are efficient producers of many key inflammatory cytokines in response to a variety of stimuli, including as nitric oxide (NO)/inducible NO synthase (iNOS), ATP-reactive P2RX7 purinergic receptor (P2X7), and inflammatory cytokines [12, 13]. Several studies have reported that mitogen-activated protein kinases (MAPK), such as extracellular signal-regulated protein kinase 1/2 (ERK1/2) and p38 MAPK (p38), are crucial mediators of inflammation in inflammatory bowel disease (IBD) [14, 15]. Recently, we reported that PAR4 activation suppresses the

inflammatory cytokines associated with the phosphorylation of ERK1/2 and p38 in MCs [16]. However, the function of PAR4 on MCs in visceral hypersensitivity is relatively unknown. Therefore, a better understanding of the role of PAR4 activation on MCs in the gut in visceral hyperalgesia is needed.

In the present study, we examined potential influence of PAR4 activation on colonic sensations in a visceral hyperalgesia rat model and the expressions of iNOS, P2X7, IL-1β, and tryptase in MCs, which might regulate sensitization and the consequent heightened pain behavior in IBS. We also investigated whether the activation of PAR4 affects the MAPK pathway, which involved in the expressions of iNOS, P2X7, IL-1β, and tryptase in MCs.

2. Materials and Methods

2.1. Induction of Chronic Visceral Hyperalgesia. The rat model of visceral hyperalgesia was induced as previously described [17]. Briefly, daily 60 mmHg colorectal distension (CRD) was performed on neonatal rats between 8 and 21 postnatal days after birth. The distention was applied using a vascular reconstruction balloon (length 20.0 mm, diameter 2.5 mm) that was inserted into the descending colon through the rectums of awake rats. The balloon was quickly distended at 60 mmHg for 1 min and then deflated and withdrawn. The control rats received the same procedure except for the CRD. The experiments were performed in these rats at the age of at least 8 weeks old.

2.2. Intracolonic Administration. The visceral hyperalgesia rats received an intracolonic administration of 100 μg PAR4-activating peptide (PAR4-AP) or control peptide diluted in 150 μl 0.9% NaCl. The visceral sensitivity measurements began 60 min following the end of the intracolonic administration.

2.3. Colorectal Distension and Electromyographic Recording. We used the electromyographic (EMG) recordings of the external oblique muscle and abdominal withdraw reflex (AWR) scores to evaluate visceral hypersensitivity 8 weeks after treatment according to the responses of rats to CRD as described previously [17, 18]. Briefly, the rats were anesthetized with ether, and CRD was performed via the insertion of a plastic balloon (5 cm) into the descending colon and rectum to 1 cm from the anus. Silver bipolar electrodes were inserted above the inguinal ligament on the side of the external oblique muscle 1.5 cm away from the midline. After the rats recovered from the inhalation anesthesia, the balloon was inflated to 20, 40, 60, and 80 mmHg for 10 s followed by 4 min of rest. The magnitude of the EMG activity was measured with a RM6240BD multichannel physiological signal acquisition and processing system (Chengdu, China). The EMG signals were amplified, filtered (×10,000, 300–5000 Hz), digitized, and rectified as previously detailed [18]. The area under the curve (AUC) values of the EMGs during the first and second distensions was computed, and the basal AUC was subtracted to obtain the net AUC in response to CRD.

2.4. Bone Marrow MC (BMMC) Preparation and Induction. BMMCs were cultured from the bone marrow cells (BMCs) of rats as previously described [19]. Briefly, BMCs were cultured for up to 10 weeks in enriched RPMI-1640 medium (containing 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mmol/l HEPES, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 0.1 mmol/l nonessential amino acids, 0.05 mmol/l β-ME, and 10% FBS) in the presence of both recombinant rat IL-3 (5 ng/ml, R&D Systems Inc.) and recombinant mouse stem cell factor (SCF, 5 ng/ml, PeproTech). The nonadherent cells were hemidepleted twice each week with enriched medium containing the cytokines mentioned above. After 3 weeks, >98% of the cells in the culture were MCs as determined by staining with toluidine blue.

2.5. Drug Administration. Cultured BMMCs that were harvested at 4 weeks were continuously stimulated with PAR4-AP (100 μmol/l) for 60 min. To assess the possible effects of ERK1/2 and p38 on the regulation of tryptase, iNOS, P2X7, and IL-1β expression following PAR4-AP stimulation, PD98059 (10 μmol/l), SB203580 (10 μM), tBHQ (50 nmol/l), or U-46619 (10 nmol/l) was added to six-well plates 60 min prior to the addition of PAR4-AP. Cells stimulated with PAR4 control peptide (100 μmol/l) without ERK1/2 or p38 inhibitors or activators were used as controls. Sister six-well plates of BMMC cultures were used to compare the control cells with the cells that were treated with PAR4-AP.

2.6. Immunohistochemistry. All colonic samples from the visceral hyperalgesia rats and controls were fixed in buffered 4% paraformaldehyde for 30 min. After overnight cryoprotection in 20% buffered sucrose, 8-μm-thick cryostat sections were mounted on poly-L-lysine-coated slides. The sections were preincubated with 1% BSA, 0.5% Triton X-100, and 10% normal donkey serum for 60 min at room temperature. The slides were incubated in a moist chamber with AA1 mouse monoclonal antibody (anti-mast cell tryptase antibody; 1 : 500, Abcam) at 4°C overnight. The slides were then washed and incubated with horseradish peroxidase- (HRP-) labeled goat anti-mouse antibody (1 : 100) for 45 min at 37°C. Next, the slides were developed in 0.05% freshly prepared 3,3′-diaminobenzedine (DAB) solution with 0.03% hydrogen peroxide for 8 min and then counterstained with toluidine blue, dehydrated, air-dried, and mounted in neutral resins. Immunoreactivity was quantified as previously detailed [13].

2.7. Western Blotting. Tissues or cultured BMMCs were lysed, and the protein was extracted. The protein lysate from each sample was separated electrophoretically on a sodium dodecyl sulfate-polyacrylamide gel and then transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 5% nonfat milk in TBS-T (containing 0.1% Tween-20) for 2 hrs, the membranes were incubated with iNOS, P2X7, IL-1β (Novus Biologicals), and tryptase (AA1) antibodies in 5% nonfat milk in TBS-T overnight at 4°C. After washes with TBS-T, the membranes were incubated with the appropriate secondary antibodies for 2 hrs. The results were visualized using an ECL chemiluminescence system. GAPDH rabbit

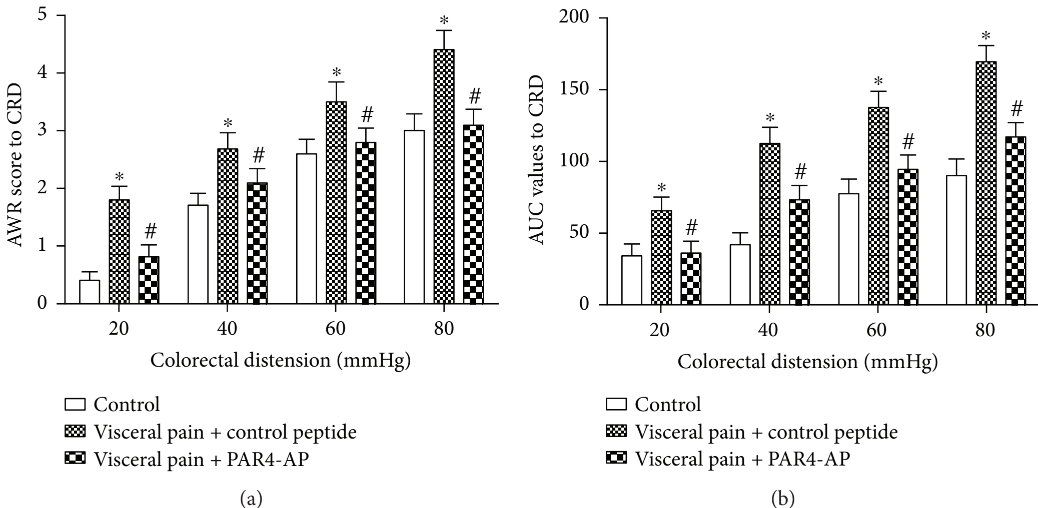

Figure 1: Effect of PAR4-AP on colorectal distension- (CRD-) induced visceral pain in the visceral hyperalgesia rats. (a) Abdominal withdrawal reflex (AWR) scores were used as an index of the response to CRD. (b) Area under the curve (AUC) of the electromyographic (EMG) activity in the external oblique muscle in response to CRD. All values are presented as the mean ± SEM ($n = 6$). $^*P < 0.05$ versus control; $^{\#}P < 0.05$ versus control peptide group.

mAb antibody (Cell Signaling Technology) was also used as a probed control to ensure the loading of equivalent amounts of the sample proteins. The band densities were compared in TotalLab software (version 2.01; Bio-Rad, Hercules, CA).

2.8. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). Total RNA was isolated from the colonic tissues or BMMCs using TRIzol reagent (Invitrogen). The RNA concentrations were determined spectrophotometrically. Subsequently, cDNA was synthesized using a cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. The synthetic oligonucleotide primer sequences were as follows: P2X7: 5′-TTACGGCACCATCAAGTGGA-3′ (sense) and 5′-GCAAAGGGAGGGTGTAGTCG-3′ (antisense); iNOS: 5′-TTCAGTATCACAACCTCAGCAAG-3′ (sense) and 5′-TGGACCTGCAAGTTAAAATCCC-3′ (antisense); IL-1β: 5′-ATGATGGCTTATTACAGTGGCAA-3′ (sense) and 5′-GTCGGAGATTCGTAGCTGGA-3′(antisense); tryptase: 5′-TACCGCTATGTCCCCAAGGA-3′ (sense) and 5′-GAGGGACACAAGTGGTCAGG-3′ (antisense); and β-actin: 5′-ATCGTGCGTGACATTAAGGAGAAG-3′ (sense) and 5′-AGGAAGGAAGGCTGGAAGAGTG-3′ (antisense). Following reverse transcription, quantitative RT-PCR was performed using a 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. In the control reactions, the reverse transcriptase was omitted. A comparative cycle threshold fluorescence (ΔCt) method was used, and the relative transcript amount of the target gene was normalized to that of β-actin using the $2^{-\Delta\Delta CT}$ method. The final results of the real-time PCR are expressed as the ratio of the test mRNA to the control. All PCR product sizes were confirmed by electrophoresis on a 1.5% agarose gel and visualization using ethidium bromide.

2.9. Flow Cytometry. The profile of anti-AA1, PAR4, P2X7, and iNOS reactivities in the cultured BMMCs was analyzed by flow cytometry using FACSCalibur (BD Biosciences). Suspended cells were harvested from the culture plates at 4 weeks and washed with PBS by centrifugation. The cell suspensions were incubated with AA1, PAR4, P2X7, and iNOS antibodies for 30 min on ice. The cells were washed twice with ice-cold PBS and then incubated with fluorescence-conjugated secondary antibody for 60 min at 4°C in the dark. A matched isotope control was set to establish the background fluorescence. The cells were washed 3 times and then analyzed by flow cytometry. The experiment was repeated three times.

2.10. Statistical Analysis. All experiments were independently repeated at least three times. The values are expressed as the means ± SEMs, and the results were analyzed using an ANOVA followed by Bonferroni's post hoc test for comparisons between groups. Significance was defined by P values < 0.05.

3. Results

3.1. A PAR4 Agonist Inhibits the Nociceptive Response to Colorectal Distension. The visceral hyperalgesia rat model was established by neonatal colorectal distention. The visceral sensitivity to CRD was determined at 8 weeks of age in the visceral hyperalgesia rats. The visceral hyperalgesia rats exhibited higher mean AWR scores and AUC values for the abdominal EMG activity at all tested distension pressures compared with the control groups ($P < 0.05$; Figures 1(a) and 1(b)). The intracolonic administration of PAR4-AP to the visceral hyperalgesia rats for 60 min elicited showed lower AWR scores and EMG activities at all tested distension pressures compared with the control peptide treatment ($P < 0.05$; Figures 1(a) and 1(b)).

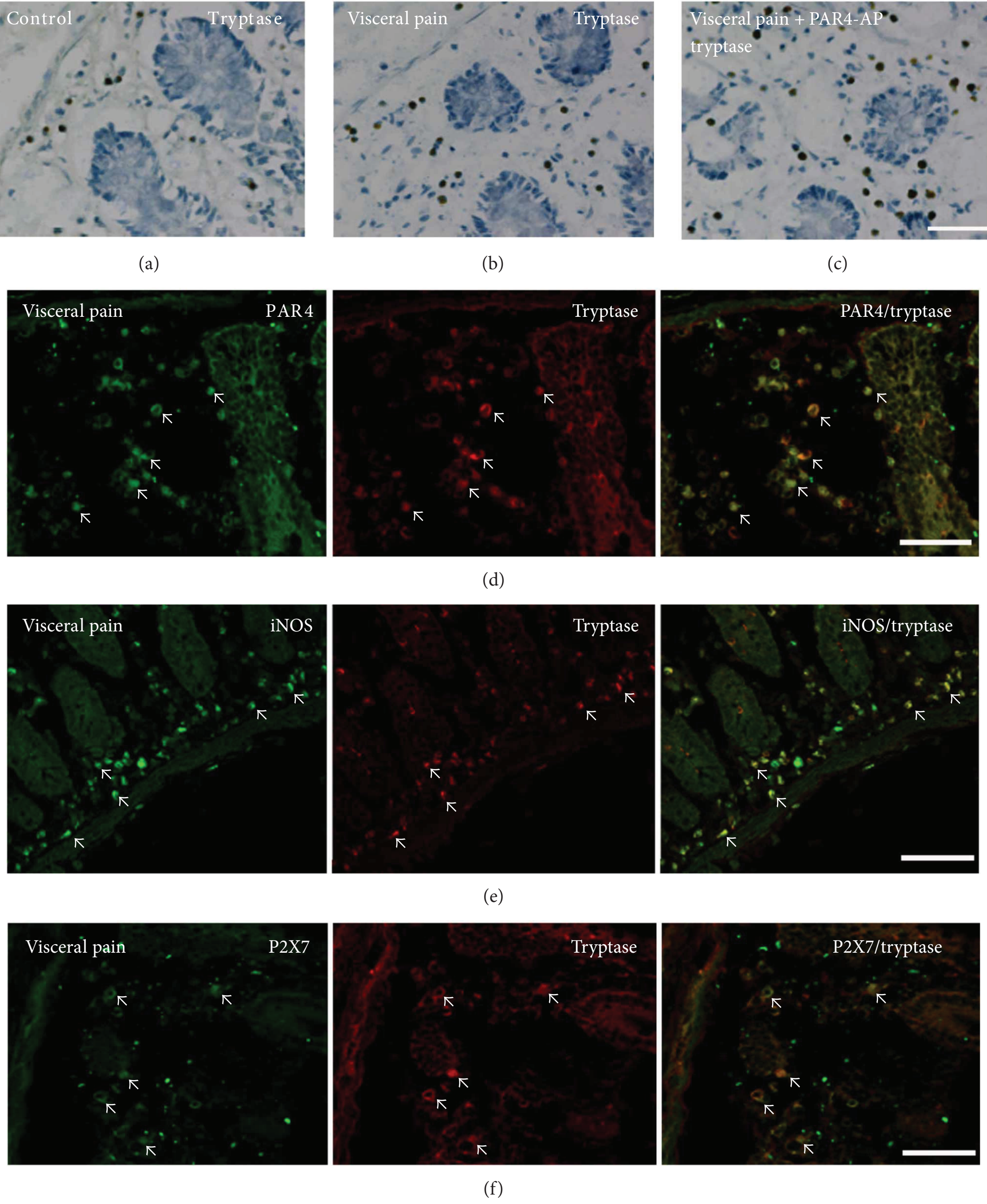

Figure 2: Expression of tryptase (AA1) and its colocalization with PAR4, iNOS, and P2X7 in the colonic mucosae of the visceral hyperalgesia rats. (a–c) Representative immunostainings for tryptase- (AA1-) positive MCs in the colonic sections are shown. The colonic sections were counterstained with toluidine blue. (d–f) Colonic sections from the visceral hyperalgesia rats costained with tryptase (AA1) and PAR4, iNOS, or P2X7 antibodies showing that the majority of the tryptase-positive MCs expressed PAR4, iNOS, or P2X7 (bar 100 μm).

3.2. MCs Expressing PAR4, iNOS, and P2X7 Immunoreactivity in the Colon. We then analyzed the tryptase (AA1) immunopositive MCs in the colonic mucosae of the visceral hyperalgesia rats with immunohistochemistry. The number of tryptase-immunopositive MCs in the colon was significantly higher in the visceral hyperalgesia rats than in the controls ($P < 0.05$; Figures 2(a) and 2(b)). The intracolonic administration of PAR4-AP for 60 min elicited no significant difference in the number of tryptase-immunopositive MCs between the visceral hyperalgesia rats that were treated with PAR4-AP and those that were treated with the control peptide (Figures 2(b), 2(c), and 3(a)). Double labeling revealed that the tryptase-immunopositive MCs extensively expressed PAR4, iNOS, and P2X7 in the colons of the visceral hyperalgesia rats (Figures 2(d)–2(f)).

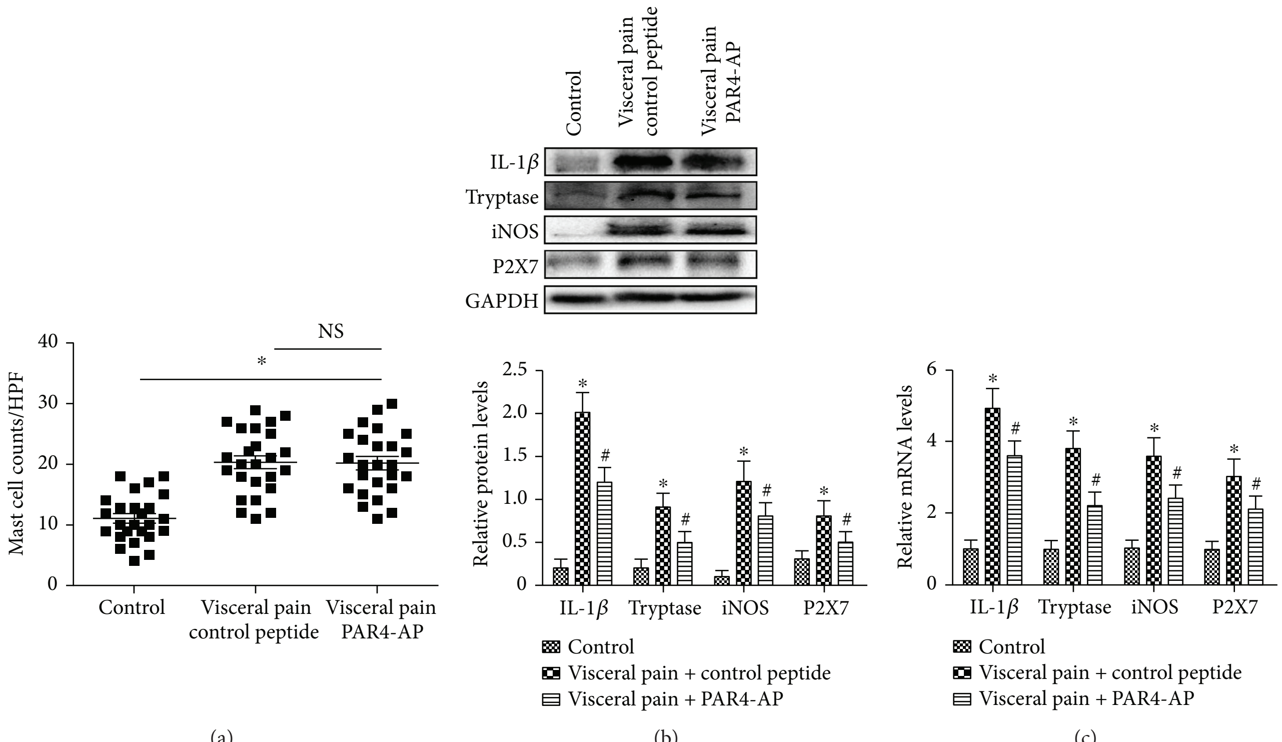

Figure 3: Effects of PAR4-AP on the expressions of tryptase, iNOS, P2X7, and IL-1β in the colons of visceral hyperalgesia rats. (a) Graph showing the numbers of tryptase- (AA1-) positive MCs in the colonic mucosae of the visceral hyperalgesia rats that were treated with PAR4-AP or control peptide ($n = 25$). HPF: high-power field. NS: no statistical significance. (b) The tryptase, iNOS, P2X7, and IL-1β protein levels were assessed by Western blotting. The mean optic densities of the protein were calculated by normalizing to GADPH. (c) The relative levels of tryptase, iNOS, P2X7, and IL-1β mRNA were measured by quantitative real-time PCR (qRT-PCR). The results were calculated by normalizing to β-actin in the same sample with the ΔCt method. The data are presented as the mean ± SEM ($n = 3$), $^*P < 0.05$ versus controls; $^\#P < 0.05$ versus the control peptide group.

3.3. Effect of PAR4-AP on the Expressions of the Tryptase, iNOS, P2X7, and IL-1β Proteins and mRNAs in the Colon. Western blotting and qRT-PCR results revealed that the tryptase, iNOS, IL-1β, and P2X7 protein and mRNA levels were elevated in the colons of the visceral hyperalgesia rats compared with the controls ($P < 0.05$). Moreover, the upregulations of the tryptase, iNOS, IL-1β, and P2X7 protein and mRNA levels were significantly suppressed in the visceral hyperalgesia rats that were treated with PAR4-AP compared with those that were treated with the control peptide ($P < 0.05$; Figure 3).

3.4. Cultured Rat BMMCs Expressed Tryptase, PAR4, iNOS, and P2X7. Cultured BMMCs, which share some similar morphological and phenotypic properties with mucosal MCs, were prepared from the bone marrow cells of rats [19]. Immunohistochemistry for mast cell tryptase (AA1) demonstrated that 99% to 100% of the cultured BMMCs that were harvested at 4 weeks exhibited characteristics typical of MCs. Double immunofluorescence staining indicated that the vast majority of the cultured BMMCs that were harvested at 4 weeks expressed both tryptase and PAR4, iNOS, or P2X7 (Figures 4(a)–4(c)). Flow cytometric analysis indicated that cultured BMMCs that were harvested at 4 weeks expressed relatively high levels of tryptase, PAR4, P2X7 or iNOS (Figure 4(d)).

3.5. Effects of MAPK on Tryptase, iNOS, IL-1β, and P2X7 Expressions Induced by PAR4-AP in BMMCs. The Western blotting and quantitative RT-PCR results revealed that the tryptase, iNOS, IL-1β, and P2X7 mRNA and protein levels in the BMMCs were decreased by the PAR4-AP treatment (Figure 5), which indicated that PAR4 activation decreased the tryptase, iNOS, IL-1β, and P2X7 expressions at both the protein and mRNA levels.

Compared with PAR4-AP alone, PD98059 and SB203580 pretreatment induced much lower iNOS and IL-1β protein and mRNA levels, which in turn markedly reversed the suppressive effect of PAR4 activation on the tryptase and P2X7 protein and mRNA expressions. Furthermore, tBHQ and U-46619 pretreatment induced much lower tryptase and P2X7 protein and mRNA expressions, which in turn markedly reversed the suppressive effect of PAR4 activation on the iNOS and IL-1β protein and mRNA levels (Figure 5). These data suggest that the role of PAR4 in suppressing the expressions of tryptase, iNOS, IL-1β, and P2X7 at the mRNA and protein levels was mediated by the MAPK signaling pathway.

4. Discussion

Our study demonstrated that the nociceptive response to CRD and the number of MCs with extensive PAR4, P2X7,

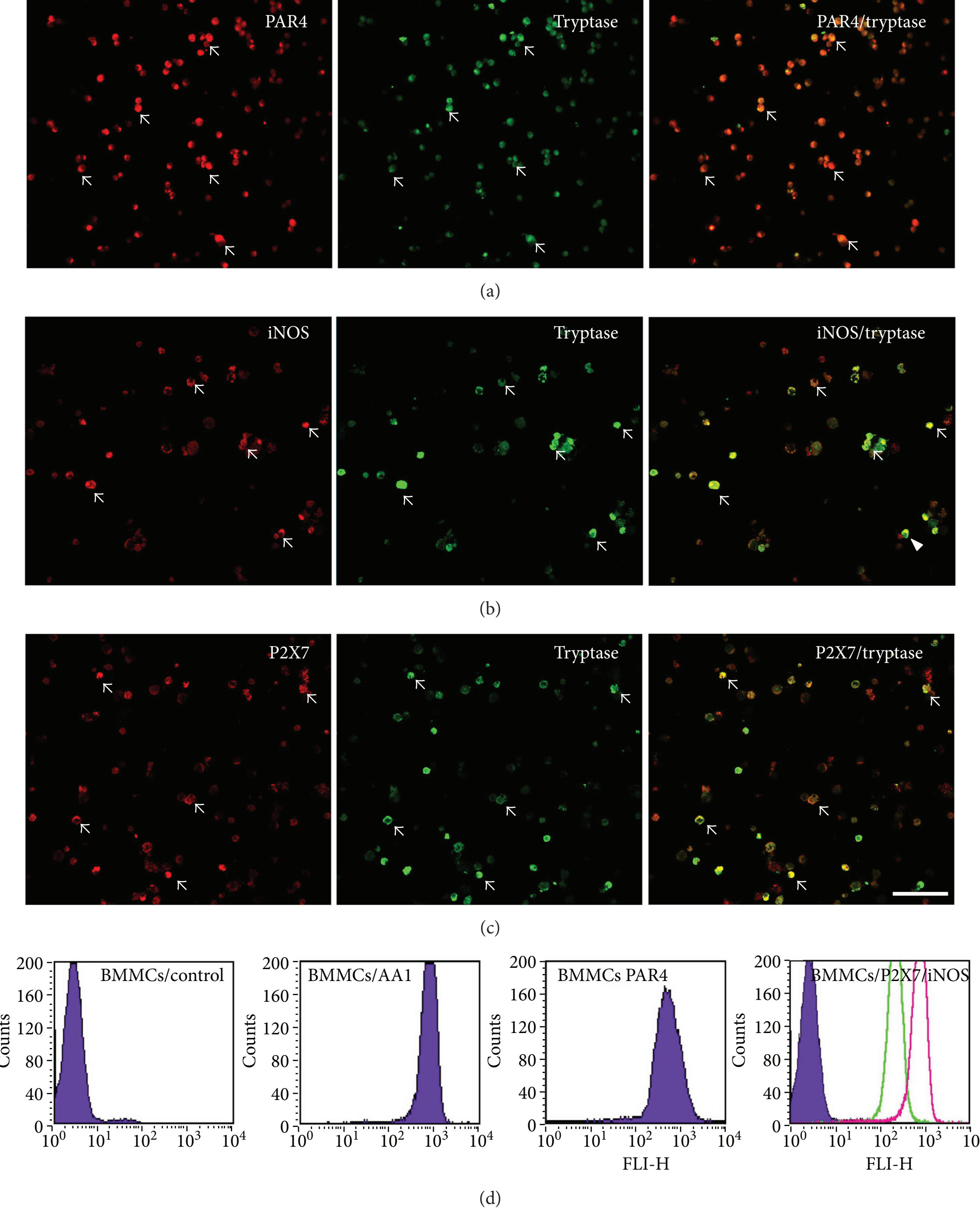

Figure 4: Cultured rat BMMCs expressed tryptase, PAR4, iNOS, and P2X7. (a–c) Expression of tryptase (AA1) and its colocalization with PAR4, iNOS, or P2X7 in cultured BMMCs (bar 100 μm). (d) Flow cytometric analysis showed that the BMMCs expressed relatively high levels of tryptase, PAR4, iNOS, and P2X7.

and iNOS expression were increased in the colons of the visceral hyperalgesia rats. The intracolonic administration of PAR4-AP inhibited colonic hypersensitivity and reduced the expressions of tryptase, iNOS, IL-1β, and P2X7 in the colons of the visceral hyperalgesia rats. These effects were associated with MAPK signals that were induced by PAR4 activation. These findings provide evidence that the visceral analgesia associated with PAR4-AP may involve in downregulations of tryptase, iNOS, IL-1β, and P2X7 expression via MAPK signals in MCs.

Previous research suggests that PAR4 activation exerts an analgesic effect in visceral hyperalgesia through the inhibition of colonic sensory neuron excitability [9]. Furthermore, PAR4 activation has been demonstrated to reverse the PAR2 or transient receptor potential vanilloid-4 (TRPV4) activation that is evoked in colorectal hypersensitivity [8].

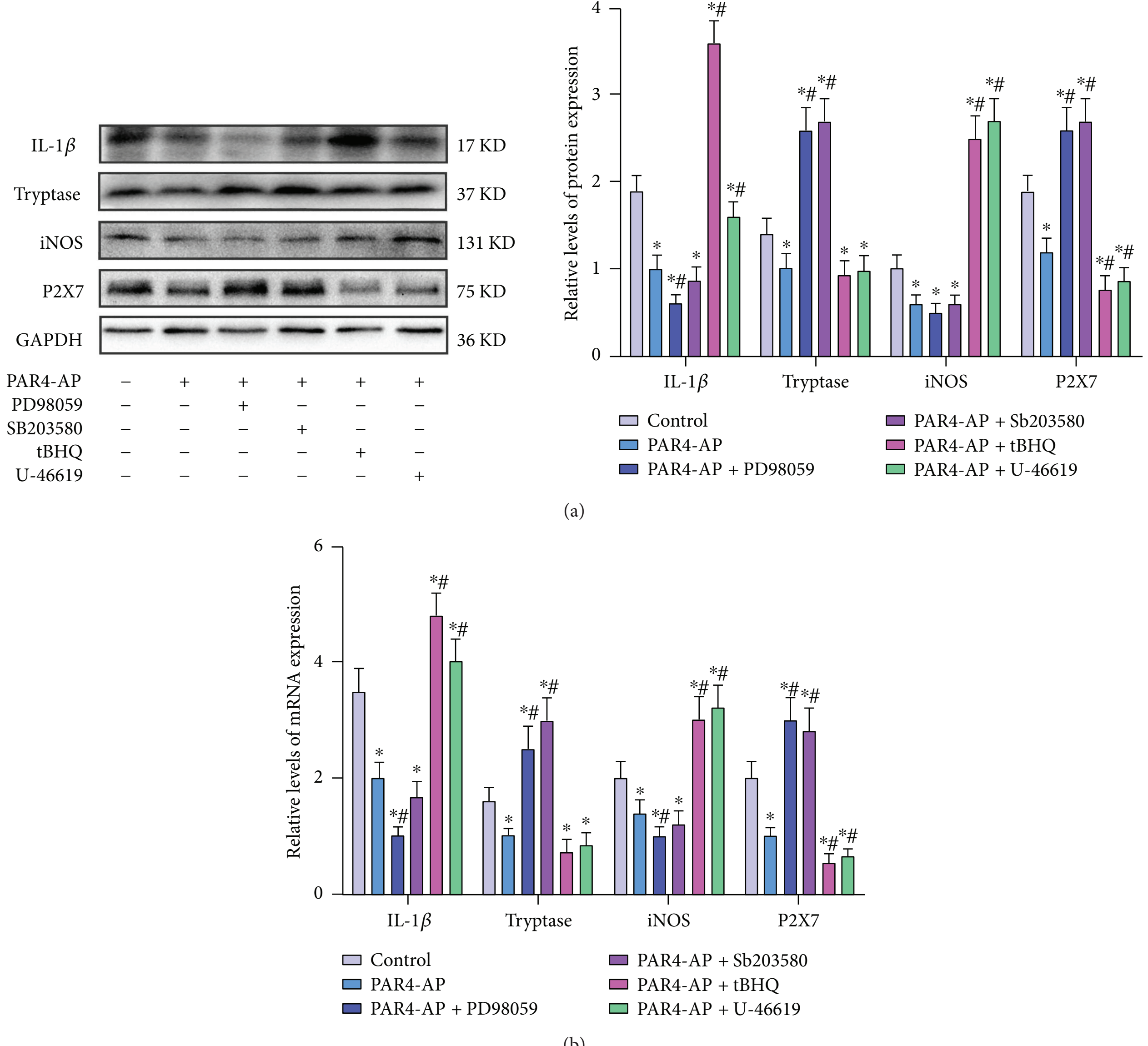

Figure 5: Effect of MAPK on PAR4-AP-evoked tryptase, iNOS, IL-1β, and P2X7 protein and mRNA expressions in BMMCs. (a) Western blotting analyses for tryptase, iNOS, IL-1β, and P2X7 protein expression in BMMCs following treatment with PAR4-AP and pretreatment with U-46619 (an activator of ERK1/2 and p38), tBHQ (an activator of ERK1/2), PD98059 (an inhibitor of ERK1/2), or SB203580 (an inhibitor of p38). The mean optic densities of the proteins were calculated by normalizing to GADPH. (b) Quantitative RT-PCR analyses of tryptase, iNOS, IL-1β, and P2X7 mRNA expression in BMMCs following treatment of the BMMCs with PAR4-AP and pretreatment with U-46619, tBHQ, PD98059, or SB203580. The results were calculated by normalizing to β-actin in the same sample with the ΔCt method. The changes in the relative mRNA levels are expressed as fold changes compared with the controls. All values are expressed as the means ± SEMs ($n = 3$). $^*P < 0.05$ versus controls; $^\#P < 0.05$ versus PAR4-AP-only groups.

Moreover, the activation of PAR4 has been demonstrated to attenuate inflammatory colonic hyperalgesia in response to CRD [20]. Herein, we demonstrated that the activation of PAR4 in colonic mucosa MCs suppresses their expression of inflammatory mediators, such as tryptase, iNOS, IL-1β, and P2X7, which suggests that these receptors could provide additional important targets for modifying pain in colonic GI disorders, such as IBS and IBD.

Hypersensitivity is followed by the activation of the colonic MCs that are responsible for colonic barrier dysfunction [21]. Tryptase is the most abundant secretory product of MCs, an important marker of MC activation, and an important mediator of inflammation [22]. Tryptase and its cognate receptor PAR2 are involved in activating proinflammatory cytokines and colon hypersensitivity in animal models of IBD [23]. Our results demonstrated that the activation of PAR4 inhibited the expression of tryptase by colonic mucosal MCs, which might suppress the tryptase-PAR2 axis to regulate sensitization and the consequently heightened pain behavior in IBS [24].

Previous studies have demonstrated that the production of proinflammatory cytokines, such as IL-1β, is associated

with P2X7 and NO through the iNOS pathway in MCs [25–27]. Several reports have found that increased numbers of MCs infiltrating the mucosa of the colon are correlated with the expressions of iNOS and IL-1β in IBS patients [13, 27], and similar findings were observed in the visceral hyperalgesia rats in the present study. The PAR4 expression in the MCs of the colonic mucosae of the visceral hyperalgesia rats that we observed was consistent with the existence of this receptor in MCs obtained from IBS patients [10, 11]. In addition to the inhibition of visceral hypersensitivity, the intracolonic administration of PAR4-AP elicited clear downregulations of iNOS, IL-1β, and P2X7 expression, which might have mediated the visceral hypersensitivity [12, 13]. These results illuminate a novel regulatory role of PAR4 during visceral hyperalgesia as demonstrated by the inhibited nociceptive response to colorectal distension, which was mediated by this receptor's control of inflammatory mediators (such as IL-1β) that were induced by NO/iNOS and P2X7 activation [25–27].

The phosphorylation of MAPK is involved in the secretion of cytokines by MCs that is induced by PAR4 activation [28]. Several studies have reported that p38 plays an important role in inflammation, and blocking p38 suppresses the transcriptional activity of NF-κB and downregulates the expression of iNOS [29]. The activation of P2X7 with BZATP in murine MCs can lead to the rapid phosphorylations of ERK and p38 [30]. A previous study demonstrated that IL-1β can stimulate ERK1/2 and p38 phosphorylations, which upregulate proinflammatory cytokine expression [31]. The inhibition of MAPK phosphorylation by PAR4 has been found to mediate the production of inflammatory cytokines, such as IL-1β, in MCs, which leads to changes in nociceptive response sensitivity [32]. Our results demonstrated that PAR4 activation decreased tryptase, IL-1β, iNOS, and P2X7 at the protein and mRNA levels in association with the regulation of p38 and ERK1/2 phosphorylation. The suppression of these inflammatory mediators through regulation by MAPK signals after the PAR4-AP treatment of the BMMCs provided a molecular mechanism for the inhibition of visceral hypersensitivity [33, 34]. However, additional evidence is needed, and related interesting questions, for example, questions concerning interactions or crosstalk with inflammatory mediators, are worthy of further investigation.

5. Conclusion

This study has provided new insight into the mechanisms involved in the antinociceptive effect of PAR4 activation. A crucial role for colonic mucosal MCs in this process has been revealed. The confirmation of PAR4 expression in these cells and its role in the inhibition of tryptase, iNOS, IL-1β, and P2X7 expression indicates that the antinociceptive effects of PAR4-AP are linked, directly or indirectly, to MCs located in the gastrointestinal tract.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Yanli Hao and Hao Niu contributed equally to this work.

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References

[1] K. Kovacic, "Current concepts in functional gastrointestinal disorders," *Current Opinion in Pediatrics*, vol. 27, no. 5, pp. 619–624, 2015.

[2] A. Farhadi, J. Z. Fields, and A. Keshavarzian, "Mucosal mast cells are pivotal elements in inflammatory bowel disease that connect the dots: stress, intestinal hyperpermeability and inflammation," *World Journal of Gastroenterology*, vol. 13, no. 22, pp. 3027–3030, 2007.

[3] W. Sohn, O. Y. Lee, S. P. Lee et al., "Mast cell number, substance P and vasoactive intestinal peptide in irritable bowel syndrome with diarrhea," *Scandinavian Journal of Gastroenterology*, vol. 49, no. 1, pp. 43–51, 2014.

[4] M. O'Sullivan, N. Clayton, N. P. Breslin et al., "Increased mast cells in the irritable bowel syndrome," *Neurogastroenterology & Motility*, vol. 12, no. 5, pp. 449–457, 2000.

[5] F. Doener, A. Michel, S. Reuter et al., "Mast cell-derived mediators promote murine neutrophil effector functions," *International Immunology*, vol. 25, no. 10, pp. 553–561, 2013.

[6] B. Tunc, L. Filik, E. Altintaş, N. Turhan, A. Ulker, and U. Dağli, "Mucosal mast cells in irritable bowel syndrome and inflammatory bowel disease," *Acta Medica (Hradec Králové)*, vol. 48, no. 3-4, pp. 163-164, 2005.

[7] S. Asfaha, N. Cenac, S. Houle et al., "Protease-activated receptor-4: a novel mechanism of inflammatory pain modulation," *British Journal of Pharmacology*, vol. 150, no. 2, pp. 176–185, 2007.

[8] C. Auge, D. Balz-Hara, M. Steinhoff, N. Vergnolle, and N. Cenac, "Protease-activated receptor-4 (PAR_4): a role as inhibitor of visceral pain and hypersensitivity," *Neurogastroenterology & Motility*, vol. 21, no. 11, pp. 1189–e107, 2009.

[9] R. Karanjia, I. Spreadbury, F. Bautista-Cruz, M. E. Tsang, and S. Vanner, "Activation of protease-activated receptor-4 inhibits the intrinsic excitability of colonic dorsal root ganglia neurons," *Neurogastroenterology & Motility*, vol. 21, no. 11, pp. 1218–1221, 2009.

[10] J. H. Zhao, L. Dong, H. T. Shi, Z. Y. Wang, H. Y. Shi, and H. Ding, "The expression of protease-activated receptor 2 and 4 in the colon of irritable bowel syndrome patients," *Digestive Diseases and Sciences*, vol. 57, no. 1, pp. 58–64, 2012.

[11] W. Han, Z. Wang, X. Lu, and C. Guo, "Protease activated receptor 4 status of mast cells in post infectious irritable bowel syndrome," *Neurogastroenterology & Motility*, vol. 24, no. 2, pp. 113–e82, 2012.

[12] Y. Kurashima, T. Amiya, T. Nochi et al., "Extracellular ATP mediates mast cell-dependent intestinal inflammation through P2X7 purinoceptors," *Nature Communications*, vol. 3, no. 1, p. 1034, 2012.

[13] S. An, G. Zong, Z. Wang, J. Shi, H. du, and J. Hu, "Expression of inducible nitric oxide synthase in mast cells contributes to the regulation of inflammatory cytokines in irritable bowel syndrome with diarrhea," *Neurogastroenterology & Motility*, vol. 28, no. 7, pp. 1083–1093, 2016.

[14] X. Li, L. Cai, H. Xu et al., "Somatostatin regulates NHE8 protein expression via the ERK1/2 MAPK pathway in DSS-induced colitis mice," *American Journal of Physiology-Gastrointestinal and Liver Physiology*, vol. 311, no. 5, pp. G954–G963, 2016.

[15] R. K. Singh, A. K. Najmi, and S. G. Dastidar, "Biological functions and role of mitogen-activated protein kinase activated protein kinase 2 (MK2) in inflammatory diseases," *Pharmacological Reports*, vol. 69, no. 4, pp. 746–756, 2017.

[16] S. An, G. Zong, Z. Wang, J. Shi, H. du, and J. Hu, "Activation of protease-activated receptor 4 of mast cells could downregulate proinflammatory cytokines in irritable bowel syndrome," *Gut*, vol. 66, no. 11, pp. 2040–2042, 2017.

[17] E. D. Al-Chaer, M. Kawasaki, and P. J. Pasricha, "A new model of chronic visceral hypersensitivity in adult rats induced by colon irritation during postnatal development," *Gastroenterology*, vol. 119, no. 5, pp. 1276–1285, 2000.

[18] F. Y. Jia, X. L. Li, T. N. Li, J. Wu, B. Y. Xie, and L. Lin, "Role of nesfatin-1 in a rat model of visceral hypersensitivity," *World Journal of Gastroenterology*, vol. 19, no. 22, pp. 3487–3493, 2013.

[19] H. N. Lee, C. H. Kim, G. G. Song, and S. W. Cho, "Effects of IL-3 and SCF on histamine production kinetics and cell phenotype in rat bone marrow-derived mast cells," *Immune Network*, vol. 10, no. 1, pp. 15–25, 2010.

[20] A. Annaházi, M. Dabek, K. Gecse et al., "Proteinase-activated receptor-4 evoked colorectal analgesia in mice: an endogenously activated feed-back loop in visceral inflammatory pain," *Neurogastroenterology & Motility*, vol. 24, no. 1, pp. 76–85.e13, 2012.

[21] K. N. Lee and O. Y. Lee, "The role of mast cells in irritable bowel syndrome," *Gastroenterology Research and Practice*, vol. 2016, Article ID 2031480, 11 pages, 2016.

[22] L. B. Schwartz, D. D. Metcalfe, J. S. Miller, H. Earl, and T. Sullivan, "Tryptase levels as an indicator of mast-cell activation in systemic anaphylaxis and mastocytosis," *The New England Journal of Medicine*, vol. 316, no. 26, pp. 1622–1626, 1987.

[23] M. J. Hamilton, M. J. Sinnamon, G. D. Lyng et al., "Essential role for mast cell tryptase in acute experimental colitis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 1, pp. 290–295, 2011.

[24] K. Roman, J. D. Done, A. J. Schaeffer, S. F. Murphy, and P. Thumbikat, "Tryptase-PAR2 axis in experimental autoimmune prostatitis, a model for chronic pelvic pain syndrome," *Pain*, vol. 155, no. 7, pp. 1328–1338, 2014.

[25] D. L. Su, Z. M. Lu, M. N. Shen, X. Li, and L. Y. Sun, "Roles of pro- and anti-inflammatory cytokines in the pathogenesis of SLE," *Journal of Biomedicine and Biotechnology*, vol. 2012, Article ID 347141, 15 pages, 2012.

[26] K. J. Wareham and E. P. Seward, "P2X7 receptors induce degranulation in human mast cells," *Purinergic Signalling*, vol. 12, no. 2, pp. 235–246, 2016.

[27] V. Shah, G. Lyford, G. Gores, and G. Farrugia, "Nitric oxide in gastrointestinal health and disease," *Gastroenterology*, vol. 126, no. 3, pp. 903–913, 2004.

[28] M. Vivinus-Nébot, R. Dainese, R. Anty et al., "Combination of allergic factors can worsen diarrheic irritable bowel syndrome: role of barrier defects and mast cells," *The American Journal of Gastroenterology*, vol. 107, no. 1, pp. 75–81, 2012.

[29] H. H. Arab, S. A. Salama, T. M. Abdelghany et al., "Camel milk attenuates rheumatoid arthritis via inhibition of mitogen activated protein kinase pathway," *Cellular Physiology and Biochemistry*, vol. 43, no. 2, pp. 540–552, 2017.

[30] S. Chen, Q. Ma, P. R. Krafft et al., "P2X7 receptor antagonism inhibits p38 mitogen-activated protein kinase activation and ameliorates neuronal apoptosis after subarachnoid hemorrhage in rats," *Critical Care Medicine*, vol. 41, no. 12, pp. e466–e474, 2013.

[31] R. N. Saha, M. Jana, and K. Pahan, "MAPK p38 regulates transcriptional activity of NF-κB in primary human astrocytes via acetylation of p65," *The Journal of Immunology*, vol. 179, no. 10, pp. 7101–7109, 2007.

[32] H. T. Yang, P. Cohen, and S. Rousseau, "IL–1β-stimulated activation of ERK1/2 and p38α MAPK mediates the transcriptional up-regulation of IL–6, IL–8 and GRO-α in HeLa cells," *Cellular Signalling*, vol. 20, no. 2, pp. 375–380, 2008.

[33] S. Tan, Y. Wang, K. Chen, Z. Long, and J. Zou, "Ketamine alleviates depressive-like behaviors via down-regulating inflammatory cytokines induced by chronic restraint stress in mice," *Biological and Pharmaceutical Bulletin*, vol. 40, no. 8, pp. 1260–1267, 2017.

[34] A. Önal, D. Coşkunsever, F. G. Çelenk, R. S. Şentürk, S. Nalbant, and S. Ülker Göksel, "Effect of ceftiofur on hyperalgesia and allodynia in a rat neuropathic pain model: the role of immune processes," *NeuroImmunoModulation*, vol. 24, no. 1, pp. 21–28, 2017.

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Research Article

The Relationship between a New Biomarker of Vagal Neuroimmunomodulation and Survival in Two Fatal Cancers

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Background. The vagus nerve may slow tumor progression because it inhibits inflammation. This study examined the relationship between a new vagal neuroimmunomodulation (NIM) index and survival in fatal cancers. *Method*. We retroactively derived markers of vagal nerve activity indexed by heart rate variability (HRV), specifically the root mean square of successive differences (RMSSD), from patients' electrocardiograms near diagnosis. The NIM index was the ratio of RMSSD to C-reactive protein levels (RMSSD/CRP). Sample 1 included 202 Belgian patients with advanced pancreatic cancer (PC), while sample 2 included 71 Belgian patients with non-small cell lung cancer (NSCLC). In both samples, we examined the overall survival, while in sample 2, we additionally examined the survival time in deceased patients. *Results*. In PC patients, in a multivariate Cox regression controlling for confounders, the NIM index had a protective relative risk (RR) of 0.68 and 95% confidence interval (95% CI) of 0.51–0.92. In NSCLC patients, the NIM index also had a protective RR of 0.53 and 95% CI of 0.32–0.88. Finally, in NSCLC, patients with a higher NIM index survived more days (475.2) than those with lower NIM (285.1) ($p < 0.05$). *Conclusions*. The NIM index, reflecting vagal modulation of inflammation, may be a new independent prognostic biomarker in fatal cancers.

1. Introduction

Despite progress made in treating various cancers, the ability to improve the prognosis in some cancers such as pancreatic cancer (PC) or non-small cell lung cancer (NSCLC) remains grim [1]. For example, the one-year survival rate in stages IIIB–IV lung cancer is poor (30–36%; [2]). Such a situation calls for searching new and modifiable prognostic biomarkers which are also related to key processes affecting prognosis, making such prognostic biomarkers potential therapeutic targets. Recent research suggests that the autonomic nervous system, and particularly the vagus nerve, may affect tumorigenesis. While oxidative stress [3], excessive inflammation [4–6], and sympathetic overactivity [7] all contribute to tumorigenesis, the vagus nerve in contrast inhibits oxidative stress [8], inflammation [9], and sympathetic activity [10]. Due to inhibiting these three tumorigenic factors, the vagus nerve is thought to possibly slow tumor growth [11, 12]. Indeed, several experimental studies in mice, though not all, show that vagotomised tumor-bearing animals had more metastases [13, 14]. Furthermore, one study demonstrated that an anti-inflammatory drug, which depends on the vagus nerve, reduced metastases in tumor-bearing mice [15]. These studies clearly indicate the possible causal relationship between adequate vagal nerve activity and reduced tumor progression, though not all studies point at this direction [16].

In humans, heart rate variability (HRV) is a noninvasive marker of vagal nerve activity [17]. HRV reflects fluctuations in normal R-R interbeat intervals. High HRV predicts lower levels of tumor makers over time [18, 19] and longer survival

times in various cancers (e.g., [19, 20]), in most cases independent of known prognostic factors. However, HRV may not predict all outcomes (e.g., presence of metastases), possibly because HRV alone does not consider vagal modulation of inflammation directly. Vagal nerve modulation of inflammation may be the critical link between vagal activity and possible slowing down of carcinogenesis [11]. Indeed, a recent study found that levels of the general inflammatory marker C-reactive protein (CRP) statistically mediated the relationship between HRV and survival time in patients with PC [21]. But can there be one single index which considers both vagal nerve activity and its modulation of inflammation, and does such an index have a prognostic value in cancer? To the best of our knowledge, no study has developed and examined the prognostic value of an index which reflects vagal modulation of inflammation.

Given the possible role of the vagus nerve in modulating cancer via inhibiting inflammation [21], we developed a simple numerical ratio indexing vagal neuroimmunomodulation (NIM) of inflammation. The NIM index considers in one number two systems: vagal nerve activity (the autonomic nervous system in the numerator: HRV) and an inflammatory marker (the immune system response in the denominator: CRP). This study examined in two fatal cancers whether the NIM index (HRV/CRP) could predict survival, independent of confounders. Based on the literature mentioned above, we hypothesized that higher NIM predicts better prognosis, independent of confounders.

Table 1: Descriptive statistics of the pancreatic cancer (PC) and non-small cell lung cancer (NSCLC) patient samples.

(a) Categorical variables

Variable	PC (%)	NSCLC (%)
Gender		
Men	53.0%	63.4%
Women	47.0%	36.6%
Radiotherapy	13.4%	69.0%
Chemotherapy	73.3%	76.0%
Surgery	40.1%	10.0%
Stage		
Stage 1		11.3%
Stage 2		7.0%
Stage 3		21.1%
Stage 4		60.6%

(b) Continuous variables

Variable	PC Mean (SD)	NSCLC Mean (SD)
Age (years)	66.2 (11.8)	62.5 (11.5)
HRV (RMSSD)	27.4 (20.5)	21.6 (24.0)
CRP	4.7 (18.8)	43.1 (63.3)

PC: pancreatic cancer; NCSLC: non-small cell lung cancer; HRV: heart rate variability; RMSSD: root mean square of successive differences; CRP: C-reactive protein.

2. Materials and Methods

2.1. Patients. After the approval of the Medical Ethics Committee, medical records of 620 patients with histologically proven advanced (locally advanced and metastatic) pancreatic ductal adenocarcinoma (PDAC) treated at the University Hospital Erasme, Brussels, between 1998 and 2011, and medical records of 650 patients with NSCLC treated at the UZ Brussels hospital between January 2005 and December 2009 were reviewed. Exclusion criteria included conditions known to alter HRV or influence inflammation, such as inflammatory diseases (e.g., arthritis), cardiovascular disease (for the NCSLC patients only), implanted pacemaker, or prescribed cardiologic medication (β-blockers, antiarrhythmics for the NSCLC patients only). Following these exclusion criteria and the (un)availability of ECG, CRP, and survival data, 202 PC and 71 NSCLC patients were included. Table 1 depicts the characteristics of these samples.

2.2. Design. The study included a historical prospective design. We collected existing archival electronic patient records (historical) and examined the prospective relationship between the NIM index (see below) derived from these records and measured at baseline (time of diagnosis, time 1), and prognosis at time 2 (e.g., overall survival and survival time). This design is commonly used in the reanalysis of existing data sets.

2.3. Measures

2.3.1. Background Variables and Confounders. In both samples, we considered the prognostic roles of patients' age, gender, stage (only in NSCLC), and treatments (chemotherapy, radiotherapy, surgery), as well as whether patients had cardiac problems (only in PC). In PC patients, stage was not considered since all patients had advanced cancer, but we considered whether the tumor spread was locally advanced versus metastatic disease.

2.3.2. Neuroimmunomodulation Index. Heart rate variability (HRV) is a common noninvasive index of vagal nerve activity [17]. We derived the measure of HRV retroactively from 10-second electrocardiographs present in patients' files. Similar ultrashort HRV measures were found to predict tumor markers in previous studies [18, 19] and overall survival in the general population [22]. To index neuroimmunomodulation (NIM), we divided the HRV index of the root mean square of successive RR intervals (RMSSD) by patients' C-reactive protein (CRP), a general marker of inflammation, to yield our NIM index = RMSSD/CRP. Both HRV and CRP were obtained near diagnosis, in both patient cohorts.

2.3.3. Outcomes. In both the PC and NSCLC cohorts, the outcome was the overall survival, defined as the time from the date of histologically proven cancer diagnosis to death or till the end of follow-up for surviving patients. However, in the NSCLC cohort, an additional outcome was the survival time among deceased patients because fewer patients survived. These data were obtained from patients' medical files and the Belgian national registry.

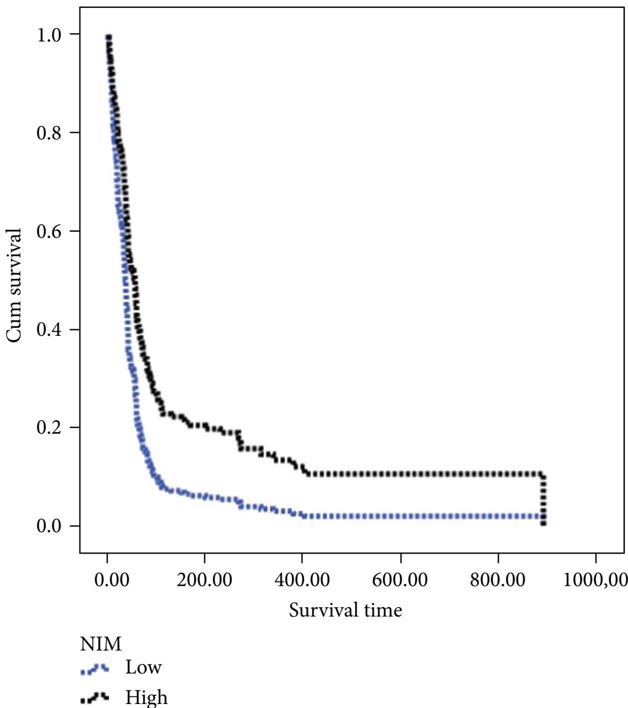

Figure 1: Cumulative survival curves of patients with pancreatic cancer with a high versus low neuroimmunomodulation (NIM) index.

2.4. Statistical Analysis. In both cancer samples, the main analysis was a Cox regression survival analysis. After identifying the significant confounders univariately, we entered them together with the categorical NIM index, split at its median value. Time till death or till the end of follow-up (for surviving patients) was also considered as the time variable, and the outcome variable was the vital status. Additionally, in the NSCLC sample, because few patients survived to follow up, we further examined only in the deceased patients the partial correlation between continuous levels of the NIM index and the survival time, controlling statistically for confounders that were significantly related to the survival time.

3. Results

Table 1 presents the background characteristics of each patient cohort. In the PC cohort, 56.9% had locally advanced disease while 43.1% had metastatic tumors. Among all confounders tested, surgery ($p < 0.001$), local versus metastatic disease ($p < 0.001$), cardiac problem ($p < 0.025$), age at diagnosis ($p < 0.001$), and presence of metastatic diseases ($p < 0.001$) were predictive of the overall survival, while radiotherapy and chemotherapy were not (both p values > 0.05). Categorical NIM was highly significantly predictive of the overall survival ($p < 0.001$; see Figure 1). In the final multivariate Cox regression (see Table 2), categorical NIM was still significantly predictive of overall survival ($p = 0.011$), independent of the significant confounders mentioned above (for NIM, the relative risk (RR) and 95% confidence interval (CI) were 0.68 and 0.51–0.92, resp.).

Table 2: Multivariate Cox regression survival analysis of relative risks for death in patients with advanced pancreatic cancer.

Variable	B	Sig.	RR	95% CI
Surgery	−0.84	0.00	0.43	0.30–0.62
Local/advanced	−0.09	0.71	0.91	0.55–1.51
Cardiac problem	0.13	0.41	1.14	0.83–1.57
Age	0.02	0.01	1.02	1.00-1.03
Metastases	0.28	0.30	1.33	0.77–2.28
NIM index	−0.38	0.01	0.68	0.51–0.92

Sig.: significance; RR: relative risk; 95% CI: 95% confidence interval; NIM index: neuroimmunomodulation index.

In the NSCLC cohort, among all tested confounders, chemotherapy tended to predict the overall survival ($p < 0.055$), while surgery ($p < 0.001$) and cancer stage ($p < 0.001$) significantly predicted the overall survival. Categorical NIM also significantly predicted the overall survival ($p < 0.005$; see Figure 2). In a multivariate Cox regression (see Table 3), categorical NIM remained a significant predictor of overall survival (for NIM, RR = 0.53; 95% CI: 0.32–0.88). Since many patients in the NSCLC cohort died, we tested the correlation between continuous NIM levels and survival time, in the deceased patients only. Controlling for the above confounders, log-transformed NIM index scores were significantly positively correlated with the survival time ($r = 0.31$, $p = 0.010$). Among these deceased patients, those with a relatively high NIM index (above the median) lived significantly longer (mean (SD) = 475.2 (383.9) days) than those with a low NIM index (mean = 285.1 (282.2) days; t (69) = 2.40, $p < 0.05$).

4. Discussion

To the best of our knowledge, this may be the first study to develop and test the prognostic value of a new composite biomarker which reflects the autonomic and immune (inflammatory) systems together, in relation to cancer prognosis. Two studies revealed that a new neuroimmunomodulation (NIM) index, which reflects vagal nerve activity (RMSSD) over general systemic inflammation (CRP) significantly predicted the overall survival in patients with advanced PC and in patients with NSCLC. These relationships remained significant also after statistically controlling for confounders which were univariately predictive of survival and which reflected the severity of cancer and its treatment. Patients with NSCLC and a relatively high NIM index had a nearly double survival time compared to those with low NIM.

These results are in line with one experimental study showing that an anti-inflammatory drug (CNI-1493), which depends on and activates the vagus nerve, led to reduced metastases in tumor-bearing mice [15]. These results also support many correlational studies showing that high vagal nerve activity, indexed by HRV, predicts reduced tumor marker levels over time or longer survival, in different cancers including young NSCLC patients (e.g., [18, 19]). These results also support those showing that CRP statistically mediated the relationship between HRV and survival in PC

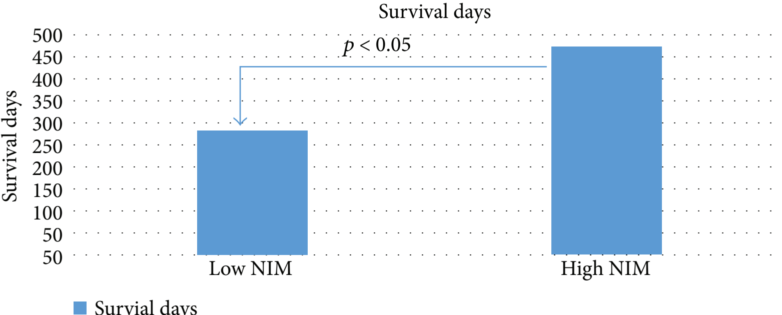

Figure 2: Survival days in deceased patients with non-small cell lung cancer with a high versus low neuroimmunomodulation (NIM) index.

Table 3: Multivariate Cox regression survival analysis of relative risks for death in patients with non-small cell lung cancer.

Variable	B	Sig.	RR	95% CI
Chemotherapy	−0.44	0.25	0.64	0.30–1.38
Surgery	−1.20	0.01	0.30	0.11–0.79
Stage	0.48	0.01	1.61	1.24–2.10
NIM index	−0.63	0.01	0.53	0.32–0.88

Sig.: significance; RR: relative risk; 95% CI: 95% confidence interval; NIM index: neuroimmunomodulation index.

[21]. However, the results of the present study extend these past observations to incorporate the *balance* between vagal activity (RMSSD) and inflammation (CRP) in one ratio and reveal its prognostic value. The novelty in these results is that this new NIM index reveals that a shift in balance from inflammation to vagal activity (a higher NIM ratio) predicts longer survival in two very fatal cancers, independent of relevant prognostic factors. These results are in line with our model of vagal nerve neuroimmunomodulation of cancer [11, 12]. Furthermore, these results are in line with the known anti-inflammatory functions of this important cranial nerve [9]. This index includes HRV derived from ECGs and includes CRP levels, both which are routinely available worldwide in hospitals. This availability makes the new NIM index highly feasible for use to estimate cancer patients' prognosis. Finally, using noninvasive vagal nerve activating methods (e.g., electric vagal nerve stimulation; [23]), NIM is a marker which could be therapeutically modified, making this index also a possible therapeutic target on its own, to possibly then influence prognosis. Future controlled intervention studies need to test such treatment avenues.

This study had several limitations. First, we did not employ formal prospective studies, rather historical prospective designs. However, previous studies demonstrating prognostic values of HRV used similar designs (e.g., [19]). Furthermore, the EGCs, from which HRV was derived, and CRP were both measured near diagnosis, while follow-ups were done later, maintaining a longitudinal aspect in the design. Second, the ECGs were very brief—lasting only 10 seconds. However, multiple studies including one large population study have demonstrated that such brief HRV measures have a prognostic value [18, 19, 21, 22] and that this brief measure correlates with HRV measures in longer tests [24]. In addition, we showed here the prognostic value of the NIM index in two cancer samples, attesting to the reliability of this new observation. Nevertheless, these results should be replicated in a formal prospective study, with longer ECG readings. Should the results be replicated, and should the new NIM index be found to be predictive in other cancers (and chronic diseases), this NIM index may be proven clinically important for prognostication and for identifying patients who require more close medical care. Finally, the relationship between NIM and prognosis only reflects an association, not a causal relationship between these variables. Some of the animal studies conducted to date do propose a causal relationship between vagal nerve activation and improved cancer prognosis [13–15]. To test this important issue in people, investigators need to test whether activating the vagus nerve shifts the balance from inflammatory to vagal activity and whether this shift could then improve cancer treatment and patients' prognosis, the ultimate goal. These would have both scientific and potential clinical significances.

Conflicts of Interest

There are no conflicts of interest.

Acknowledgments

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References

[1] A. Teague, K. H. Lim, and A. Wang-Gillam, "Advanced pancreatic adenocarcinoma: a review of current treatment strategies and developing therapies," *Therapeutic Advances in Medical Oncology*, vol. 7, no. 2, pp. 68–84, 2015.

[2] J. H. Schiller, D. Harrington, C. P. Belani et al., "Comparison of four chemotherapy regimens for advanced non–small-cell lung cancer," *The New England Journal of Medicine*, vol. 346, no. 2, pp. 92–98, 2002.

[3] M. Valko, M. Izakovic, M. Mazur, C. J. Rhodes, and J. Telser, "Role of oxygen radicals in DNA damage and cancer incidence," *Molecular and Cellular Biochemistry*, vol. 266, no. 1/2, pp. 37–56, 2004.

[4] T. Lawrence, "Macrophages and NF-κB in cancer," in *Current Topics in Microbiology and Immunology*, M. Karin, Ed., vol. 349, pp. 171–184, Springer, Berlin, Heidelberg, 2011.

[5] A. Mantovani, P. Allavena, A. Sica, and F. Balkwill, "Cancer-related inflammation," *Nature*, vol. 454, no. 7203, pp. 436–444, 2008.

[6] E. Voronov, D. S. Shouval, Y. Krelin et al., "IL-1 is required for tumor invasiveness and angiogenesis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 5, pp. 2645–2650, 2003.

[7] F. Entschladen, T. L. Drell, K. Lang, J. Joseph, and K. S. Zaenker, "Tumour-cell migration, invasion, and metastasis: navigation by neurotransmitters," *The Lancet Oncology*, vol. 5, no. 4, pp. 254–258, 2004.

[8] T. Tsutsumi, T. Ide, M. Yamato et al., "Modulation of the myocardial redox state by vagal nerve stimulation after experimental myocardial infarction," *Cardiovascular Research*, vol. 77, no. 4, pp. 713–721, 2008.

[9] K. J. Tracey, "Reflex control of immunity," *Nature Reviews Immunology*, vol. 9, no. 6, pp. 418–428, 2009.

[10] K. Saku, T. Kishi, K. Sakamoto et al., "Afferent vagal nerve stimulation resets baroreflex neural arc and inhibits sympathetic nerve activity," *Physiological Reports*, vol. 2, no. 9, article e12136, 2014.

[11] Y. Gidron, H. Perry, and M. Glennie, "Does the vagus nerve inform the brain about preclinical tumours and modulate them?," *The Lancet Oncology*, vol. 6, no. 4, pp. 245–248, 2005.

[12] M. De Couck, B. Mravec, and Y. Gidron, "You may need the vagus nerve to understand pathophysiology and to treat diseases," *Clinical Science*, vol. 122, no. 7, pp. 323–328, 2012.

[13] N. Erin, P. J. Boyer, R. H. Bonneau, G. A. Clawson, and D. R. Welch, "Capsaicin-mediated denervation of sensory neurons promotes mammary tumor metastasis to lung and heart," *Anticancer Research*, vol. 24, no. 2B, pp. 1003–1009, 2004.

[14] N. Erin, G. Akdas Barkan, J. F. Harms, and G. A. Clawson, "Vagotomy enhances experimental metastases of 4THMpc breast cancer cells and alters substance P level," *Regulatory Peptides*, vol. 151, no. 1-3, pp. 35–42, 2008.

[15] N. Erin, O. Duymuş, S. Oztürk, and N. Demir, "Activation of vagus nerve by semapimod alters substance P levels and decreases breast cancer metastasis," *Regulatory Peptides*, vol. 179, no. 1-3, pp. 101–108, 2012.

[16] N. A1, K. Ryberg, J. Heiman Ullmark et al., "Is acetylcholine a signaling molecule for human colon cancer progression?," *Scandinavian Journal of Gastroenterology*, vol. 46, no. 4, pp. 446–455, 2011.

[17] T. B. Kuo, C. J. Lai, Y. T. Huang, and C. C. Yang, "Regression analysis between heart rate variability and baroreflex-related vagus nerve activity in rats," *Journal of Cardiovascular Electrophysiology*, vol. 16, no. 8, pp. 864–869, 2005.

[18] C. Mouton, A. Ronson, D. Razavi et al., "The relationship between heart rate variability and time-course of carcinoembryonic antigen in colorectal cancer," *Autonomic Neuroscience: Basic & Clinical*, vol. 166, no. 1-2, pp. 96–99, 2012.

[19] M. De Couck, D. van Brummelen, D. Schallier, J. De Grève, and Y. Gidron, "The relationship between vagal nerve activity and clinical outcomes in prostate and non-small cell lung cancer patients," *Oncology Reports*, vol. 30, no. 5, pp. 2435–2441, 2013.

[20] J. K. Chiang, T. B. Kuo, C. H. Fu, and M. Koo, "Predicting 7-day survival using heart rate variability in hospice patients with non-lung cancers," *PLoS One*, vol. 8, no. 7, article e69482, 2013.

[21] M. De Couck, R. Maréchal, S. Moorthamers, J.-L. Van Laethem, and Y. Gidron, "Vagal nerve activity predicts overall survival in metastatic pancreatic cancer, mediated by inflammation," *Cancer Epidemiology*, vol. 40, pp. 47–51, 2015.

[22] J. M. Dekker, E. G. Schouten, P. Klootwijk, J. Pool, C. A. Swenne, and D. Kromhout, "Heart rate variability from short electrocardiographic recordings predicts mortality from all causes in middle-aged and elderly men: the Zutphen Study," *American Journal of Epidemiology*, vol. 145, no. 10, pp. 899–908, 1997.

[23] K. T1, K. Hösl, O. Kiess, A. Schanze, J. Kornhuber, and C. Forster, "BOLD fMRI deactivation of limbic and temporal brain structures and mood enhancing effect by transcutaneous vagus nerve stimulation," *Journal of Neural Transmission*, vol. 114, no. 11, pp. 1485–1493, 2007.

[24] R. M. Hamilton, P. S. McKechnie, and P. W. Macfarlane, "Can cardiac vagal tone be estimated from the 10-second ECG?," *International Journal of Cardiology*, vol. 95, no. 1, pp. 109–115, 2004.

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Review Article

Roles of Host Immunity in Viral Myocarditis and Dilated Cardiomyopathy

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The pathogenesis of viral myocarditis includes both the direct damage mediated by viral infection and the indirect lesion resulted from host immune responses. Myocarditis can progress into dilated cardiomyopathy that is also associated with immunopathogenesis. T cell-mediated autoimmunity, antibody-mediated autoimmunity (autoantibodies), and innate immunity, working together, contribute to the development of myocarditis and dilated cardiomyopathy.

1. Introduction

The International Society and Federation of Cardiology of the World Health Organization (WHO) defined myocarditis in 1995 as an inflammatory disease of the heart muscle, diagnosed by established histological, immunological, and immunohistochemical criteria [1]. Most cases of myocarditis are of viral origin [2–4]. Many viruses have been implicated as causes of myocarditis, including coxsackieviruses group B, parvoviruses, echoviruses, adenovirus, influenza virus H1N1, Epstein–Barr virus, rubella (German measles) virus, varicella (chickenpox) virus, mumps virus, measles virus, yellow fever virus, dengue fever virus, polio virus, rabies virus, hepatitis A and C viruses, human immunodeficiency virus (HIV), and Zika virus; while parvovirus B19 (PVB19) has recently be demonstrated by endomyocardial biopsy (combined with polymerase chain reaction and in situ hybridization) as the most frequently detected virus in myocarditis, coxsackievirus B3 (CVB3) remains the most extensively studied virus that causes myocarditis both in human beings and in animal models [5–10]. Viral myocarditis usually progresses on two stages although the exact pathophysiology mechanism in humans is still not completely understood: first, the viral infection generates direct damages to the myocardium (virus-mediated lysis of myocardial cells), and then host immune responses produce indirect lesions of the cardiac muscle by killing virus-infected (antiviral immunity) and uninfected (autoimmunity) cardiomyocytes; some cases progress to dilated cardiomyopathy, and some may result in heart failure or sudden death [11–15]. Researchers found several years ago in animal experiments that the infection of BALB/c mice with coxsackievirus B3 (an RNA virus) and murine cytomegalovirus (a DNA virus) led to essentially the same pathophysiological outcomes in the heart [16]; in addition, immunosuppressive and immunoadsorption therapies have been reported to alleviate symptoms and ameliorate heart function in myocarditis and dilated cardiomyopathy patients [17–20]; these results strongly suggested the importance of the immunopathological process in the disease(s). The roles of host immunity in the development of viral myocarditis and in dilated cardiomyopathy are summarized.

2. T Cell-Mediated Autoimmunity

In cell-mediated immune responses, T cells are the most important immune-competent cells, and they also play a crucial role in the pathogenesis of viral myocarditis. Woodruff and Woodruff [21] first established the implication of T cells

in the pathogenesis of this disease using CD1 and BALB/c mice in 1974. They demonstrated that pretreatment of CD1 mice with rabbit anti-thymocyte serum greatly suppressed the production of inflammation and tissue injury in the hearts after CVB3 infection, and deprivation of T cells by thymectomy and lethal irradiation led to a decrease in mortality and a decrease in cardiac inflammation and necrosis in CVB3-infected BALB/c mice. Following that, numerous researches were conducted to demonstrate the involvement of T cells in the pathogenesis of viral myocarditis and to determine T cell subtypes that take part in the immunopathogenesis. Kishimoto et al. [22] examined the changes in percentages of T and B cells in peripheral blood and the heart of DBA/2 mice inoculated with encephalomyocarditis virus. They found a marked decrease in T cell number in peripheral blood on day 14 but no significant changes in B cell number throughout the entire period. T cells accounted for about 80% of the cells in the myocardial tissue on days 7 and 14. Huber and Pfaeffle [23] found that the Th1 cell response required the activation of γ/δ T cells. In addition, male and female BALB/c mice differ in response to CVB3 infection. The viral infection resulted in substantial infiltration of inflammatory cells and lymphocytes in the myocardium; while male mice gave predominantly a Th1 cell response, female mice gave predominantly a Th2 cell response [24].

Later, Opavsky et al. [25] used knockout mice lacking an individual component of the T cell receptor (TCR) or coreceptor ($CD4^{-/-}$, $CD8^{-/-}$, $CD4^{-/-}$, and $CD8^{-/-}$, or $TCR\beta^{-/-}$) to observe the contribution of T cell subpopulations to host susceptibility to CVB3 myocarditis. The disease was more severe in $CD8^{-/-}$ mice but reduced in $CD4^{-/-}$ mice. Removal of both CD4 and CD8 molecules from T cells by genetic knockout protected the mice from the disease. In $TCR\beta^{-/-}$ (T cell receptor β chain knockout) mice, prolonged survival and minimal myocardial lesion were observed after CVB3 infection. In $CD4^{-/-}CD8^{-/-}$ mice, increased interferon-γ (IFN-γ) and decreased tumor necrosis factor-α (TNF-α) expression and reduced myocardial damage were observed. These results indicated that the presence of $TCR\alpha\beta^{+}$ T cells could boost host susceptibility to viral myocarditis. One mechanism by which $CD4^{+}$ and $CD8^{+}$ T cell subsets mediate the pathogenesis of viral myocarditis may be related to the expression of specific cytokines. The findings in the mice with genetical CD4 and/or CD8 deficiency supported the conclusion that the cellular inflammatory infiltration following viral infection in susceptible mouse strains contributes substantially to the mortality and myocardium lesion associated with viral infection [26].

Increasing evidence supports the earlier findings that myocarditis is an autoimmune disease that involves the participation of $CD4^{+}$ T cells [27–36]. The activation of T cells requires costimulatory signals and the respective binding of CD28 and CD40 ligands on the surface of T cells to B7 and CD40 molecules on the surface of antigen-presenting cells; otherwise, the T cells will be in a state of anergy [37–44]. Matsui et al. [45] using transgenic technology effectively prevented the transmission of costimulatory signals and thus greatly reduced the severity of experimental autoimmune myocarditis, confirming that the activation of T cells could promote the development of viral myocarditis. More and more researches demonstrated that the cognate interaction between the inducible costimulatory molecule (ICOS) and ICOS ligand (ICOSL), a member of the CD28 family, is an indispensable signaling for the activation of T cells. Blocking the ICOS–ICOSL signaling with anti-ICOS antibodies can block or attenuate myocarditis resulting from autoimmunity [46–49].

Most recently, the role of Th17 cells in viral myocarditis and dilated cardiomyopathy has drawn much attention [50–53]. The Th17 cell is a $CD4^{+}$ T cell subpopulation distinct from IFN-γ-producing Th1 and IL-4-producing Th2 and is characterized by secreting IL-17, a proinflammatory cytokine [54]; many studies over the past decade have been focusing on IL-17 and Th17 cell participation in the inflammatory process of the autoimmune diseases. Earlier studies in EAM showed that IL-17 might be the critical effector cytokine responsible for EAM because neutralization of IL-17 could reduce myocarditis and heart autoantibody responses and that IL-17 promoted the recruitment of monocytes, the major heart-infiltrating cells in EAM, to the heart [55]. Yuan et al. [56] reported that IL-17 was related to the progression of acute viral myocarditis (AVMC) in a mouse model through regulating autoantibody production and neutralization of IL-17 could inhibit autoantibody production in CVB3-induced AVMC. In the CVB3-induced AVMC mouse model, Yuan et al. [57] also showed that Th17 cells contributed to viral replication and that IL-17 was important for the regulation. Using IL-17 monoclonal antibody-treated viral myocarditis mice, Fan et al. [58] showed that IL-17 was critically complicated in the pathogenesis of murine viral myocarditis and inhibition of IL-17 could alleviate the myocardium inflammation. Zhu et al. [59] reported that inhibition of Th17 cells by the newly discovered cytokine IL-27 could effectively ameliorate CVB3-induced viral myocarditis. Myers et al. [60] recently identified a Th17 cell phenotype of human myocarditis/dilated cardiomyopathy that had raised $CD4^{+}$ $IL\text{-}17^{+}$ T cells and Th17-promoting cytokines including IL-6, IL-23, and TGF-β as well as GM-CSF (granulocyte-macrophage colony-stimulating factor) secreting $CD4^{+}$ T cells. They found that the Th17 phenotype was associated with the effects of cardiac myosin on $CD14^{+}$ monocytes and heart failure. Persistent heart failure was linked with high proportions of IL-17-producing T cells and IL-17-promoting cytokines and the phenotype contained within a significantly low proportion of $FOXP3^{+}$ Tregs; these may be related to disease severity.

Studies pointed out [61] that Th17 cell differentiation is mediated by the interaction between the costimulatory signal CD28 and ICOS, but interaction between cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and B7 inhibits Th17 cell differentiation. Martín et al. [62] showed that the C-type lectin receptor CD69 inhibited Th17 cell differentiation by promoting the activation of the Jak3 signal transducer and activator of the transcription 5 signaling pathway. IL-23 is required for Th17 cell's maintenance and pathogenic function. The IL-23/Th17 pathway is involved in the pathogenesis of several autoimmune diseases. Using a CVB3-induced murine model of viral myocarditis, Yang et al. [63] showed

that the IL-23/Th17 axis is involved in the viral myocarditis. A study by Yamashita et al. [64] showed that IL-6 mediated Th17 differentiation in the onset (but not the progression) of EAM through RORγt, an isoform of retinoic acid receptor-related orphan receptor, and RORγt has been suggested as a master regulator for Th17 differentiation. Lately, Liu et al. [65] showed that microRNA-21 and -146b are related to the pathogenesis of viral myocarditis in mice through regulation of Th17 differentiation. Using microarrays, the authors detected the upregulation of miRNA-21 and -146b in a murine model of viral myocarditis. Silencing of miRNA-21 and -146b reduced RORγt expression, decreased Th17 level, and ameliorated the severity of viral myocarditis.

It has been well established that CD80 (or B7-1) and CD86 (or B7-2) are crucial costimulatory molecules in T cell activation, inducing Th1 and Th2 differentiation, respectively, in host immune responses. Most recently, Huang et al. [66] investigated the role of CD80 and CD86 in Th17 differentiation in AVMC. The authors infected C57BL/6 mice with CVB3 and examined its effects on Th17 differentiation with anti-CD80 and anti-CD86 monoclonal antibodies (McAbs). The results revealed that treatment with anti-CD80 McAb induced significant suppression of Th17 differentiation and RORγt mRNA expression both in vivo and in vitro, while anti-CD86 McAb treatment did not show such effect. Thus, it is CD80 not CD86 that regulates Th17 cell differentiation.

Regulatory T cells (Treg cells or Tregs) belong to a subset of CD4 T cells that express the biomarkers FOXP3 and CD25 in addition to CD4 and function to keep immune homeostasis by suppressing the development of effector T cells in particular Th17 cells that participate in the pathogenesis of a number of autoimmune diseases by producing IL-17 which promotes inflammation [67–70]. The balance between the immunosuppressive Treg cells and the proinflammatory Th17 cells is very important in host immune responses, and an imbalance between them plays a critical role in many inflammatory and autoimmune diseases. It has been established that the differentiation and proliferation of Treg cells are controlled by the transcriptional factor Forkhead box protein P3 (Foxp3) in combination with transforming growth factor beta (TGF-β), and the immunosuppressive function of Tregs is depending on the anti-inflammatory cytokines IL-10 and IL-35, as well as TGF-β, while the differentiation and proliferation of Th17 cells are regulated by the transcriptional factor RORγt in combination with IL-6 and the function of Tregs is dependent on the proinflammatory cytokines IL-17, IL-21, and IL-22. The interleukin-1 family member 7 or IL-37, the seventh member of the IL-1 family of eleven members, has recently been recognized as one of the few anti-inflammatory cytokines and is capable of suppressing a wide spectrum of proinflammatory responses. An et al. [71] reported that IL-37 suppressed Th17 response and enhanced Treg response in the spleen of a CVB3-induced murine viral myocarditis model. IL-37 downregulated the expression of Th17-related cytokines IL-17 and IL-6 but upregulated the expression of Treg-related cytokine IL-10 in the murine heart. Thus, IL-37 may exert an anti-inflammatory function in the mouse model of viral myocarditis through mediating a balance between Th17 cells and Treg cells,

Using an EAM mouse model, Yan et al. [72] revealed that the expression miR-155, a type of microRNA that is closely related to the immune system, was greatly upraised in $CD4^+$ T cells and in the cardiac muscle tissue of the EAM mice; meanwhile, there was a proliferative and functional imbalance between the Tregs and Th17 cells resulting from the active induction and proliferation of Th17 cells and an elevated resistance of Th17 cells to Treg-exerted suppression. On the other hand, inhibition of miR-155 in EAM mice resulted in lessened disease severity and cardiac damage, attenuated Th17 immune response, and reduced secretion of Th17-polarizing cytokines by dendritic cells. These findings demonstrated that miR-155 could promote the development of EAM by driving an imbalance between Tregs and Th17 cells that favors the development of Th17 cells.

3. Antibody-Mediated Autoimmunity

Studies have demonstrated that autoantibodies play an important role in the pathogenesis of myocarditis and in dilated cardiomyopathy [73–80]. Passive transfer of anti-heart autoantibodies can induce myocarditis and dilated cardiomyopathy in experimental animals while removal of autoantibodies with immunoadsorption or other methods can improve cardiac function of myocarditis and dilated cardiomyopathy patients [81–88]. Autoantibodies may be produced by molecular mimicry, in which viral proteins possess homologous amino acid sequences with cardiomyocyte proteins (or the virus and the host share the same antigenic determinant or epitope) and the induction of an immune response to the viral antigen thus leads to a cross-reaction with self-antigens (the antibodies produced against the viral antigen bind to or react with antigens of the cardiac muscle cells that share the same antigenic determinant with the viral antigen), resulting in autoimmunity. Alternatively, autoantibodies may be generated through the initial damage to myocardial cells by viral infection that releases a large quantity of self-antigens into the circulation, in which case the viral infection is followed by a second phase of immune process of the disease, recruiting active immunocompetent cells into the cardiac tissue that favor B cell activation and subsequent autoantibody production, resulting in myocardium damage and progression of heart disease [89–115].

A wide spectrum of autoantibodies associated with human or murine myocarditis has been described in the literature, of which the most important one is directed against the contractile protein myosin [116] (For an inclusive list of the autoantibodies, please refer to Dörner et al. [74], page 334, Table 1.) The presence of the cardiac myosin-specific autoantibodies in mice with myocarditis was first described in 1987 by Rose et al. [117], Alvarez et al. [118], and Neu et al. [119]. Neu et al. [120] reported that the infection of a susceptible mouse strain with CVB3 resulted in myocarditis associated with a high titer of myosin autoantibody specific for the cardiac myosin isoform. The injection of cardiac myosin itself in certain strains of mice also produces severe myocarditis with high titers of cardiac

myosin autoantibodies. The mouse strains that are resistant to cardiac myosin induced-myocarditis did not develop increased cardiac myosin autoantibodies. On the other hand, injection of the mice with skeletal muscle myosin did not produce the expected effect, suggesting that the immunogenic epitopes or determinants are specific to the cardiac myosin isoform.

In human beings, the α-myosin isoform exists exclusively in cardiac myocytes, whereas the β-myosin isoform is found both in cardiac myocytes and in slow skeletal muscle cells. Pummerer et al. [121] mapped the pathogenic epitopes on the myosin molecules in 1996. They found that α-myosin is the dominant immunogenic isoform that induces myocarditis (with high severity and prevalence) whereas β-myosin seldom causes the disease. So, the immunogenic epitopes of α-myosin (amino acid sequences) must reside in regions different from β-myosin. Three immunogenic amino sequences were identified. One sequence (AA 614–643) located in the head of the α-myosin heavy chain induced severe myocarditis, whereas two others (AA 735–747 and AA 947–960) that reside in the rod of the α-myosin heavy chain only induced minor pathologic changes in BALB/c mice. The autoimmunogenic epitope is located in a different region in A/J mice: between amino acid residue 334 and 352 of the α-myosin molecule [122]. Schwimmbeck et al. [123] identified the immunogenic epitopes of human myosin using synthetic peptides. More than 44% of the seral samples from patients with myocarditis or dilated cardiomyopathy bound to a region corresponding to amino acids 345 to 352 of the human myosin heavy chain, while only 4% of the sera from healthy controls reacted with this peptide. Circulating autoantibodies to whole myosin molecules were detected in 26%–46% of the patients who had myocarditis or dilated cardiomyopathy; in these patients, the autoimmune reactivity did not show significant difference between α-myosin and β-myosin [124–126].

Caforio et al. [127] investigated whether anti-heart autoantibodies are directly pathogenic to the host by passive transfer of affinity-purified anti-heart autoantibodies from sera of patients with myocarditis to normal BALB/c mice to induce experimental myocarditis. The results showed that myocarditis was present in 52% of the mice that received purified sera from patients; in contrast, only 2% of the control mice were complicated with the disease. Yuan et al. [128] examined whether the immune tolerance to swine cardiac myosin could protect BALB/c mice that have myosin-induced myocarditis from myocardial injury. The results showed that myocardial degeneration, necrosis, and inflammatory cell infiltration were found in the nontolerance mouse group but not in the immune tolerance group, suggesting the protective effect of immune tolerance on the development of autoimmune myocarditis.

Lately, the role of β1-adrenoreceptor autoantibodies in the pathogenesis of dilated cardiomyopathy attracted much attention [129–139]. Dilated cardiomyopathy is a common cause of heart failure which remains a main health problem because of its high prevalence and the sudden cardiac death. The cardiopathogenic role played by autoantibodies directed against β1-adrenoreceptors has been established in the last two decades. Experimental mouse models have shown that β1-adrenoreceptor autoantibodies caused progressive dilated cardiomyopathy. Clinical studies also revealed that β1-adrenoreceptor autoantibodies are frequently detected in sera from dilated cardiomyopathy patients and are closely associated with the disease. Autoantibodies with β-adrenergic effects were first isolated from sera of idiopathic dilated cardiomyopathy patients [140]. Not long after, the presence of autoantibodies (a γ-globulin) against the β1 adrenoreceptor in sera of idiopathic dilated cardiomyopathy patients was confirmed [141].

The first step in the development of idiopathic dilated cardiomyopathy might be a viral myocarditis. Several viruses might be implicated in the pathogenesis of dilated cardiomyopathy [142]. One hypothesis was that the destruction of cardiac myocytes releases the β1 adrenoreceptor as an autoantigen that induces the autoimmune response. An alternative hypothesis was that β1-adrenoreceptor autoantibodies are produced in antimicrobial immune responses as the microbes have cross-reacting antigens with the receptor. Levin and Hoebecke [143] recently put forward a "bystander" explanation. Certain viruses, bacteria, and fungi have sequence similarity with the second extracellular loop of the β1 adrenoreceptor. Nearly all sequences show homology with either the epitope targeted by β1-adrenoreceptor autoantibodies detectable in Chagas disease patients or the epitope recognized by β1-adrenoreceptor autoantibodies from idiopathic dilated cardiomyopathy patients. In consideration of the fact that the microbial flora of the human intestinal tract as well as most of the symbiotic microorganisms habiting in the human body are mostly unknown, it is most possibility that similar or common epitopes are present among them and that under pathological conditions these epitopes may be presented to the adaptive immune system and induce pathological immune responses [143].

Compared with autoantibody-negative cases, dilated cardiomyopathy patients who are positive in β1-adrenoreceptor autoantibodies showed a poorer left ventricle function, a higher occurrence of severe ventricular arrhythmias, and a higher incidence of sudden cardiac death [144]. It was found that the existence of stimulating β1-adrenoreceptor autoantibodies is independently associated with a roughly threefold increase in cardiac death risk in dilated cardiomyopathy patients [145]. The pathogenic role of β1-adrenoreceptor autoantibodies was confirmed in animal experiments in which peptides corresponding to the second extracellular loop of β1 adrenoreceptors could trigger similar changes in the myocardium to those observed in dilated cardiomyopathy patients [146]. Some researchers have proposed that even though β1-adrenoreceptor autoantibodies are not the only autoantibodies detectable in sera of dilated cardiomyopathy patients, they tended to play a more important part in the initiation and development of dilated cardiomyopathy than did other autoantibodies [147].

4. Innate Immunity in Viral Myocarditis

Innate immune responses also play an important role in the development of myocarditis and are responsible for the

progression to dilated cardiomyopathy. The roles of Toll-like receptors (TLRs) are discussed below.

When host cells are faced with pathogens, pattern recognition receptors (PRRs), most importantly TLRs, recognize pathogen-associated molecular patterns (PAMPs) and activate an intracellular network of signaling pathways that results in the production of numerous cytokines that may exert protection and may also cause inflammation [148–151].

Of all PRRs, TLRs are first described and the most intensively studied. A total of thirteen TLRs (TLR1 through TLR13) have been identified, of which the latter two (TLR12 and TLR13) are not found in humans [152–155]. TLRs are able to recognize different PAMPs presented on various microorganisms including viruses, bacteria, and fungi [156–164]. In addition to immune cells, TLRs are expressed in various tissues, in particular in the myocardial tissue; the latter may account for the links between the immune system and cardiac injury [165–167].

TLR3, which recognizes viral double-stranded RNA, is important in the early response to virus infection. Hardarson et al. [168] examined the role of TLR3 in protection from encephalomyocarditis virus (EMCV) infection. They infected TLR3-deficient ($TLR3^{-/-}$) mice with EMCV. These $TLR3^{-/-}$ mice were more susceptible to EMCV infection and had a much higher viral load in their heart tissue than $TLR3^{+/+}$ mice. Histopathological examination indicated that myocardial inflammation was less obvious in $TLR3^{-/-}$ mice than in $TLR3^{+/+}$ mice. $TLR3^{-/-}$ mice also produced less proinflammation cytokines and chemokines after EMCV infection. Gorbea et al. [169] reported that individuals carrying mutated TLR3 (genetic variant P554S or L412F) had a reduced innate immune response to enteroviruses and increased viral replication through a mechanism involving inhibited NF-κB and type I interferon signaling, which diminished viral clearance and increased the chance of cardiac pathology. Gorbea et al. [170] also found that depletion of extracellular mutant 29 (Ecm29), an adaptor protein of a proteasome subset, increased the abundance of TLR3 in HEK-293 cells and in HeLa cells. The absence of Ecm29 increased TLR3 signaling, increased phosphorylation/activation of effector kinases downstream of TLR3, and enhanced nuclear localization of interferon regulatory factor 3 (IRF3) and the building up of signaling molecules in juxtanuclear recycling endosomes. Thus, Ecm proteasomes are related to the trafficking of TLR3 and the attenuation of TLR3-dependent signaling.

TLR4, the first TLR to be found in human beings, has been reported to perform a variety of functions in a number of pathological conditions, including myocarditis, and its level in the heart is the highest compared with other TLRs [171–173]. TLR4 recognizes lipopolysaccharide (LPS), which leads to the activation of numerous transcription factors via two signaling pathways: MYD88- (myeloid differentiation primary response 88-) dependent pathway and MYD88-independent pathway [174–177].

Studies have shown that inhibition of the TLR4 system could reduce the severity of myocardial inflammation. Fairweather et al. [178] studied the effects of TLR4 deficiency in CVB3 infection and myocarditis. They found that the severity of myocarditis, degree of viral replication, and levels of IL-1β/IL-18 expression were significantly reduced in TLR4-deficient mice and that TLR4 as well as IL-12Rβ1 aggravated CVB3 infection and myocarditis but IFN-γ inhibited viral replication. TLR4 and IL-12Rβ1 might share common downstream pathways that directly affected IL-1β and IL-18 production, and IL-1beta and IL-18 played an important part in the pathogenesis of CVB3-induced myocarditis.

Fuse et al. [179] examined the role of MYD88, an important adaptor protein in the TLR4 signaling pathway, in the pathogenesis of CVB3-induced myocarditis. They found that the MYD88 level in cardiac tissue was increased significantly in wild-type mice after infection of CVB3. $MYD88^{-/-}$ mice showed a significantly higher survival rate than did $MYD88^{+/+}$ mice after exposure to CVB3. Pathological examination displayed a significant decrease of heart inflammation in $MYD88^{-/-}$ mice. Cardiac viral concentrations were significantly decreased in $MYD88^{-/-}$ mice. The levels of mRNAs for IL-1β, TNF-α, IFN-γ, and IL-18 were significantly decreased in the heart of $MYD88^{-/-}$ mice, and serum levels of Th1 cytokines were significantly decreased in these mice as well. By contrast, cardiac levels of activated IRF3 and IFN-β (but not other usual upstream signals of IRF3) were significantly increased in these $MYD88^{-/-}$ mice. These results indicated that MYD88 may be a very important mediator in cardiac inflammation, inducing cytokine production and maintaining Th1/Th2 cytokine balance. Deficiency of MYD88 may provide protection to the host heart through direct activation of IRF3 and IFN-β.

5. Differing Perspectives or Interpretations

5.1. Roles of $CD4^+$ Regulatory T Cells. $CD4^+$ and $CD8^+$ T cells have been reported to be involved in the pathogenesis of myocarditis by many authors; however, regulatory T cells (Tregs), a subset of $CD4^+$ T cells, have been shown to have protective effects [180, 181]. Shi et al. [181] recently demonstrated that the adoptive transfer of Treg cells protected the mice intraperitoneally challenged with CVB3 from myocarditis through the TGF-β–CAR (transforming growth factor β-coxsackie virus and adenovirus receptor) pathway, which maintained the antiviral immune response against CVB3 and thus suppressed the immune response to the cardiac tissue.

5.2. Viruses as Pathogens or Passengers. A large number of viruses have been detected in cardiac tissue, but the interpretation of their role in myocarditis is controversial. Nielsen et al. [182] examined the prevalence of three strains of viruses (adenovirus, enterovirus, and PVB19) in myocardial autopsy specimens from deceased individuals with myocarditis and in noninflammatory control hearts. They found that adenovirus, enterovirus, and PVB19 were rare causes of myocarditis. The detection of PVB19 in myocardial autopsy specimens, in particular, most likely represents a persistent infection with no or limited association with myocardial inflammation.

6. Summary

Immune responses in viral myocarditis function as a double-edged sword: it may be beneficial to the host by limiting viral spread and eliminating the viruses; nevertheless, excessive immune responses can damage cardiac muscle cells and contribute to destructive consequences which could lead to dilated cardiomyopathy. The boundary between the protective antiviral effects and the harmful immunopathological process is usually not clear [183–186]. Careful clinical and laboratory examinations could help doctors to make good judgment and to choose proper medications.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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References

[1] P. Richardson, W. McKenna, M. Bristow et al., “Report of the 1995 World Health Organization/International Society and Federation of Cardiology Task Force on the definition and classification of cardiomyopathies,” *Circulation*, vol. 93, no. 5, pp. 841-842, 1996.

[2] A. L. P. Caforio, G. Malipiero, R. Marcolongo, and S. Iliceto, “Myocarditis: a clinical overview,” *Current Cardiology Reports*, vol. 19, no. 7, p. 63, 2017.

[3] S. Sagar, P. P. Liu, and L. T. Cooper Jr., “Myocarditis,” *The Lancet*, vol. 379, no. 9817, pp. 738–747, 2012.

[4] L. T. Cooper Jr., “Myocarditis,” *The New England Journal of Medicine*, vol. 360, no. 15, pp. 1526–1538, 2009.

[5] D. Fairweather, K. A. Stafford, and Y. K. Sung, “Update on coxsackievirus B3 myocarditis,” *Current Opinion in Rheumatology*, vol. 24, no. 4, pp. 401–407, 2012.

[6] M. Spartalis, E. Tzatzaki, E. Spartalis, C. Damaskos, S. Mavrogeni, and V. Voudris, “Parvovirus B19 myocarditis of fulminant evolution,” *Cardiology Research*, vol. 8, no. 4, pp. 172–175, 2017.

[7] J. Verdonschot, M. Hazebroek, J. Merken et al., “Relevance of cardiac parvovirus B19 in myocarditis and dilated cardiomyopathy: review of the literature,” *European Journal of Heart Failure*, vol. 18, no. 12, pp. 1430–1441, 2016.

[8] M. L. S. Lobo, Â. Taguchi, H. A. Gaspar, J. F. Ferranti, W. B. d. Carvalho, and A. F. Delgado, “Fulminant myocarditis associated with the H1N1 influenza virus: case report and literature review,” *Revista Brasileira de Terapia Intensiva*, vol. 26, no. 3, pp. 321–326, 2014.

[9] N. A. B. Ntusi, “HIV and myocarditis,” *Current Opinion in HIV and AIDS*, vol. 12, no. 6, pp. 561–565, 2017.

[10] A. M. Minhas, A. Nayab, S. Iyer et al., “Association of Zika virus with myocarditis, heart failure, and arrhythmias: a literature review,” *Cureus*, vol. 9, no. 6, article e1399, 2017.

[11] G. Fung, H. Luo, Y. Qiu, D. Yang, and B. McManus, “Myocarditis,” *Circulation Research*, vol. 118, no. 3, pp. 496–514, 2016.

[12] I. Kindermann, C. Barth, F. Mahfoud et al., “Update on myocarditis,” *Journal of the American College of Cardiology*, vol. 59, no. 9, pp. 779–792, 2012.

[13] B. Javadi and A. Sahebkar, “Natural products with anti-inflammatory and immunomodulatory activities against autoimmune myocarditis,” *Pharmacological Research*, vol. 124, pp. 34–42, 2017.

[14] R. G. Weintraub, C. Semsarian, and P. Macdonald, “Dilated cardiomyopathy,” *The Lancet*, vol. 390, no. 10092, pp. 400–414, 2017.

[15] M. Nikolaou, G. Lazaros, A. Karavidas, S. Hatzianastasiou, D. Miliopoulos, and S. Adamopoulos, “Recurrent viral myocarditis: the emerging link toward dilated cardiomyopathy,” *Hellenic Journal Cardiology*, no. 17, pp. 30231-30232, 2017.

[16] N. R. Rose, “Viral myocarditis,” *Current Opinion in Rheumatology*, vol. 28, no. 4, pp. 383–389, 2016.

[17] C. Lu, F. Qin, Y. Yan, T. Liu, J. Li, and H. Chen, “Immunosuppressive treatment for myocarditis: a meta-analysis of randomized controlled trials,” *Journal of Cardiovascular Medicine*, vol. 17, no. 8, pp. 631–637, 2016.

[18] P. R. Camargo, T. S. Okay, L. Yamamoto, G. M. B. del Negro, and A. A. Lopes, “Myocarditis in children and detection of viruses in myocardial tissue: implications for immunosuppressive therapy,” *International Journal of Cardiology*, vol. 148, no. 2, pp. 204–208, 2011.

[19] S. B. Felix, D. Beug, and M. Dörr, “Immunoadsorption therapy in dilated cardiomyopathy,” *Expert Review of Cardiovascular Therapy*, vol. 13, no. 2, pp. 145–152, 2015.

[20] J. Pei, N. Li, J. Chen et al., “The predictive values of beta$_1$-adrenergic and M$_2$ muscarinic receptor autoantibodies for sudden cardiac death in patients with chronic heart failure,” *European Journal of Heart Failure*, vol. 14, no. 8, pp. 887–894, 2012.

[21] J. F. Woodruff and J. J. Woodruff, “Involvement of T lymphocytes in the pathogenesis of coxsackie virus B$_3$ heart disease,” *The Journal of Immunology*, vol. 113, no. 6, pp. 1726–1734, 1974.

[22] C. Kishimoto, K. Kuribayashi, T. Masuda, N. Tomioka, and C. Kawai, “Immunologic behavior of lymphocytes in experimental viral myocarditis: significance of T lymphocytes in the severity of myocarditis and silent myocarditis in BALB/c-nu/nu mice,” *Circulation*, vol. 71, no. 6, pp. 1247–1254, 1985.

[23] S. A. Huber, A. Moraska, and M. Choate, “T cells expressing the gamma delta T-cell receptor potentiate coxsackievirus B3-induced myocarditis,” *Journal of Virology*, vol. 66, no. 11, pp. 6541–6546, 1992.

[24] S. A. Huber and B. Pfaeffle, “Differential Th1 and Th2 cell responses in male and female BALB/c mice infected with coxsackievirus group B type 3,” *Journal of Virology*, vol. 68, no. 8, pp. 5126–5132, 1994.

[25] M. A. Opavsky, J. Penninger, K. Aitken et al., “Susceptibility to myocarditis is dependent on the response of $\alpha\beta$ T lymphocytes to coxsackieviral infection,” *Circulation Research*, vol. 85, no. 6, pp. 551–558, 1999.

[26] K. U. Knowlton and C. Badorff, “The immune system in viral myocarditis: maintaining the balance,” *Circulation Research*, vol. 85, no. 6, pp. 559–561, 1999.

[27] W. Bracamonte-Baran and D. Čiháková, “Cardiac autoimmunity: myocarditis,” in *The Immunology of Cardiovascular Homeostasis and Pathology*, S. Sattler and T. Kennedy-Lydon,

Eds., vol. 1003 of Advances in Experimental Medicine and Biology, pp. 187–221, Springer, Cham, 2017.

[28] X. Gao, B. Wei, Y. Deng, Y. L. Huang, and W. Wu, "Increased mobilization of CD45+CD34+VLA-4+ cells in acute viral myocarditis induced by coxsackievirus B3," *Cardiology*, vol. 138, no. 4, pp. 238–248, 2017.

[29] C. Comarmond and P. Cacoub, "Myocarditis in autoimmune or auto-inflammatory diseases," *Autoimmunity Reviews*, vol. 16, no. 8, pp. 811–816, 2017.

[30] K. Tajiri, Y. Yasutomi, and K. Aonuma, "Recent advances in the management of autoimmune myocarditis: insights from animal studies," *Current Pharmaceutical Design*, vol. 22, no. 4, pp. 427–439, 2016.

[31] A. Valaperti, "Drugs targeting the canonical NF-κB pathway to treat viral and autoimmune myocarditis," *Current Pharmaceutical Design*, vol. 22, no. 4, pp. 440–449, 2016.

[32] A. M. Muller, A. Fischer, H. A. Katus, and Z. Kaya, "Mouse models of autoimmune diseases - autoimmune myocarditis," *Current Pharmaceutical Design*, vol. 21, no. 18, pp. 2498–2512, 2015.

[33] J. Reddy, C. Massilamany, I. Buskiewicz, and S. A. Huber, "Autoimmunity in viral myocarditis," *Current Opinion in Rheumatology*, vol. 25, no. 4, pp. 502–508, 2013.

[34] F. Leuschner, H. A. Katus, and Z. Kaya, "Autoimmune myocarditis: past, present and future," *Journal of Autoimmunity*, vol. 33, no. 3-4, pp. 282–289, 2009.

[35] N. R. Rose, "Myocarditis: infection versus autoimmunity," *Journal of Clinical Immunology*, vol. 29, no. 6, pp. 730–737, 2009.

[36] M. Afanasyeva and N. Rose, "Viral infection and heart disease: autoimmune mechanisms," *Infection and Autoimmunity*, vol. 3, no. 4, pp. 299–318, 2004.

[37] E. Hui, J. Cheung, J. Zhu et al., "T cell costimulatory receptor CD28 is a primary target for PD-1–mediated inhibition," *Science*, vol. 355, no. 6332, pp. 1428–1433, 2017.

[38] R. Tian, H. Wang, G. D. Gish et al., "Combinatorial proteomic analysis of intercellular signaling applied to the CD28 T-cell costimulatory receptor," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 112, no. 13, pp. E1594–E1603, 2015.

[39] J. S. Maltzman and L. A. Turka, "T-cell costimulatory blockade in organ transplantation," *Cold Spring Harbor Perspectives in Medicine*, vol. 3, no. 12, article a015537, 2013.

[40] A. H. Lichtman, "T cell costimulatory and coinhibitory pathways in vascular inflammatory diseases," *Frontiers in Physiology*, vol. 3, p. 18, 2012.

[41] T. Ito, T. Ueno, M. R. Clarkson et al., "Analysis of the role of negative T cell costimulatory pathways in CD4 and CD8 T cell-mediated alloimmune responses in vivo," *The Journal of Immunology*, vol. 174, no. 11, pp. 6648–6656, 2005.

[42] M. D. Vu and X. C. Li, "Study of T-cell costimulatory blockade in vivo at a single-cell level," in *Adoptive Immunotherapy: Methods and Protocols*, B. Ludewig and M. W. Hoffmann, Eds., vol. 109 of Methods in Molecular Medicine, pp. 409–420, Humana Press, Totowa, NJ, USA, 2005.

[43] N. Najafian and S. J. Khoury, "T cell costimulatory pathways: blockade for autoimmunity," *Expert Opinion on Biological Therapy*, vol. 3, no. 2, pp. 227–236, 2003.

[44] M. Sho, S. E. Sandner, N. Najafian et al., "New insights into the interactions between T-cell costimulatory blockade and conventional immunosuppressive drugs," *Annals of Surgery*, vol. 236, no. 5, pp. 667–675, 2002.

[45] Y. Matsui, M. Inobe, H. Okamoto et al., "Blockade of T cell costimulatory signals using adenovirus vectors prevents both the induction and the progression of experimental autoimmune myocarditis," *Journal of Molecular and Cellular Cardiology*, vol. 34, no. 3, pp. 279–295, 2002.

[46] L. Marisa, M. Svrcek, A. Collura et al., "The balance between cytotoxic T-cell lymphocytes and immune checkpoint expression in the prognosis of colon tumors," *Journal of the National Cancer Institute*, vol. 110, no. 1, pp. 68–77, 2018.

[47] L. Mo, Q. Chen, X. Zhang et al., "Depletion of regulatory T cells by anti-ICOS antibody enhances anti-tumor immunity of tumor cell vaccine in prostate cancer," *Vaccine*, vol. 35, no. 43, pp. 5932–5938, 2017.

[48] Z. Brzoza, W. Grzeszczak, W. Trautsolt, and D. Moczulski, "Inducible T-cell costimulator (*ICOS*) and *CD28* polymorphisms possibly play a role in the pathogenesis of chronic autoreactive urticaria," *Clinical and Experimental Dermatology*, vol. 42, no. 8, pp. 863–867, 2017.

[49] H. Futamatsu, J. Suzuki, H. Kosuge et al., "Attenuation of experimental autoimmune myocarditis by blocking activated T cells through inducible costimulatory molecule pathway," *Cardiovascular Research*, vol. 59, no. 1, pp. 95–104, 2003.

[50] Q. Long, Y. H. Liao, Y. Xie et al., "Coxsackievirus B3 directly induced Th17 cell differentiation by inhibiting Nup98 expression in patients with acute viral myocarditis," *Frontiers in Cellular and Infection Microbiology*, vol. 6, p. 171, 2016.

[51] H. Jin and X. Guo, "Valproic acid ameliorates coxsackievirus-B3-induced viral myocarditis by modulating Th17/Treg imbalance," *Virology Journal*, vol. 13, no. 1, p. 168, 2016.

[52] L. Li, L. Li, L. Xiao, and J. Shangguan, "Progranulin ameliorates coxsackievirus-B3-induced viral myocarditis by downregulating Th1 and Th17 cells," *Experimental Cell Research*, vol. 367, no. 2, pp. 241–250, 2018.

[53] H. Cheng, Y. Xi, X. Chi, Y. Wu, and G. Liu, "Fenofibrate treatment of rats with experimental autoimmune myocarditis by alleviating Treg/Th17 disorder," *Central European Journal of Immunology*, vol. 1, no. 1, pp. 64–70, 2016.

[54] J. G. Barin, G. C. Baldeviano, M. V. Talor et al., "Fatal eosinophilic myocarditis develops in the absence of IFN-γ and IL-17A," *The Journal of Immunology*, vol. 191, no. 8, pp. 4038–4047, 2013.

[55] A. Valaperti, R. R. Marty, G. Kania et al., "$CD11b^{+}$ monocytes abrogate Th17 $CD4^{+}$ T cell-mediated experimental autoimmune myocarditis," *The Journal of Immunology*, vol. 180, no. 4, pp. 2686–2695, 2008.

[56] J. Yuan, M. Yu, Q. W. Lin et al., "Neutralization of IL-17 inhibits the production of anti-ANT autoantibodies in CVB3-induced acute viral myocarditis," *International Immunopharmacology*, vol. 10, no. 3, pp. 272–276, 2010.

[57] J. Yuan, M. Yu, Q. W. Lin et al., "Th17 cells contribute to viral replication in coxsackievirus B3-induced acute viral myocarditis," *The Journal of Immunology*, vol. 185, no. 7, pp. 4004–4010, 2010.

[58] Y. Fan, W. Weifeng, Y. Yuluan, K. Qing, P. Yu, and H. Yanlan, "Treatment with a neutralizing anti-murine interleukin-17 antibody after the onset of coxsackievirus b3-induced viral myocarditis reduces myocardium inflammation," *Virology Journal*, vol. 8, no. 1, p. 17, 2011.

[59] H. Zhu, C. Lou, and P. Liu, "Interleukin-27 ameliorates coxsackievirus-B3-induced viral myocarditis by inhibiting Th17 cells," *Virology Journal*, vol. 12, no. 1, article 189, 2015.

[60] J. M. Myers, L. T. Cooper, D. C. Kem et al., "Cardiac myosin-Th17 responses promote heart failure in human myocarditis," *JCI Insight*, vol. 1, article e85851, no. 9, 2016.

[61] H. Ying, L. Yang, G. Qiao et al., "Cutting edge: CTLA-4–B7 interaction suppresses Th17 cell differentiation," *The Journal of Immunology*, vol. 185, no. 3, pp. 1375–1378, 2010.

[62] P. Martín and F. Sánchez-Madrid, "CD69: an unexpected regulator of T_H17 cell–driven inflammatory responses," *Science Signaling*, vol. 4, no. 165, article pe14, 2011.

[63] F. Yang, W. F. Wu, Y. L. Yan, Y. Pang, Q. Kong, and Y. L. Huang, "Expression of IL-23/Th17 pathway in a murine model of coxsackie virus B3-induced viral myocarditis," *Virology Journal*, vol. 8, no. 1, p. 301, 2011.

[64] T. Yamashita, T. Iwakura, K. Matsui et al., "IL-6-mediated Th17 differentiation through RORγt is essential for the initiation of experimental autoimmune myocarditis," *Cardiovascular Research*, vol. 91, no. 4, pp. 640–648, 2011.

[65] Y. L. Liu, W. Wu, Y. Xue et al., "MicroRNA-21 and -146b are involved in the pathogenesis of murine viral myocarditis by regulating TH-17 differentiation," *Archives of Virology*, vol. 158, no. 9, pp. 1953–1963, 2013.

[66] Y. Huang, Y. Li, B. Wei, W. Wu, and X. Gao, "CD80 regulates Th17 cell differentiation in coxsackie virus B3-induced acute myocarditis," *Inflammation*, vol. 41, no. 1, pp. 232–239, 2017.

[67] N. A. J. Dawson, J. Vent-Schmidt, and M. K. Levings, "Engineered tolerance: tailoring development, function, and antigen-specificity of regulatory T cells," *Frontiers in Immunology*, vol. 8, article 1460, 2017.

[68] H. Yu, R. Paiva, and R. A. Flavell, "Harnessing the power of regulatory T-cells to control autoimmune diabetes: overview and perspective," *Immunology*, vol. 153, no. 2, pp. 161–170, 2017.

[69] P. Kraj and L. Ignatowicz, "The mechanisms shaping the repertoire of $CD4^+$ $Foxp3^+$ regulatory T cells," *Immunology*, vol. 153, no. 3, pp. 290–296, 2017.

[70] Y. Kitagawa and S. Sakaguchi, "Molecular control of regulatory T cell development and function," *Current Opinion in Immunology*, vol. 49, pp. 64–70, 2017.

[71] B. An, X. Liu, G. Li, and H. Yuan, "Interleukin-37 ameliorates coxsackievirus B3-induced viral myocarditis by modulating the Th17/regulatory T cell immune response," *Journal of Cardiovascular Pharmacology*, vol. 69, no. 5, pp. 305–313, 2017.

[72] L. Yan, F. Hu, X. Yan et al., "Inhibition of microRNA-155 ameliorates experimental autoimmune myocarditis by modulating Th17/Treg immune response," *Journal of Molecular Medicine*, vol. 94, no. 9, pp. 1063–1079, 2016.

[73] K. E. Simpson, M. W. Cunningham, C. K. Lee et al., "Autoimmunity against the heart and cardiac myosin in children with myocarditis," *Journal of Cardiac Failure*, vol. 22, no. 7, pp. 520–528, 2016.

[74] A. Dörner, A. Kallwellis-Opara, M. Pauschinger, U. Kühl, and H. P. Schultheiss, "Cardiac autoantibodies in viral myocarditis," *Heart Failure Clinics*, vol. 1, no. 3, pp. 333–343, 2005.

[75] A. L. P. Caforio, F. Tona, S. Bottaro et al., "Clinical implications of anti-heart autoantibodies in myocarditis and dilated cardiomyopathy," *Autoimmunity*, vol. 41, no. 1, pp. 35–45, 2008.

[76] T. Yoshikawa, A. Baba, and Y. Nagatomo, "Autoimmune mechanisms underlying dilated cardiomyopathy," *Circulation Journal*, vol. 73, no. 4, pp. 602–607, 2009.

[77] J. Latva-Hirvelä, V. Kytö, A. Saraste et al., "Development of troponin autoantibodies in experimental coxsackievirus B3 myocarditis," *European Journal of Clinical Investigation*, vol. 39, no. 6, pp. 457–462, 2009.

[78] A. Matsumori, "Lessons learned from experimental myocarditis," *Herz*, vol. 37, no. 8, pp. 817–821, 2012.

[79] B. Maisch, A. D. Ristić, G. Hufnagel, and S. Pankuweit, "Pathophysiology of viral myocarditis: the role of humoral immune response," *Cardiovascular Pathology*, vol. 11, no. 2, pp. 112–122, 2002.

[80] E. Saygili, F. Noor-Ebad, J. W. Schröder et al., "Autoantibodies in dilated cardiomyopathy induce vascular endothelial growth factor expression in cardiomyocytes," *Biochemical and Biophysical Research Communications*, vol. 465, no. 1, pp. 119–124, 2015.

[81] G. Bhardwaj, M. Dörr, P. K. Sappa et al., "Endomyocardial proteomic signature corresponding to the response of patients with dilated cardiomyopathy to immunoadsorption therapy," *Journal of Proteomics*, vol. 150, pp. 121–129, 2017.

[82] M. Dandel, G. Wallukat, A. Englert, and R. Hetzer, "Immunoadsorption therapy for dilated cardiomyopathy and pulmonary arterial hypertension," *Atherosclerosis Supplements*, vol. 14, no. 1, pp. 203–211, 2013.

[83] C. Trimpert, L. R. Herda, L. G. Eckerle et al., "Immunoadsorption in dilated cardiomyopathy: long-term reduction of cardiodepressant antibodies," *European Journal of Clinical Investigation*, vol. 40, no. 8, pp. 685–691, 2010.

[84] N. P. Becker, J. Müller, P. Göttel, G. Wallukat, and I. Schimke, "Cardiomyopathy — an approach to the autoimmune background," *Autoimmunity Reviews*, vol. 16, no. 3, pp. 269–286, 2017.

[85] G. Wallukat, J. Müller, A. Haberland et al., "Aptamer BC007 for neutralization of pathogenic autoantibodies directed against G-protein coupled receptors: a vision of future treatment of patients with cardiomyopathies and positivity for those autoantibodies," *Atherosclerosis*, vol. 244, pp. 44–47, 2016.

[86] A. Haberland, M. Holtzhauer, A. Schlichtiger et al., "Aptamer BC 007 – a broad spectrum neutralizer of pathogenic autoantibodies against G-protein-coupled receptors," *European Journal of Pharmacology*, vol. 789, pp. 37–45, 2016.

[87] M. Dandel, A. Englert, G. Wallukat et al., "Immunoadsorption can improve cardiac function in transplant candidates with non-ischemic dilated cardiomyopathy associated with diabetes mellitus," *Atherosclerosis Supplements*, vol. 18, pp. 124–133, 2015.

[88] S. N. Pokrovsky, M. V. Ezhov, M. S. Safarova et al., "Ig apheresis for the treatment of severe DCM patients," *Atherosclerosis Supplements*, vol. 14, no. 1, pp. 213–218, 2013.

[89] S. Benvenga and F. Guarneri, "Molecular mimicry and autoimmune thyroid disease," *Reviews in Endocrine and Metabolic Disorders*, vol. 17, no. 4, pp. 485–498, 2016.

[90] N. R. Rose, "Learning from myocarditis: mimicry, chaos and black holes," *F1000Prime Reports*, vol. 6, p. 25, 2014.

[91] C. Massilamany, A. Koenig, J. Reddy, S. Huber, and I. Buskiewicz, "Autoimmunity in picornavirus infections," *Current Opinion in Virology*, vol. 16, pp. 8–14, 2016.

[92] R. Root-Bernstein and D. Fairweather, "Unresolved issues in theories of autoimmune disease using myocarditis as a framework," *Journal of Theoretical Biology*, vol. 375, pp. 101–123, 2015.

[93] R. Root-Bernstein, "Rethinking molecular mimicry in rheumatic heart disease and autoimmune myocarditis: laminin, collagen IV, CAR, and B1AR as initial targets of disease," *Frontiers Pediatrics*, vol. 2, p. 85, 2014.

[94] C. Massilamany, S. A. Huber, M. W. Cunningham, and J. Reddy, "Relevance of molecular mimicry in the mediation of infectious myocarditis," *Journal of Cardiovascular Translational Research*, vol. 7, no. 2, pp. 165–171, 2014.

[95] C. Massilamany, A. Gangaplara, D. Steffen, and J. Reddy, "Identification of novel mimicry epitopes for cardiac myosin heavy chain-α that induce autoimmune myocarditis in A/J mice," *Cellular Immunology*, vol. 271, no. 2, pp. 438–449, 2011.

[96] S. A. Huber, "Autoimmunity in coxsackievirus B3 induced myocarditis," *Autoimmunity*, vol. 39, no. 1, pp. 55–61, 2006.

[97] R. S. Fujinami, M. G. von Herrath, U. Christen, and J. L. Whitton, "Molecular mimicry, bystander activation, or viral persistence: infections and autoimmune disease," *Clinical Microbiology Reviews*, vol. 19, no. 1, pp. 80–94, 2006.

[98] F. N. Paleev, A. A. Kotova, S. V. Suchkov, and N. R. Paleev, "Modern aspects of immunopathogenesis of autoimmune myocarditis," *Russian Journal of Immunology*, vol. 7, no. 4, pp. 323–327, 2002.

[99] C. M. Lawson, "Evidence for mimicry by viral antigens in animal models of autoimmune disease including myocarditis," *Cellular and Molecular Life Sciences*, vol. 57, no. 4, pp. 552–560, 2000.

[100] J. M. Davies, "Molecular mimicry: can epitope mimicry induce autoimmune disease?," *Immunology & Cell Biology*, vol. 75, no. 2, pp. 113–126, 1997.

[101] C. J. Gauntt, S. M. Tracy, N. Chapman et al., "Coxsackievirus-induced chronic myocarditis in murine models," *European Heart Journal*, vol. 16, Supplement O, pp. 56–58, 1995.

[102] C. J. Gauntt, H. M. Arizpe, A. L. Higdon et al., "Molecular mimicry, anti-coxsackievirus B3 neutralizing monoclonal antibodies, and myocarditis," *The Journal of Immunology*, vol. 154, no. 6, pp. 2983–2995, 1995.

[103] B. Maisch, E. Bauer, M. Cirsi, and K. Kochsiek, "Cytolytic cross-reactive antibodies directed against the cardiac membrane and viral proteins in coxsackievirus B3 and B4 myocarditis. Characterization and pathogenetic relevance," *Circulation*, vol. 87, Supplement 5, pp. IV49–IV65, 1993.

[104] C. Gauntt, A. Higdon, D. Bowers, E. Maull, J. Wood, and R. Crawley, "What lessons can be learned from animal model studies in viral heart disease?," *Scandinavian Journal of Infectious Diseases Supplementum*, vol. 88, pp. 49–65, 1993.

[105] C. M. Lawson, H. L. O'Donoghue, and W. D. Reed, "Mouse cytomegalovirus infection induces antibodies which cross-react with virus and cardiac myosin: a model for the study of molecular mimicry in the pathogenesis of viral myocarditis," *Immunology*, vol. 75, no. 3, pp. 513–519, 1992.

[106] K. W. Beisel, J. Srinivasappa, and B. S. Prabhakar, "Identification of a putative shared epitope between Coxsackie virus B4 and alpha cardiac myosin heavy chain," *Clinical & Experimental Immunology*, vol. 86, no. 1, pp. 49–55, 1991.

[107] I. S. Aranda-Uribe, E. Ortega, and E. Martínez-Cordero, "Immunization of BALB/c mice with pigeon IgY induces the production of anti-IgG autoantibodies," *Autoimmunity*, vol. 50, no. 6, pp. 336–345, 2017.

[108] J. R. Kerr, "The role of parvovirus B19 in the pathogenesis of autoimmunity and autoimmune disease," *Journal of Clinical Pathology*, vol. 69, no. 4, pp. 279–291, 2016.

[109] J. Suurmond and B. Diamond, "Autoantibodies in systemic autoimmune diseases: specificity and pathogenicity," *The Journal of Clinical Investigation*, vol. 125, no. 6, pp. 2194–2202, 2015.

[110] C. Backes, N. Ludwig, P. Leidinger et al., "Immunogenicity of autoantigens," *BMC Genomics*, vol. 12, no. 1, p. 340, 2011.

[111] O. Barzilai, M. Ram, and Y. Shoenfeld, "Viral infection can induce the production of autoantibodies," *Current Opinion in Rheumatology*, vol. 19, no. 6, pp. 636–643, 2007.

[112] H. Nakamura, A. Kawakami, and K. Eguchi, "Mechanisms of autoantibody production and the relationship between autoantibodies and the clinical manifestations in Sjögren's syndrome," *Translational Research*, vol. 148, no. 6, pp. 281–288, 2006.

[113] R. E. Paque and R. Miller, "Adoptively transferred anti-idiotype pulsed B cells mediate autoimmune myocarditis," *Infection and Immunity*, vol. 60, no. 8, pp. 3396–3404, 1992.

[114] I. Siloşi, C. A. Siloşi, M. V. Boldeanu et al., "The role of autoantibodies in health and disease," *Romanian Journal of Morphology and Embryology*, vol. 57, Supplement 2, pp. 633–638, 2016.

[115] W. T. Ma, C. Chang, M. E. Gershwin, and Z. X. Lian, "Development of autoantibodies precedes clinical manifestations of autoimmune diseases: a comprehensive review," *Journal of Autoimmunity*, vol. 83, pp. 95–112, 2017.

[116] U. Nussinovitch and Y. Shoenfeld, "The clinical and diagnostic significance of anti-myosin autoantibodies in cardiac disease," *Clinical Reviews in Allergy & Immunology*, vol. 44, no. 1, pp. 98–108, 2013.

[117] N. R. Rose, K. W. Beisel, A. Herskowitz et al., "Cardiac myosin and autoimmune myocarditis," in *Ciba Foundation Symposium 129 - Autoimmunity and Autoimmune Disease*, vol. 129, pp. 3–24, John Wiley & Sons, Ltd, Chichester, UK, 1987.

[118] F. L. Alvarez, N. Neu, N. R. Rose, S. W. Craig, and K. W. Beisel, "Heart-specific autoantibodies induced by Coxsackievirus B_3: identification of heart autoantigens," *Clinical Immunology and Immunopathology*, vol. 43, no. 1, pp. 129–139, 1987.

[119] N. Neu, K. W. Beisel, M. D. Traystman, N. R. Rose, and S. W. Craig, "Autoantibodies specific for the cardiac myosin isoform are found in mice susceptible to coxsackievirus B3-induced myocarditis," *The Journal of Immunology*, vol. 138, no. 8, pp. 2488–2492, 1987.

[120] N. Neu, N. R. Rose, K. W. Beisel, A. Herskowitz, G. Gurri-Glass, and S. W. Craig, "Cardiac myosin induces myocarditis in genetically predisposed mice," *The Journal of Immunology*, vol. 139, no. 11, pp. 3630–3636, 1987.

[121] C. L. Pummerer, K. Luze, G. Grässl et al., "Identification of cardiac myosin peptides capable of inducing autoimmune myocarditis in BALB/c mice," *The Journal of Clinical Investigation*, vol. 97, no. 9, pp. 2057–2062, 1996.

[122] D. L. Donermeyer, K. W. Beisel, P. M. Allen, and S. C. Smith, "Myocarditis-inducing epitope of myosin binds constitutively and stably to I-Ak on antigen-presenting cells in the heart," *Journal of Experimental Medicine*, vol. 182, no. 5, pp. 1291–1300, 1995.

[123] P. L. Schwimmbeck, N. K. Bland, H. P. Schultheiss, and B. E. Strauer, "The possible value of synthetic peptides in the diagnosis and therapy of myocarditis and dilated cardiomyopathy," *European Heart Journal*, vol. 12, Supplement D, pp. 76–80, 1991.

[124] A. L. Caforio, M. Grazzini, J. M. Mann et al., "Identification of alpha- and beta-cardiac myosin heavy chain isoforms as major autoantigens in dilated cardiomyopathy," *Circulation*, vol. 85, no. 5, pp. 1734–1742, 1992.

[125] Z. Wang, Y. Liao, J. Dong, S. Li, J. Wang, and M. L. Fu, "Clinical significance and pathogenic role of anti-cardiac myosin autoantibody in dilated cardiomyopathy," *Chinese Medical Journal*, vol. 116, no. 4, pp. 499–502, 2003.

[126] N. Latif, C. S. Baker, M. J. Dunn, M. L. Rose, P. Brady, and M. H. Yacoub, "Frequency and specificity of antiheart antibodies in patients with dilated cardiomyopathy detected using SDS-PAGE and western blotting," *Journal of the American College of Cardiology*, vol. 22, no. 5, pp. 1378–1384, 1993.

[127] A. L. P. Caforio, A. Angelini, M. Blank et al., "Passive transfer of affinity-purified anti-heart autoantibodies (AHA) from sera of patients with myocarditis induces experimental myocarditis in mice," *International Journal of Cardiology*, vol. 179, pp. 166–177, 2015.

[128] H. T. Yuan, Y. H. Liao, Z. Wang et al., "Prevention of myosin-induced autoimmune myocarditis in mice by anti-L_3T_4 monoclonal antibody," *Canadian Journal of Physiology and Pharmacology*, vol. 81, no. 2, pp. 84–88, 2003.

[129] J. Müller, G. Wallukat, and I. Schimke, "Autoantibodies directed against the β_1-adrenergic receptor in patients with dilated cardiomyopathy," *Journal of the American College of Cardiology*, vol. 70, no. 6, pp. 808-809, 2017.

[130] M. Park, G. R. Reddy, G. Wallukat, Y. K. Xiang, and S. F. Steinberg, "β_1-Adrenergic receptor O-glycosylation regulates N-terminal cleavage and signaling responses in cardiomyocytes," *Scientific Reports*, vol. 7, no. 1, article 7890, 2017.

[131] Y. Nagatomo, D. M. McNamara, and W. H. W. Tang, "Reply: understanding subclass diversity of detectable β_1-adrenergic receptor autoantibodies and their clinical impact," *Journal of the American College of Cardiology*, vol. 70, no. 6, p. 809, 2017.

[132] W. Gao, W. J. Guo, D. Y. Hou et al., "Autoantibodies against β_1-adrenergic receptor: response to induction therapy with bortezomib-containing regimens for multiple myeloma patients," *Leukemia & Lymphoma*, vol. 59, no. 3, pp. 717–724, 2017.

[133] Z. X. Lei, B. T. Li, H. A. Li et al., "Relationship between catecholamine level and gene polymorphism of β1 adrenergic receptor G1165C in children with EV71 infection in hand foot and mouth disease," *Asian Pacific Journal of Tropical Medicine*, vol. 10, no. 5, pp. 473–477, 2017.

[134] K. Hayashi, M. Gotou, T. Matsui, K. Imahashi, T. Nishimoto, and H. Kobayashi, "Identification of phosphorylation sites on β1-adrenergic receptor in the mouse heart," *Biochemical and Biophysical Research Communications*, vol. 488, no. 2, pp. 362–367, 2017.

[135] O. Magvanjav, C. McDonough, Y. Gong et al., "Pharmacogenetic associations of β1-adrenergic receptor polymorphisms with cardiovascular outcomes in the SPS3 trial (Secondary Prevention of Small Subcortical Strokes)," *Stroke*, vol. 48, no. 5, pp. 1337–1343, 2017.

[136] L. Vanhoutte, C. Guilbaud, R. Martherus et al., "MRI assessment of cardiomyopathy induced by β1-adrenoreceptor autoantibodies and protection through β3-adrenoreceptor overexpression," *Scientific Reports*, vol. 7, article 43951, 2017.

[137] J. K. Patel, "The β_1-adrenergic receptor IgG subclass 3 autoantibody in dilated cardiomyopathy: *Friend or Foe?*," *Journal of the American College of Cardiology*, vol. 69, no. 8, pp. 978–980, 2017.

[138] B. Bornholz, B. Hanzen, Y. Reinke, S. B. Felix, and F. Boege, "Impact of common β_1-adrenergic receptor polymorphisms on the interaction with agonistic autoantibodies in dilated cardiomyopathy," *International Journal of Cardiology*, vol. 214, pp. 83–85, 2016.

[139] S. Dalal, B. Connelly, M. Singh, and K. Singh, "NF2 signaling pathway plays a pro-apoptotic role in β-adrenergic receptor stimulated cardiac myocyte apoptosis," *PLoS One*, vol. 13, no. 4, article e0196626, 2018.

[140] G. Wallukat and A. Wollenberger, "Effects of the serum gamma globulin fraction of patients with allergic asthma and dilated cardiomyopathy on chronotropic beta adrenoceptor function in cultured neonatal rat heart myocytes," *Biomedica Biochimica Acta*, vol. 46, no. 8-9, pp. S634–S639, 1987.

[141] C. J. Limas, I. F. Goldenberg, and C. Limas, "Autoantibodies against beta-adrenoceptors in human idiopathic dilated cardiomyopathy," *Circulation Research*, vol. 64, no. 1, pp. 97–103, 1989.

[142] Y. Maekawa, M. Ouzounian, M. A. Opavsky, and P. P. Liu, "Connecting the missing link between dilated cardiomyopathy and viral myocarditis: virus, cytoskeleton, and innate immunity," *Circulation*, vol. 115, no. 1, pp. 5–8, 2007.

[143] M. J. Levin and J. Hoebeke, "Cross-talk between anti-β_1-adrenoceptor antibodies in dilated cardiomyopathy and Chagas' heart disease," *Autoimmunity*, vol. 41, no. 6, pp. 429–433, 2008.

[144] M. Iwata, T. Yoshikawa, A. Baba, T. Anzai, H. Mitamura, and S. Ogawa, "Autoantibodies against the second extracellular loop of $beta_1$-adrenergic receptors predict ventricular tachycardia and sudden death in patients with idiopathic dilated cardiomyopathy," *Journal of the American College of Cardiology*, vol. 37, no. 2, pp. 418–424, 2001.

[145] S. Störk, V. Boivin, R. Horf et al., "Stimulating autoantibodies directed against the cardiac β_1-adrenergic receptor predict increased mortality in idiopathic cardiomyopathy," *American Heart Journal*, vol. 152, no. 4, pp. 697–704, 2006.

[146] R. Jahns, V. Boivin, L. Hein et al., "Direct evidence for a β_1-adrenergic receptor-directed autoimmune attack as a cause of idiopathic dilated cardiomyopathy," *The Journal of Clinical Investigation*, vol. 113, no. 10, pp. 1419–1429, 2004.

[147] J. M. Lappé, C. M. Pelfrey, and W. H. W. Tang, "Recent insights into the role of autoimmunity in idiopathic dilated cardiomyopathy," *Journal of Cardiac Failure*, vol. 14, no. 6, pp. 521–530, 2008.

[148] U. Ohto, "Conservation and divergence of ligand recognition and signal transduction mechanisms in toll-like receptors," *Chemical and Pharmaceutical Bulletin*, vol. 65, no. 8, pp. 697–705, 2017.

[149] L. A. J. O'Neill, D. Golenbock, and A. G. Bowie, "The history of toll-like receptors — redefining innate immunity," *Nature Reviews Immunology*, vol. 13, no. 6, pp. 453–460, 2013.

[150] D. H. Kono, R. Baccala, and A. N. Theofilopoulos, "TLRs and interferons: a central paradigm in autoimmunity," *Current Opinion in Immunology*, vol. 25, no. 6, pp. 720–727, 2013.

[151] A. Mohammad Hosseini, J. Majidi, B. Baradaran, and M. Yousefi, "Toll-like receptors in the pathogenesis of autoimmune diseases," *Advanced Pharmaceutical Bulletin*, vol. 5, no. 5, pp. 605–614, 2015.

[152] M. J. Jiménez-Dalmaroni, M. E. Gerswhin, and I. E. Adamopoulos, "The critical role of toll-like receptors — from microbial recognition to autoimmunity: a comprehensive review," *Autoimmunity Reviews*, vol. 15, no. 1, pp. 1–8, 2016.

[153] L. N. Dong, J. P. Wang, P. Liu, Y. F. Yang, J. Feng, and Y. Han, "Faecal and mucosal microbiota in patients with functional gastrointestinal disorders: correlation with toll-like receptor 2/toll-like receptor 4 expression," *World Journal of Gastroenterology*, vol. 23, no. 36, pp. 6665–6673, 2017.

[154] M. Shah, M. A. Anwar, J. H. Kim, and S. Choi, "Advances in antiviral therapies targeting toll-like receptors," *Expert Opinion on Investigational Drugs*, vol. 25, no. 4, pp. 437–453, 2016.

[155] A. Achek, D. Yesudhas, and S. Choi, "Toll-like receptors: promising therapeutic targets for inflammatory diseases," *Archives of Pharmacal Research*, vol. 39, no. 8, pp. 1032–1049, 2016.

[156] M. Farrugia and B. Baron, "The role of toll-like receptors in autoimmune diseases through failure of the self-recognition mechanism," *International Journal of Inflammation*, vol. 2017, Article ID 8391230, 12 pages, 2017.

[157] S. Ntoufa, M. G. Vilia, K. Stamatopoulos, P. Ghia, and M. Muzio, "Toll-like receptors signaling: a complex network for NF-κB activation in B-cell lymphoid malignancies," *Seminars in Cancer Biology*, vol. 39, pp. 15–25, 2016.

[158] K. Paarnio, S. Väyrynen, K. Klintrup et al., "Divergent expression of bacterial wall sensing Toll-like receptors 2 and 4 in colorectal cancer," *World Journal of Gastroenterology*, vol. 23, no. 26, pp. 4831–4838, 2017.

[159] Y. Guo, Q. Chai, Y. Zhao, P. Li, J. Qiao, and J. Huang, "Increased activation of toll-like receptors-7 and -8 of peripheral blood mononuclear cells and upregulated serum cytokines in patients with pediatric systemic lupus erythematosus," *International Journal of Clinical and Experimental Medicine*, vol. 8, no. 11, pp. 20472–20480, 2015.

[160] J. Zhang, J. Zhou, B. Xu, C. Chen, and W. Shi, "Different expressions of TLRs and related factors in peripheral blood of preterm infants," *International Journal of Clinical and Experimental Medicine*, vol. 8, no. 3, pp. 4108–4114, 2015.

[161] J. Qiao, B. Luan, H. Gu, and Y. Zhang, "Effect of different 1, 25-(OH)2D3 doses on high mobility group box1 and toll-like receptors 4 expression in lung tissue of asthmatic mice," *International Journal of Clinical and Experimental Medicine*, vol. 8, no. 3, pp. 4016–4023, 2015.

[162] A. Tiwari, P. Singh, P. Jaitley et al., "*Eucalyptus robusta* leaves methanolic extract suppresses inflammatory mediators by specifically targeting TLR4/TLR9, MPO, COX2, iNOS and inflammatory cytokines in experimentally-induced endometritis in rats," *Journal of Ethnopharmacology*, vol. 213, pp. 149–158, 2017.

[163] L. Feng, N. Yang, C. Li et al., "Pudilan xiaoyan oral liquid alleviates LPS-induced respiratory injury through decreasing nitroxidative stress and blocking TLR4 activation along with NF-KB phosphorylation in mice," *Journal of Ethnopharmacology*, vol. 214, pp. 292–300, 2017.

[164] Y. Chen, H. Li, M. Li et al., "*Salvia miltiorrhiza* polysaccharide activates T lymphocytes of cancer patients through activation of TLRs mediated -MAPK and -NF-κB signaling pathways," *Journal of Ethnopharmacology*, vol. 200, pp. 165–173, 2017.

[165] S. Frantz, G. Ertl, and J. Bauersachs, "Mechanisms of disease: Toll-like receptors in cardiovascular disease," *Nature Clinical Practice Cardiovascular Medicine*, vol. 4, no. 8, pp. 444–454, 2007.

[166] X. Guo, M. Xue, C. J. Li et al., "Protective effects of triptolide on TLR4 mediated autoimmune and inflammatory response induced myocardial fibrosis in diabetic cardiomyopathy," *Journal of Ethnopharmacology*, vol. 193, pp. 333–344, 2016.

[167] D. M. Adamczak, "The role of toll-like receptors and vitamin d in cardiovascular diseases—a review," *International Journal of Molecular Sciences*, vol. 18, no. 11, p. 2252, 2017.

[168] H. S. Hardarson, J. S. Baker, Z. Yang et al., "Toll-like receptor 3 is an essential component of the innate stress response in virus-induced cardiac injury," *American Journal of Physiology Heart and Circulatory Physiology*, vol. 292, no. 1, pp. H251–H258, 2007.

[169] C. Gorbea, K. A. Makar, M. Pauschinger et al., "A role for Toll-like receptor 3 variants in host susceptibility to enteroviral myocarditis and dilated cardiomyopathy," *Journal of Biological Chemistry*, vol. 285, no. 30, pp. 23208–23223, 2010.

[170] C. Gorbea, M. Rechsteiner, J. G. Vallejo, and N. E. Bowles, "Depletion of the 26S proteasome adaptor Ecm29 increases Toll-like receptor 3 signaling," *Science Signaling*, vol. 6, no. 295, article ra86, 2013.

[171] Y. Yang, J. Lv, S. Jiang et al., "The emerging role of Toll-like receptor 4 in myocardial inflammation," *Cell Death & Disease*, vol. 7, no. 5, article e2234, 2016.

[172] L. Zuliani-Alvarez, A. M. Marzeda, C. Deligne et al., "Mapping tenascin-C interaction with toll-like receptor 4 reveals a new subset of endogenous inflammatory triggers," *Nature Communications*, vol. 8, no. 1, p. 1595, 2017.

[173] J. Yang, R. Zhang, X. Jiang et al., "Toll-like receptor 4–induced ryanodine receptor 2 oxidation and sarcoplasmic reticulum Ca^{2+} leakage promote cardiac contractile dysfunction in sepsis," *Journal of Biological Chemistry*, vol. 293, no. 3, pp. 794–807, 2017.

[174] M. E. Lopes Pires, S. R. Clarke, S. Marcondes, and J. M. Gibbins, "Lipopolysaccharide potentiates platelet responses via toll-like receptor 4-stimulated Akt-Erk-PLA_2 signalling," *PLoS One*, vol. 12, no. 11, article e0186981, 2017.

[175] J. C. Jang, J. Li, L. Gambini et al., "Human resistin protects against endotoxic shock by blocking LPS–TLR4 interaction," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 114, no. 48, pp. E10399–E10408, 2017.

[176] F. X. Zhang and R. S. Xu, "Juglanin ameliorates LPS-induced neuroinflammation in animal models of Parkinson's disease and cell culture via inactivating TLR4/NF-κB pathway," *Biomedicine & Pharmacotherapy*, vol. 97, pp. 1011–1019, 2018.

[177] F. Cochet and F. Peri, "The role of carbohydrates in the lipopolysaccharide (LPS)/toll-like receptor 4 (TLR4) signalling," *International Journal of Molecular Sciences*, vol. 18, no. 11, p. 2318, 2017.

[178] D. Fairweather, S. Yusung, S. Frisancho et al., "IL-12 receptor β1 and toll-like receptor 4 increase IL-1β- and IL-18-associated myocarditis and coxsackievirus replication," *The Journal of Immunology*, vol. 170, no. 9, pp. 4731–4737, 2003.

[179] K. Fuse, G. Chan, Y. Liu et al., "Myeloid differentiation factor-88 plays a crucial role in the pathogenesis of Coxsackievirus B3–induced myocarditis and influences type I interferon production," *Circulation*, vol. 112, no. 15, pp. 2276–2285, 2005.

[180] D. J. Marchant and B. M. McManus, "Regulating viral myocarditis: allografted regulatory T cells decrease immune infiltration and viral load," *Circulation*, vol. 121, no. 24, pp. 2609–2611, 2010.

[181] Y. Shi, M. Fukuoka, G. Li et al., "Regulatory T cells protect mice against coxsackievirus-induced myocarditis through the transforming growth factor β–coxsackie-adenovirus receptor pathway," *Circulation*, vol. 121, no. 24, pp. 2624–2634, 2010.

[182] T. S. Nielsen, J. Hansen, L. P. Nielsen, U. T. Baandrup, and J. Banner, "The presence of enterovirus, adenovirus, and parvovirus B19 in myocardial tissue samples from autopsies: an evaluation of their frequencies in deceased individuals with myocarditis and in non-inflamed control hearts," *Forensic Science, Medicine, and Pathology*, vol. 10, no. 3, pp. 344–350, 2014.

[183] S. A. Huber, "Viral myocarditis and dilated cardiomyopathy: etiology and pathogenesis," *Current Pharmaceutical Design*, vol. 22, no. 4, pp. 408–426, 2016.

[184] G. Sinagra, M. Anzini, N. L. Pereira et al., "Myocarditis in clinical practice," *Mayo Clinic Proceedings*, vol. 91, no. 9, pp. 1256–1266, 2016.

[185] J. Krejci, D. Mlejnek, D. Sochorova, and P. Nemec, "Inflammatory cardiomyopathy: a current view on the pathophysiology, diagnosis, and treatment," *BioMed Research International*, vol. 2016, Article ID 4087632, 11 pages, 2016.

[186] B. H. Trachtenberg and J. M. Hare, "Inflammatory cardiomyopathic syndromes," *Circulation Research*, vol. 121, no. 7, pp. 803–818, 2017.

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Research Article

How to Distinguish Patients with pSS among Individuals with Dryness without Invasive Diagnostic Studies

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In the course of pSS, inflammatory cell infiltration consists mainly of lymphocytes infiltrating exocrine glands, which leads to their impaired function. The characteristic feature is generalized dryness. The aim of this study was to attempt to answer the question whether it is possible to distinguish between patients with pSS and individuals with dryness caused by other pathologies without applying invasive studies. The study included 68 patients with pSS and 43 healthy controls with dryness. $FS \geq 1$ was observed in 90% of patients with pSS (with or without dryness), and only in 23% of the control group (only with xerostomia). In the pSS group, anaemia ($p = 0.0085$), lymphocytopenia ($p = 0.0006$), elevated ERS ($p = 0.001$), higher RF titer, and ANA antibodies were noted. Configuration of anti-SSA + SSB + Ro52 antibodies was characteristic for the pSS group. Considering the clinical symptoms, statistically significant differences were noted between pSS patients and the control group in frequency ($p = 0.02$) and severity ($p = 0.042$) of fatigue, lymphadenopathy, major salivary gland involvement, and photosensitivity to UV light. In conclusion, invasive methods are pivotal in pSS diagnosis in this salivary gland biopsy. Chronic fatigue syndrome is more common in pSS patients and can be subjective distinguishing factor in the group of people with dryness.

1. Introduction

In the course of primary Sjögren's syndrome (pSS), inflammatory cell infiltration consists mainly of lymphocytes infiltrating exocrine glands, which leads to their impaired function. The characteristic feature is generalized dryness [1]. Inflammation may involve many organs evoking clinical symptoms depending on the exact location [2]. The disease develops slowly, and months can pass before the patient presents full spectrum of clinical symptoms. Insufficient treatment without inhibiting the autoimmune response leads to severe complications. The aim of this study was to attempt to answer the question whether it is possible to distinguish between patients with pSS and individuals with dryness caused by other pathologies without applying invasive studies.

2. Materials and Methods

The study included 68 patients (66 females and 2 males) diagnosed with pSS based on 2002 American-European Consensus Classification Criteria for pSS [3] after obtaining their informed consent (Bioethics Committee number 357/2010), who received medical care in the Department of Rheumatology and Internal Medicine between the years 2010 and 2013. Retrospectively, all patients met current (2016) classification criteria [4], which were unavailable at the time of the study.

The control group consisted of 43 individuals (5 males and 38 females), who had been observed and diagnosed for pSS due to dryness but finally were not diagnosed neither with pSS nor with any other rheumatic disorder.

The exclusion criteria encompassed the following: other autoimmune disease (e.g., rheumatoid arthritis, systemic

lupus erythematosus, and systemic scleroderma), hepatitis C virus (HCV) and human immunodeficiency virus (HIV) infection, sarcoidosis, history of lymphoma, amyloidosis, hyperlipoproteinemia type V, graft-versus-host disease (GVHD), eosinophilia myalgia syndrome, history of head and neck irradiation, psychiatric and hypnotic drugs, uncontrolled hypertension, and uncontrolled diabetes mellitus.

We analyzed factors such as age, smoking, severity of dryness according to the EULAR Sjogren's Syndrome Patient Reported Index (ESSPRI) [5], fatigue assessed using the visual analogue scale (VAS) (0–10 cm) [6], intensity of inflammation in labial glands (LSB) [7] assessed by focus score (FS), organ pathology assessed using the EULAR Sjögren's syndrome disease activity index (ESSDAI) [8], laboratory tests such as anti-nuclear antibodies (ANA), extractable nuclear antigens (ENA), rheumatoid factor (RF), inflammatory parameters (erythrocyte sedimentation rate (ESR), C-reactive protein (CRP)), full blood count, and protein electrophoresis.

3. Statistical Analysis

For calculations, we used STATISTICA v. 9.0 software as well as Excel spreadsheet. In statistical analysis, we used Spearman's correlation rank coefficient (for paired variables with nonnormal distribution), Pearson's correlation coefficient (for paired variables with normal distribution), and linear regression. Mann–Whitney U test was used to verify differences between means for variables with normal nondistribution or nonhomogenous variances. Student t-test was used for differences between means for variables with normal distribution. Independence of quantitative and qualitative variables was using either nonparametric chi-squared or Fisher's test. All tests were conducted at the significance level of $\alpha = 0.05$, $p < 0.05$.

4. Results

Mean age of patients with pSS was 51.2 (19–82) years. Mean time since the onset of symptoms to diagnosis was 7.5 years.

Mean age of healthy individuals was 51.1 (23–73) years. In the control group, total exclusion of connective tissue disorders since the onset of symptoms was 5.4 years.

4.1. Patients with pSS versus Control Group. In the studied group, mean severity of inflammation evaluated on pathology examination of LSB in FS was 2.2 in patients with pSS and 0.3 in the control group. $FS \geq 1$ was observed in 90% of patients with pSS (with or without dryness), and only in 23% of the control group (only with xerostomia). Intensity of inflammatory infiltration expressed by the FS was much higher in patients with pSS ($p < 0.00001$, chi-squared test) (Table 1).

As far as laboratory test results are concerned, statistically significant differences (Mann–Whitney U test) between patients with pSS and the control group were noted in anaemia rate ($p = 0.0085$), lymphocytopenia ($p = 0.0006$), ERS level ($p = 0.001$), and in RF titer ($p < 0.00001$). No correlation between CRP level ($p = 0.61$) in pSS patients and the control group was found (13% versus 2%, resp.) (Table 1).

ANA antibodies were not found in 79% of healthy participants and in 19% of pSS patients. Statistical analysis (chi-squared test) showed that ANA antibodies are more common in pSS patients compared to the control group, and the difference was statistically significant ($p < 0.00001$).

Antibodies most commonly detected in pSS were anti-SSA (82% of patients), anti-Ro52 (70% of patients), and anti-SSB (69% of patients). Configuration of all three specific antibodies anti-SSA + SSB + Ro52 was the most common (among 54% of patients), while anti-SSA + SSB combination was observed in 15% of patients and anti-SSA + Ro52 in 13% of patients. None of the pSS patients was positive for anti-SSA or anti-SSB antibodies only, without any other specific antibodies (Table 2).

4.2. Clinical Symptoms in pSS Patients versus Control Group. Mean age of pSS patients at the onset of symptoms was 51 ± 14.39 years (minimum 12 and maximum 71). In the control group, mean age of participants at the onset of dryness was 42 ± 11.42 (minimum 20 and maximum 72) years.

Mean time between pSS diagnosis and first clinical symptoms was 7.5 years (SD 7.5). Mean time needed for exclusion of pSS in the control group was 5.4 years (SD 4.4).

Considering severity of xerophthalmia, xerostomia, and vaginal dryness, as well as subjective sensation of fatigue, statistically significant differences (Mann–Whitney U test) were noted between pSS patients and the control group in frequency ($p = 0.02$) and severity ($p = 0.042$) of fatigue. Female patients with pSS reported vaginal dryness more often ($p = 0.002$); however, the intensity of this symptom was comparable in both groups. pSS patients more frequently reported more intensive fatigue affecting daily activity (5.46 cm on VAS scale on average). Mean severity scores for dryness symptoms were as follows: xerophthalmia 4.47 cm for the pSS group and 4.28 cm in the control group ($p = 0.96$), xerostomia 4.76 cm for the pSS group and 3.77 cm in the control group ($p = 0.12$), and vaginal dryness in women: 3.03 cm for the pSS group and 2.82 cm in the control group ($p = 0.64$). Differences in severity of dryness symptoms between pSS patients and the control group were not observed.

Table 3 presents frequency of clinical symptoms in patients with pSS and in the control group. Physical examination and history taking revealed that skin lesions, peripheral joint swelling, as well as major salivary gland involvement (examined physically or by diagnostic ultrasound), swollen lymph nodes, and UV-light photosensitivity were statistically more common in patients with pSS.

Skin lesions were in 19 (30%) patients with pSS observed as erythema, urticaria, purpura on lower extremities, livedo reticularis, erythematous, and exfoliative lesions. Skin lesions in the control group never took the form of purpura, but rather erythema only, and were observed significantly less often than in pSS group (19 versus 2 patients). Mean age in pSS population with skin changes was 47 (SD 14) years, while

Table 1: Values of laboratory test results, immunological markers, and severity of dryness and fatigue in patients with pSS and in the control group.

Study group	Number of patients/% pSS	Number of patients/% Control	Minimum pSS	Minimum Control	Maximum pSS	Maximum Control	p value
ANA > 1 : 320 n/%	55/81%	9/21%	1 : 320	1 : 320	1 : 10000	1 : 320	<0.000
CRP mean value	2.38 ± 3.71	2.13 ± 2.84	0.19	0.00	24.38	18.30	
CRP > 5 mg/dl n/%	9/13%	1/2%					0.047
Focus-score LSB mean value	2.22 ± 1.35	0.37 ± 0.93	0.00	0.00	4.00	4.00	
FS ≥ 1 n/%	61/90%	10/23%					<0.001
Hemoglobin level mean value	12.99 ± 1.78	13.52 ± 1.23	10.40	11.00	24.80	16.50	
Anaemia n/%	15/22%	3/7%					0.029
WBC mean value	5.43 ± 2.09	5.99 ± 1.61	1.95	3.73	14.40	10.10	
WBC < 4 tys. n/%	19/28%	1/2%					0.0003
Lymphocyte mean value	1.58 ± 1.16	1.79 ± 0.46	0.48	0.70	9.99	2.70	
Lymphopenia n/%	39/57%	6/14%					<0.001
Oral dryness mean value	4.76 ± 2.99	3.77 ± 2.93	0.00	0.00	10.00	10.00	0.12
>0 in VAS n/%	60/88%	32/74%					0.05
Dry eye mean value	4.47 ± 2.69	4.28 ± 2.70	0.00	0.00	10.00	8.00	0.96
>0 in VAS n/%	62/91%	36/84%					0.18
Vaginal dryness mean value	3.03 ± 2.73	2.82 ± 2.94	0.00	0.00	9.00	9.00	0.64
>0 in VAS n/%	41/62%	11/29%					<0.000
Fatigue mean value	5.46 ± 2.37	3.84 ± 2.65	0.00	0.00	10.00	8.00	0.04
>0 in VAS n/%	67/98%	35/81%					0.002
ESR mean value	31.51 ± 24.77	16.02 ± 11.62	6.00	1.00	103.00	53.00	0.001
ESR > 20 mm/hr n/%	39/57%	10/23%					0.0004
RF mean value	79.32 ± 128.67	12.31 ± 25.94	0.00	0.00	801.00	171.80	
RF > 14 IU/ml n/%	50/73%	7/16%					<0.000

n: number of patients; normal value: ANA: antinuclear antibodies < 1 : 320 (EUROIMMUN Hep-20-10/liver Monkey set); CRP: C-reactive protein < 5 mg/dl; LSB: labial salivary gland biopsy; haemoglobin: 12–16 g/dl in women, 14–18 g/dl in men; WBC: white blood cells 4–10 k/μl; lymphocytes: 1.5–3.5 k/μl (in complete peripheral blood count); VAS: visual analogue scale (0–10 cm); ESR: erythrocyte sedimentation rate 3–15 mm/h; RF: 0–14 IU/ml.

Table 2: Detailed distribution of anti-SSA, anti-SSB, and anti-Ro52 specific antibodies and their titers (luminous intensity 0–3) in pSS patients.

Anti-SSA—number of patients/%	Anti-SSB—number of patients/%	Anti-Ro52—number of patients/%
Titer 3+—54 patients/79%	Titer 3+—37 patients/54%	Titer 3+—44 patients/65%
Titer 2+—one patient	Titer 2+—6 patients/9%	Titer 2+—0 patients
Titer 1+—one patient	Titer 1+—3 patients	Titer 1+—3 patients
	Titer 0.5+—one patient	Titer 0.5+—one patient
Only anti-SSA—0 patients	Only anti-SSB—0 patients	Only anti-Ro52—3 patients/4%
anti-SSA + SSB—10 patients/15%	anti-SSA + SSB—10 patients/15%	anti-SSA + Ro52—9 patients/13%
anti-SSA + Ro52—9 patients/13%	anti-SSB + Ro52—0 patients	anti-SSB + Ro52—0 patients
anti-SSA + SSB + Ro52—37 patients/54%	anti-SSA + SSB + Ro52—37 patients/54%	anti-SSA + SSB + Ro52—37 patients/54%

For ANA testing, the EUROIMMUN Hep-20-10/liver monkey set was used. For determination of antigen specificity of the anti-nuclear antibodies, Anti-Ena Profile Plus 1 Euroline immunoblotting set was used.

the mean time needed for final diagnosis of pSS was 11 years (SD 10); patients without skin lesions tended to be older (mean age of 53, SD 13; $p = 0.1$), and the time needed for the final diagnosis of pSS was shorter (6 years on average, SD 4). No correlation between skin lesions and CRP level ($p = 0.9$), ESR ($p = 0.1$), FS ($p = 0.05$), leukocyte count ($p = 0.1$), RF titer ($p = 0.3$), gamma globulin level ($p = 0.06$), severity of xerophthalmia ($p = 0.3$), and xerostomia ($p = 0.9$), as well as the presence of anti-SSA, anti-SSB, and anti-Ro52 specific antibodies, was observed. Among pSS patients with hypergammaglobulinemia, skin lesions were more common (66% versus 33%) and the difference was statistically significant.

Table 3: Clinical features in pSS patients and in the control group.

Clinical presentation	Primary Sjögren's syndrome (number of patients/%)	Control group (number of patients/%)
Arthralgia	48/70%	28/65%
Arthritis*	20/30%	2/5%
Large salivary gland involvement*	33/48%	4/9%
Raynaud's syndrome	8/12%	2/5%
Bone marrow infiltration	1/1%	0
Alopecia	2/3%	0
Muscle soreness	9/13%	2/5%
Hearing loss	6/9%	3/7%
Abnormal chest HRCT	18/26%	Not performed
Gastrointestinal	7/10%	2/5%
Polyneuropathy	7/10%	1/2%
Peripheral lymphadenopathy*	15/22%	0
UV-light photosensitivity*	7/10%	0

*Statistically significant. Bone marrow infiltration was defined as an abnormal percentage of plasmocytes on bone marrow biopsy (>3.5% plasmocytes). Gastrointestinal involvement included pancreatitis, enlarged lymph nodes on imaging, hepatomegaly and/or splenomegaly, diarrhea (watery stools, >200 g/d, >3 stools daily), weight loss, and nonspecific abdominal pain. Insomnia: difficulty of falling asleep or early waking up lasting for more than 2 weeks; peripheral lymphadenopathy: swollen lymph nodes > 1 cm on physical examination except for inguinal lymph nodes, where the cut-off size is >2 cm.

The chest HRCT scan revealed changes in the lung tissue in the course of 29% of all examined patients with pSS. The most common lesions were fibrosis, enlarged mediastinal lymph nodes, and nodes which was previously reported in other publication [9]. Chest high-resolution computed tomography (HRCT) was not performed in the control group due to the lack of indications for this type of diagnostic study (no pulmonary involvement).

4.3. Lymphadenopathy. Peripheral lymphadenopathy was reported in 22% of pSS patients. Lymphadenopathy occurred more frequently among younger patients ($p = 0.04$) (Figure 1) (mean age of 45 years SD 18 versus 53 years SD 12). Time to final diagnosis of pSS in patients with lymphadenopathy was 9 years on average (SD 6 years), while it took 7 years (SD 8) without that symptom.

No correlation between lymphadenopathy and CRP level ($p = 0.5$), ESR ($p = 0.2$), FS ($p = 0.2$), and leukocyte count ($p = 0.5$) was found. In patients with lymphadenopathy, RF titer was higher and the difference was statistically significant ($p = 0.003$). Mean RF titer in patients with lymphadenopathy was 110 IU/ml, while in patients who did not present that symptom was 70 IU/ml.

Among patients with pSS and lymphadenopathy, elevated level of gamma globulins (2.1 g/dl, SD 0.8) was noted,

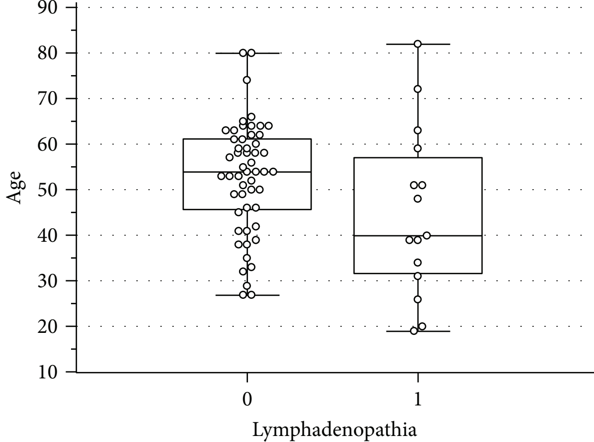


Figure 1: Correlation between the age of pSS patients and lymphadenopathy.

compared to pSS patients without lymphadenopathy, and that symptom was more prevalent in patients with hypergammaglobulinemia ($p = 0.0004$), the difference being statistically significant (Figure 2).

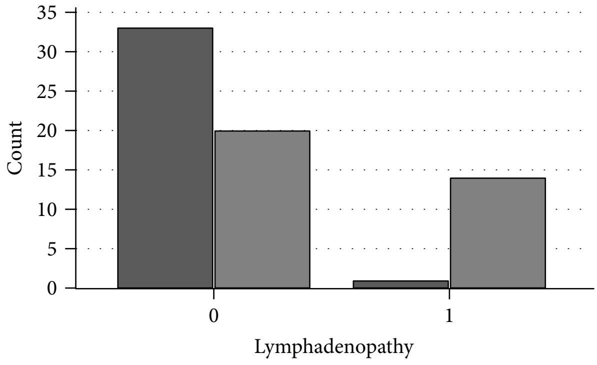
In patients with lymphadenopathy, high titer of anti-SSA specific antibodies (3.0 versus 2.2; $p = 0.02$) was observed compared to patients who did not present this symptom (Figure 2).

All pSS patients with lymph node enlargement tested positive for specific anti-SSA antibodies, while the results were positive in 77% of patients without this symptom; however, the difference was not statistically significant ($p = 0.05$).

No correlation between the presence or titer of anti-SSB specific antibodies and lymphadenopathy was observed ($p = 0.06$; $p = 0.1$).

4.4. Musculoskeletal Involvement. Arthralgia was reported by 47% of patients at the onset of the disease and by 70% during the entire course of observation. In the control group, 65% reported arthralgia. Arthritis was observed in 30% of pSS patients (in 15% at the onset of the disease) and in 5% of healthy participants. Pain sensation mostly affected minor joints of the hand and knees. Inflammation was located within minor joints of the hand and ankles. Also, muscle pain was more prevalent in pSS patients compared to the control group (13% versus 5%, resp.).

Joint swelling was observed in 30% of pSS patients (20 patients). The mean age of patients with joint swelling was 47 (SD 13) years, and the mean time required for final diagnosis of pSS was 9 years (SD 9). In patients with no musculoskeletal involvement, the mean age was 52 (SD 14) years and the mean time required for final diagnosis of pSS was 7 years (SD 7). No correlation was found between inflammation of the joints and age ($p = 0.1$), CRP level ($p = 0.6$), ESR ($p = 0.6$), FS ($p = 0.4$), leukocyte count ($p = 0.7$), RF titer ($p = 0.8$), gammaglobulin level ($p = 0.6$), severity of xerophthalmia ($p = 0.6$), and xerostomia ($p = 0.2$) as well as the presence of anti-SSA ($p = 0.8$), anti-SSB ($p = 0.1$), and anti-Ro52 ($p = 0.2$) antibodies.



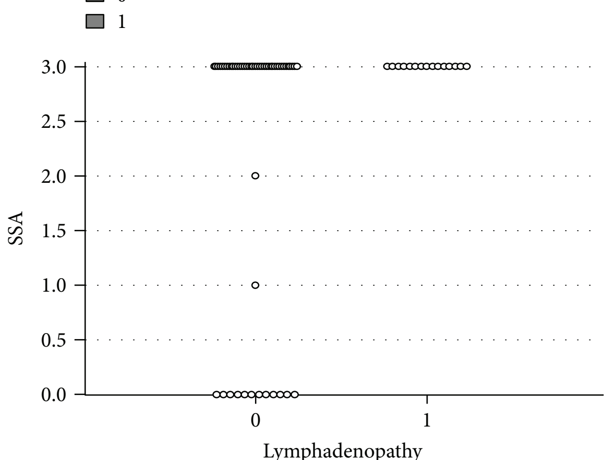


Figure 2: Lymphadenopathy rate in patients with hypergammaglobulinemia and its correlation with anti-SSA antibodies.

4.5. Time Required for Final Diagnosis of pSS. The shortest time required to make the final diagnosis of pSS was observed in the case of pulmonary involvement (7 years SD 6), and it was the longest for skin lesions (11 years SD 10). Distribution of time to final diagnosis depending on symptoms in pSS patients is shown in Table 4.

5. Discussion

In clinical practice, most patients with pSS report dryness. However, symptoms are not specific for a single disease. On the other hand, dryness observed in pSS may develop after months, while other symptoms of pSS can constitute primary manifestation, such as musculoskeletal and neurological symptoms or lymphomas. Due to this variety of clinical presentation, the time needed for the right diagnosis is longer and takes about 3 to 8 years since the onset of symptoms [10]. In the studied group, the time was 7.5 years on average (92 months) and was the shortest in the case of pulmonary involvement (7 years, SD 6 years) and the longest for skin presentation (11 years, SD 10 years). However, in individuals with rheumatologic disorders excluded, the time needed to exclude autoimmune basis of dryness was 5.4 years on average.

Table 4: Distribution of time to final diagnosis of pSS depending on symptoms.

Clinical presentation	Time required for final diagnosis of pSS	Standard deviation (SD)
Abnormal chest HRCT	7	6
Major salivary gland involvement	10	9
Lymphadenopathy	9	6
Skin lesions	11	10
Peripheral arthritis	9	9

Xerophthalmia and xerostomia in both pSS patients and the control group occurred at a similar rate (79% versus 79% and 81% versus 77% accordingly). As the primary symptom, pSS patients reported xeropthalmia (54%), while healthy individuals reported xerostomia (49%). The results were similar to those reported by other authors, who summarized the frequency of each symptom in pSS [3, 11]. Due to the fact that dryness can be caused by many factors and disorders, diagnosis of pSS is not possible solely based on the presence and severity of dryness. On the one hand, patients are observed for other diseases and they tend to ignore dryness symptoms. On the other hand, elderly patients reporting peripheral arthralgia and dryness are difficult to diagnose. In that group, more patients report dryness, which is often caused by physiology and hormonal disturbances [12].

Considering severity of xerophthalmia, xerostomia, and vaginal dryness as well as subjective feeling of excessive fatigue, there was a significant difference in intensity and rate of fatigue between pSS patients and healthy individuals. Patients with pSS complained about more pronounced fatigue impairing their daily activity compared to the control group. However, no difference in severity of dryness was observed between patients and healthy individuals, except for vaginal dryness in females, which was more frequent in pSS patients. Nevertheless, its severity (VAS scale) was comparable to healthy individuals. Therefore, based solely on dryness, it is impossible to distinguish between symptoms of pSS and other causes in an outpatient setting.

In etiopathogenesis of fatigue in pSS, psychological factors, autoimmune response, hormonal dysfunction, cytokines, and viral infections can all play an important role [13]. Ramos-Casals et al. showed that excessive fatigue relating to pSS occurs in 70–80% of patients and often interferes with work [14]. In the studied group, the rate of fatigue was even higher reaching over 90%, which indicates necessity to use fatigue-assessing clinical scales (e.g., FACID and VAS) in everyday rheumatologic assessment of pSS patients. No relationship between severity of fatigue reported by patients and laboratory test results or lymphocytic infiltration in labial glands was found. Other less specific general symptoms in pSS and related to fatigue included sleep disturbances, anxiety, and depression; the frequency of such symptoms in pSS accounts to 15%, 20%, and 40%, respectively [14].

Moreover, in about 50% of pSS patients, musculoskeletal pain can be observed [14, 15]. In our study, musculoskeletal pain was split into muscle and peripheral joint pain. It turned out that muscle pain is far less common compared to joint pain in both pSS patients and the control group. None of the symptoms was pathognomonic for the studied group. Thus, it is incorrect to state that every person with dryness and musculoskeletal symptoms must suffer from pSS. It is worth noting that a substantial percentage of individuals reported arthralgia despite exclusion of connective tissue disease and rheumatoid arthritis after full diagnostic work-up. It makes diagnosis of pSS more difficult, being one of the common but noncharacteristic symptoms.

Similar conclusions were drawn by Hackett et al. in their study. They showed the influence of pSS on daily activity in patients compared to healthy individuals with the same age and sex. It turned out that pSS patients complained about pain more frequently compared to healthy individuals ($p < 0.0001$) in a statistically significant manner; they also more often reported depression ($p < 0.0001$). Also, lower Health Assessment Questionnaire (HAQ scores) were noted ($p = 0.002$) [16].

In our study, musculoskeletal symptoms were observed at a considerable frequency in pSS patients, among whom 47% complained about peripheral arthralgia at the beginning of the disease and 70% during the entire observation period. In the control group, pain was commonly observed as well. The participants differed as to the rate of peripheral joint inflammation, which was observed in 30% of pSS patients (including 15% at the onset of the disease). Among healthy individuals, widened joint contours were observed only in 5% of participants and were associated with osteoarthritis. Pain sensation in pSS patients affected mostly minor joints of the hand and knees. Inflammation involved mostly minor joints of the hand and ankles. No correlation between peripheral joint involvement and hypergammaglobulinemia, intensity of inflammation assessed by focus score, and, interestingly, RF titer was found. It should be noted that RF itself is a relatively common occurrence found in 40–70% of pSS patients [17]. In our study, RF was present in 70% of pSS patients. RF was statistically less frequent in healthy individuals. Therefore, in differential diagnosis of musculoskeletal pain and suspicion of pSS, RF testing should be carried out routinely.

Moreover, based on a history taking and physical examination, features distinguishing individuals with dryness and pSS patients include skin lesions typical for pSS, photosensitivity to UV light, large salivary gland involvement, and peripheral lymphadenopathy (excluding infections and hematological disorders). Special attention should be given to those symptoms in diagnosis of pSS, despite the fact that not all of them (UV-light photosensitivity) are included in the disease activity scales (ESSDAI). Peripheral lymphadenopathy alone is statistically more frequent in younger individuals, and it correlates with anti-SSA antibodies and hypergammaglobulinemia.

Among invasive diagnostic studies, specific ENA antibodies and infiltration assessment on FS according to Fisher's protocol [6] remain characteristic and irreplaceable, and so they have been included in all classification criteria for pSS so far [3, 4, 18]. In the studied group, anti-SSA antibodies proved to be characteristic for pSS, and it was more commonly found than SSB antigen. It has been confirmed by results published by Baer et al. [19] and explains why anti-SSB antibodies have not been included in new current classification criteria for pSS [18]. However, the combination of anti-SSA, anti-SSB, and anti-Ro52 antibodies in the same patient increases probability of pSS diagnosis as shown in our study. Interestingly, the diagnosis rate of pSS was lower in patients, and in some cases, anti-Ro52 were not found. None of the participants was found with both anti-SSA and anti-SSB antibodies without coexisting other specific antibodies. It might be related to a low number of the studied group; nevertheless, it requires further analysis on a larger cohort. It should be remembered that specific antibodies may be predicting factors of developing pSS later [20].

Being unable to distinguish pSS patients from individuals reporting dryness solely based on xerostomia with coexisting risk factors of pSS (skin lesions, swollen salivary glands, peripheral lymphadenopathy, photosensitivity to UV light, and fatigue), it is necessary to perform LSB with pathology assessment according to pSS protocol [6]. As shown in our study, which confirmed the results of other studies from recent years [21], it is the key examination in this group of patients. Despite its invasiveness, the procedure itself is simple and lasts about 20 minutes including patient preparation; it does not require suturing and is associated with low risk of complications. Positive result narrows down the differential diagnosis and is rarely found in healthy individuals [22]. Therefore, pathology assessment of minor salivary glands has been included in classification criteria for pSS for years and plays a crucial role [3, 4].

As shown in our study, diagnosis of pSS is prolonged by a few years, 7.5 on average. The least time required for final diagnosis of pSS was observed in the case of pulmonary involvement, and it was the longest for skin lesions. Similarly, individuals with dryness symptoms were observed for 5 years on average before pSS was excluded as the cause of the symptoms. It may be due to the fact that clinical symptoms of pSS develop with time. The course of the disease is long-standing and insidious, noncharacteristic for this disease only, and dryness itself is quite common in general population. Symptoms of pSS usually develop gradually, which results in kind of becoming accustomed to, for example, dryness; physicians diagnose other diseases even in the advanced form of pSS. Thus, pSS patients are referred to many specialists before visiting rheumatologist after months, who can either exclude or confirm pSS only after invasive studies (ANA/ENA antibodies, biopsy, and LSB), which remain the gold standard.

Conflicts of Interest

This research was performed as part of the employment of the authors, Wroclaw Medical University, Department of Rheumatology and Internal Medicine, Borowska 213, 50-556 Wroclaw, Poland.

References

[1] F. Ferro, R. Vagelli, C. Bruni et al., "One year in review 2016: Sjögren's syndrome," *Clinical and Experimental Rheumatology*, vol. 34, no. 2, pp. 161–171, 2016.

[2] C. Baldini, P. Pepe, L. Quartuccio et al., "Primary Sjögren's syndrome as a multi-organ disease: impact of the serological profile on the clinical presentation of the disease in a large cohort of Italian patients," *Rheumatology*, vol. 53, no. 5, pp. 839–844, 2014.

[3] C. Vitali, S. Bombardieri, R. Jonsson et al., "Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group," *Annals of the Rheumatic Diseases*, vol. 61, no. 6, pp. 554–558, 2002.

[4] C. H. Shiboski, S. C. Shiboski, R. Seror et al., "2016 American College of Rheumatology/European League Against Rheumatism classification criteria for primary Sjögren's syndrome. A consensus and data-driven methodology involving three international patient cohorts," *Annals of the Rheumatic Diseases*, vol. 76, no. 1, pp. 9–16, 2017.

[5] R. Seror, E. Theander, J. G. Brun et al., "Validation of EULAR primary Sjögren's syndrome disease activity (ESSDAI) and patient indexes (ESSPRI)," *Annals of the Rheumatic Diseases*, vol. 74, no. 5, pp. 859–866, 2015.

[6] M. E. Cline, J. Herman, E. R. Shaw, and R. D. Morton, "Standardization of the visual analogue scale," *Nursing Research*, vol. 41, no. 6, pp. 378–380, 1992.

[7] B. A. Fisher, R. Jonsson, T. Daniels et al., "Standardisation of labial salivary gland histopathology in clinical trials in primary Sjögren's syndrome," *Annals of the Rheumatic Diseases*, vol. 76, no. 7, pp. 1161–1168, 2017.

[8] R. Seror, S. J. Bowman, P. Brito-Zeron et al., "EULAR Sjögren's syndrome disease activity index (ESSDAI): a user guide," *RMD Open*, vol. 1, no. 1, article e000022, 2015.

[9] A. Sebastian, M. Misterska-Skóra, J. Silicki, M. Sebastian, and P. Wiland, "Chest HRCT findings in patients with primary Sjögren's syndrome," *Advances in Clinical and Experimental Medicine*, vol. 26, no. 7, pp. 1101–1106, 2017.

[10] S. Khattri and P. Barland, "Primary Sjogren's syndrome and autoimmune cytopenias: a relation often overlooked," *Bulletin of the NYU Hospital for Joint Diseases*, vol. 70, no. 2, pp. 130–132, 2012.

[11] H.-J. Haga, C. Gram Gjesdal, L. M. Irgens, and M. Östensen, "Reproduction and gynaecological manifestations in women with primary Sjögren's syndrome: a case-control study," *Scandinavian Journal of Rheumatology*, vol. 34, no. 1, pp. 45–48, 2005.

[12] J. F. Williamson, K. Huynh, M. A. Weaver, and R. M. Davis, "Perceptions of dry eye disease management in current clinical practice," *Eye & Contact Lens*, vol. 40, no. 2, pp. 111–115, 2014.

[13] Y. Z. Gong, J. Nititham, K. Taylor et al., "Differentiation of follicular helper T cells by salivary gland epithelial cells in primary Sjögren's syndrome," *Journal of Autoimmunity*, vol. 51, pp. 57–66, 2014.

[14] M. Ramos-Casals, P. Brito-Zeron, A. Siso-Almirall, and X. Bosch, "Primary Sjögren syndrome," *BMJ*, vol. 344, article e3821, 2012.

[15] C. Vitali and N. Del Papa, "Pain in primary Sjögren's syndrome," *Best Practice & Research Clinical Rheumatology*, vol. 29, no. 1, pp. 63–70, 2015.

[16] K. L. Hackett, J. L. Newton, J. Frith et al., "Impaired functional status in primary Sjögren's syndrome," *Arthritis Care & Research*, vol. 64, no. 11, pp. 1760–1764, 2012.

[17] V. K. Bournia and P. G. Vlachoyiannopoulos, "Subgroups of Sjögren syndrome patients according to serological profiles," *Journal of Autoimmunity*, vol. 39, no. 1-2, pp. 15–26, 2012.

[18] S. C. Shiboski, C. H. Shiboski, L. A. Criswell et al., "American College of Rheumatology classification criteria for Sjögren's syndrome: a data-driven, expert consensus approach in the Sjögren's International Collaborative Clinical Alliance cohort," *Arthritis Care & Research*, vol. 64, no. 4, pp. 475–487, 2012.

[19] A. N. Baer, M. McAdams DeMarco, S. C. Shiboski et al., "The SSB-positive/SSA-negative antibody profile is not associated with key phenotypic features of Sjögren's syndrome," *Annals of the Rheumatic Diseases*, vol. 74, no. 8, pp. 1557–1561, 2015.

[20] R. Jonsson, E. Theander, B. Sjöström, K. Brokstad, and G. Henriksson, "Autoantibodies present before symptom onset in primary Sjögren syndrome," *JAMA*, vol. 310, no. 17, pp. 1854-1855, 2013.

[21] K. M. Leehan, N. P. Pezant, A. Rasmussen et al., "Minor salivary gland fibrosis in Sjögren's syndrome is elevated, associated with focus score and not solely a consequence of aging," *Clinical and Experimental Rheumatology*, 2017.

[22] R. Manthorpe, C. Benoni, L. Jacobsson et al., "Lower frequency of focal lip sialadenitis (focus score) in smoking patients. Can tobacco diminish the salivary gland involvement as judged by histological examination and anti-SSA/Ro and anti-SSB/La antibodies in Sjögren's syndrome?," *Annals of the Rheumatic Diseases*, vol. 59, no. 1, pp. 54–60, 2000.

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Research Article

The Role of Monocyte/Macrophage and CXCR3 in Differentiation between Recurrent Hepatitis C and Acute Cellular Rejection Postliver Transplantation

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Objective. Liver transplantation (LT) is the recommended treatment for patients with advanced liver disease and cirrhosis in all guidelines, mostly as a complication of HCV. The distinction between reinfection of the graft with HCV and acute cellular rejection (ACR) is essential because they are managed differently. Hepatic macrophages, which can either arise from circulating blood-derived monocytes (BDM) or from resident tissue Kupffer cells, are central in the pathogenesis of chronic liver injury. The aim of this work was to evaluate whether the origin of macrophages and the immune mediator CXCR3 could help in differentiating between acute recurrent HCV and ACR after liver transplantation. *Methods*. Twenty-nine cases of recurrent hepatitis C and 26 cases of ACR were included in this study. The expression of CD 68 (macrophage marker), CD11b (BDM marker), and CxCR3 in the postliver transplant biopsy using immunohistochemistry was determined. *Results*. CD11b expression highlighting macrophages of BDM origin was in favor of recurrent hepatitis C ($P < 0.001$) than in ACR ($P = 0.44$), while CXCR3 expression by hepatocytes was in favor of ACR ($P = 0.001$). *Conclusion*. Macrophage infiltrating liver tissue post LT can distinguish between ACR by upregulation of CXCR3 and recurrent hepatitis C by predominant CD11b.

1. Introduction

Chronic hepatitis C virus infection (HCV) is a major cause of end-stage liver disease that has been increasingly the important indication for liver transplantation (LT) globally. HCV reinfection of the graft occurs almost universally, leading to graft injury in the majority of patients and cirrhosis in 8–44% in 5–7 years after reinfection [1]. The viral load may be influenced by corticosteroid intake [2], and the histologic features of recurrent hepatitis C may be modified by immunosuppressive therapy, which harden its differentiation from acute cellular rejection (ACR) [3]. Allograft failure is the most common cause of death and retransplantation among those recipients [4–7].

ACR is encountered in 18%–30% of transplanted patients leading to allograft failure [8, 9]. The differential diagnosis between recurrent HCV and ACR is often difficult due to the same clinical picture, and laboratory abnormalities detected in both diseases, and even similar histological features [10]. Moreover, low interobserver and intraobserver agreement rates were found among experienced liver transplant pathologists for the histopathologic differentiation of recurrent hepatitis C from ACR [3].

IFN-free direct-acting antiviral agents (DAAs) have improved tolerability and can potentially be used in post-transplant setting, which should result in better outcomes [11]. However, incorrect diagnosis may be detrimental, as failure to increase immunosuppression in patients with

ACR may lead to acceleration of rejection, and inappropriate treatment of suspected acute rejection with high-dose pulse steroid therapy in misdiagnosed recurrent HCV can lead to aggravation of the disease, graft loss, and poor survival; hence, accurate diagnosis remains a critical issue [8, 12].

Macrophages hold a fundamental role in regulating inflammatory processes [13]. In particular, hepatic macrophages have the main role in the pathogenesis of acute and chronic liver injury through a wide range of different functions in the liver. The liver has about 80% of all body macrophages as local resident self-renewing macrophages, termed Kupffer cells. Blood monocytes can infiltrate into the liver; however, under steady-state conditions, blood monocyte-derived macrophages (BDM) do not contribute to the pool of local resident macrophages in the liver [14]. Previous work demonstrated that a reduction in number and function of circulating monocytes are strongly correlated with activation of systemic anti-inflammatory responses [15]. Macrophages in the liver can be distinguished based on their origin and certain marker expression. CD68 (cluster of differentiation 68) is a glycoprotein which binds low-density lipoprotein and is expressed on monocytes/macrophages [16]. Yang et al. [17] demonstrated that early activation of macrophages as a result of graft injury might play an important role in the accelerated ACR.

However, patients with HCV infection may have a significant increase in CD68+ expression in their portal tracts compared with normal tissue [18].

CXCL10 is well known in hepatitis C as a hepatocyte-derived chemotactic ligand and initiator of inflammatory cascades via its cognate receptor C-X-C motif receptor 3 (CXCR3). It is widely expressed on multiple cells of the innate immune system, including hepatic Kupffer cells, dendritic cells, natural killer (NK) cell, and neutrophils. Hence, these entire different innate immune cells are potential targets for CXCL10-mediated chemotaxis. [19]. Treatment with a CXCL11-neutralizing antibody reduced the number of CXCR3+ cells in the skin allograft and prolonged graft survival [20].

Till now, no precise marker for diagnosing ACR or recurrent HCV is currently available. This study aimed to determine the role of CD68, CD 11b, and CXCR3, as markers of resident Kupffer cells and BDM, in the differentiation between HCV reinfection and ACR in the postliver transplant setting using immunohistochemistry.

2. Methods

2.1. Specimens' Selection. This retrospective study was conducted on liver biopsies from 55 patients who had living donor liver transplantation (LDLT) for chronic HCV complications, whether cirrhosis and/or HCC, and who had developed elevated liver enzymes 6 months following transplantation. Twenty-nine patients developed recurrent HCV, and 26 patients were diagnosed as ACR based on histopathological examination. Laboratory investigation and histological criteria established the diagnosis which was confirmed by good response to treatment. Serological and clinical data were collected from the patients' files. The study was approved by the National Liver Institute Institutional Review Board.

Paraffin-embedded blocks of those liver biopsies were retrieved from the archive of the Pathology Department, National Liver Institute, Menoufia University, in the period between 2015 and 2017. Baseline characteristics including donor and recipient age and gender, pretransplantation HCC status, liver function tests, HCV-RNA level, and MELD score were determined.

2.2. Histopathological Evaluation. Serial liver sections in four micrometer thickness were cut from each paraffin-embedded block, for hematoxylin and eosin (H&E) staining and immunostaining. H&E staining was used for evaluation of histopathological changes including determination of the following parameters:

(i) Extent of infiltrate and the degree of portal inflammation identified by mononuclear infiltration of portal tracts

(ii) Presence of interface hepatitis, spotty necrosis, confluent necrosis, steatosis, and cholestasis

(iii) Presence or absence of fibrosis

(iv) Presence of bile duct injury, venous endothelial injury, hepatic artery injury, or perivenular necrosis

(v) The nature and number of portal tract infiltrate: plasma cells, eosinophils, neutrophils, macrophages, and immunoblast cells

2.3. Immunohistochemistry. Immunohistochemistry was carried out for all tissues mentioned in the study. After deparaffinization and rehydration, hydrogen peroxide was applied to block nonspecific background staining. Heat-induced antigen retrieval was performed using citrate buffer solution low pH (pH 6) for CD11b and CX3CR1 antibodies and high pH (pH 9) for CD68. They are anti-human antibodies that arose in animals and recognize CD68 antigens on human macrophages [21]. Antigen retrieval solution was performed in a vegetable steamer for 20 minutes at 97°C followed by incubation for an additional 20 minutes in the warm buffer. All antibodies were incubated overnight at 4°C. Sections were incubated with a monoclonal mouse CD68 (clone KP1,0, DAKO A/S, Glostrup, Denmark, dilution 1 : 50), a rabbit polyclonal primary anti CD11 b (Novus Biologicals, Littleton, CO, USA, dilution 1 : 50), or a 1 : 200 dilution of a rabbit polyclonal primary anti-CX3CR1 antibody (Novus Biologicals, Littleton, CO, USA). Detection of the immunostaining was carried out utilizing the EnVisionTM FLEX/HRP detection system (DAKO A/S, Glostrup, Denmark) with the 3-diaminobenzidine (DAKO) as chromogen. After counterstaining with Mayer's hematoxylin, the slides were independently assessed by two pathologists for detection of each antibody.

Human lymph node (stains sinusoids) was positive tissue control for CD68; benign prostatic hyperplasia was positive tissue control for CD11 and human heart tissue for CX3CR1.

Negative tissue controls were included in the protocol of staining by omitting the primary antibodies.

2.4. Interpretation of CD68, CD11b, and CXCR3. The immunoreactivity for CD68 was identified as membranous brownish discoloration of macrophages. The positive cells were quantified in three portal tracts and adjacent hepatic parenchyma per case (liver core). The positive cases were further divided according to the median number of CD68 positive cells into high expression (>40%) and low expression (<40%). The immunoreactivity for CD11b was assigned when cytoplasmic brownish discoloration was seen in mononuclear inflammatory cells. The positive cells were quantified in three portal tracts, interface, and adjacent hepatic parenchyma. The percentage of positivity was evaluated and expressed as range, mean, and median. The cases were divided into low expression when up to 30% of hepatocytes were positive and high expression when >30% of hepatocytes showed immunoreactivity. The immunoreactivity for CXCR3 was identified as cytoplasmic brownish discoloration of mononuclear inflammatory cells. The positive cells were quantified in three portal tracts and adjacent parenchyma per case (liver core). The positive cases were further divided according to the median number of CXCR3 positive cells into high expression (>20%) and low expression (<20%).

2.5. Statistical Analysis. Qualitative data was expressed in number and percentages, and quantitative data was expressed as mean and standard deviation. Fisher exact and chi-square tests were used to study the association between two qualitative variables. *t*-test was used for comparison between two quantitative variables. A P value of <0.05 was considered statistically significant.

3. Results

The baseline clinical, laboratory data of the studied patients are presented in Table 1. No significant difference was observed between the two groups regarding recipient age, gender, MELD score, and presence of HCC before transplantation ($P = 0.11$, 0.87, 0.57, and 0.54, resp.).

Histopathological features of recurrent chronic hepatitis C (CHC) and ACR are demonstrated in Table 2.

3.1. CD68 Expression. CD68 was detected in the inflammatory infiltrate in all cases of recurrent hepatitis C and ACR and was localized in portal tracts and adjacent parenchyma. The number of macrophages identified by CD68 immunostaining ranged from 10 to 60 in both groups. In recurrent hepatitis C, the mean ± SD was 32.3 ± 17.5 and a median of 30.0, while in cases of ACR, the mean ± SD was 37.7 ± 12.4 and a median of 40.0 without detectable significant difference between the two groups ($P = 0.21$) as shown in Table 3. Twelve cases (46.2%) of recurrent hepatitis C showed high expression of CD68 in comparison to 17 patients (65.4%) in ACR.

3.2. CD11b Expression. CD11b expression was detected among the mononuclear inflammatory infiltrate in portal areas, interface, or hepatic parenchyma. In recurrent hepatitis C, the number of macrophages identified by CD11b immunostaining ranged from 10 to 60; the mean ± SD was 26.5 ± 17.2 and the median was 30.0, while in ACR, ranging from 10 to 50, the mean ± SD was 17.3 ± 12.5 and a median of 10.0, with a significant difference detected between the two groups ($P = 0.03$). High expression of CD11b (≥30%) was found in 14 (53.8%) and 5 cases (19.2%) of recurrent HCV and ACR, respectively ($P = 0.01$).

Table 1: Demographic and laboratory characteristics of patients with recurrent chronic hepatitis C (CHC) and acute cellular rejection (ACR).

Parameters	Recurrent CHC ($n = 29$) %	ACR ($n = 26$)%
Recipient age (years)	47.9 ± 5.7	42.2 ± 13.4
Gender of recipient (M/F) n, %	24(82.7)/5(17.3)	26(100)/0(0)
Pretreatment HCC n, %	8 (27.6)	7 (26.9)
MELD score	15.8 ± 2.6	15.4 ± 2.0
Total bilirubin (mg/dL)	4.11 ± 5.0	5.1 ± 2.9
AST (IU/L)	127.1 ± 65.1	198.8 ± 164.1
ALT (IU/L)	165.9 ± 111.9	269.7 ± 257.6
GGT (IU/L)	721.7 ± 1041.4	1054.3 ± 1232.2
ALP (IU/L)	371.6 ± 211.3	400.9 ± 267
Serum albumin (gm/dL)	4.22 ± 0.33	3.97 ± 0.49

AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma glutamyl transferase; ALP: alkaline phosphatase.

3.3. CXCR3 Expression. CXCR3 expression was shown among the mononuclear inflammatory infiltrate in portal tracts, interface, or hepatic parenchyma. A significant difference between the two groups was detected ($P < 0.001$). The number of macrophages identified by CXCR3 immunostaining in recurrent hepatitis C ranged from 0 to 30; the mean ± SD was 6.2 ± 8.5 and the median was 1.0. While in ACR, it ranged from 0 to 40; the mean ± SD was 20.4 ± 10.4 and the median was 20.0. High expression of CXCR3 (≥20%) was found in 2 (7.7%) versus 18 (69.2%) cases of recurrent HCV and ACR, respectively ($P < 0.001$). Figure 1 demonstrated immunohistochemical staining of CD68, CD11b, and CXCR3 in recurrent hepatitis C (Figures 1(a), 1(c), and 1(e)) and acute cellular rejection (Figures 1(b), 1(d), and 1(f)).

Subgroup analysis was performed according to presence or absence of HCC and revealed the same trend, although CD11b expression was not statistically significant between both groups (Tables 4 and 5).

Histological criteria established the diagnosis which was confirmed by good response to treatment in 94% of cases. Three cases were confusing and reassessment of the biopsy or even rebiopsy was mandatory. They are misdiagnosed as recurrent HCV posttransplantation, however, during follow-up, one patient was discovered to have lymphoproliferative malignancy and improved upon receiving systemic chemotherapy. The other two patients were found to have hyperacute and chronic rejection that unfortunately did not respond to increasing dose of immunosuppressive drugs

Table 2: Histopathological characteristics of patients with recurrent chronic hepatitis C (CHC) and acute cellular rejection (ACR).

Parameters	Recurrent CHC ($n = 29$) %	ACR ($n = 26$) %
Extent of infiltrate *n*, %		
I	7 (24.13)	10 (38.5)
II	17 (58.62)	14 (53.8)
III	5 (17.24)	2 (7.7)
Spotty necrosis (absent/present) *n*, %	0 (0)/29 (100)	1 (3.8)/25 (96.2)
Confluent necrosis (absent/present) *n*, %	5 (17.24)/24 (82.76)	23 (88.5)/3 (11.5)
Perivenular necrosis (absent/present) *n*, %	20 (68.96)/9 (31.04)	8 (30.8)/18 (69.2)
Fibrosis (absent/present) *n*, %	9 (31.04)/20 (68.96)	18 (69.2)/8 (30.8)
Cholestasis (absent/present) *n*, %	23 (79.3)/6 (20.7)	17 (65.4)/9 (34.6)
Steatosis (absent/present) *n*, %	4 (13.8)/25 (86.2)	22 (84.6)/4 (15.4)
Bile duct injury (absent/present) *n*, %	16 (55.2)/13 (44.8)	3 (11.5)/23 (88.5)
Vascular injury (absent/present) *n*, %	28 (96.5)/1 (3.5)	4 (15.4)/22 (84.6)

Table 3: Comparison between recurrent chronic hepatitis C (CHC) and acute cellular rejection (ACR) regarding the studied markers (CD68, CD11b, and CXCR3).

Marker	Recurrent CHC	ACR	*t*-test	*P* value
CD68				0.21
Mean ± SD	32.3 ± 17.5	37.7 ± 12.4	−1.27	
Median	30.0	40.0		
CD11b				0.03
Mean ± SD	26.5 ± 17.2	17.3 ± 12.5	2.21	
Median	30.0	10.0		
CXCR3				0.001
Mean ± SD	6.2 ± 8.5	20.4 ± 10.4	−5.40	
Median	10.0	20.0		

and died. Another two patients diagnosed with ACR also died due to sepsis.

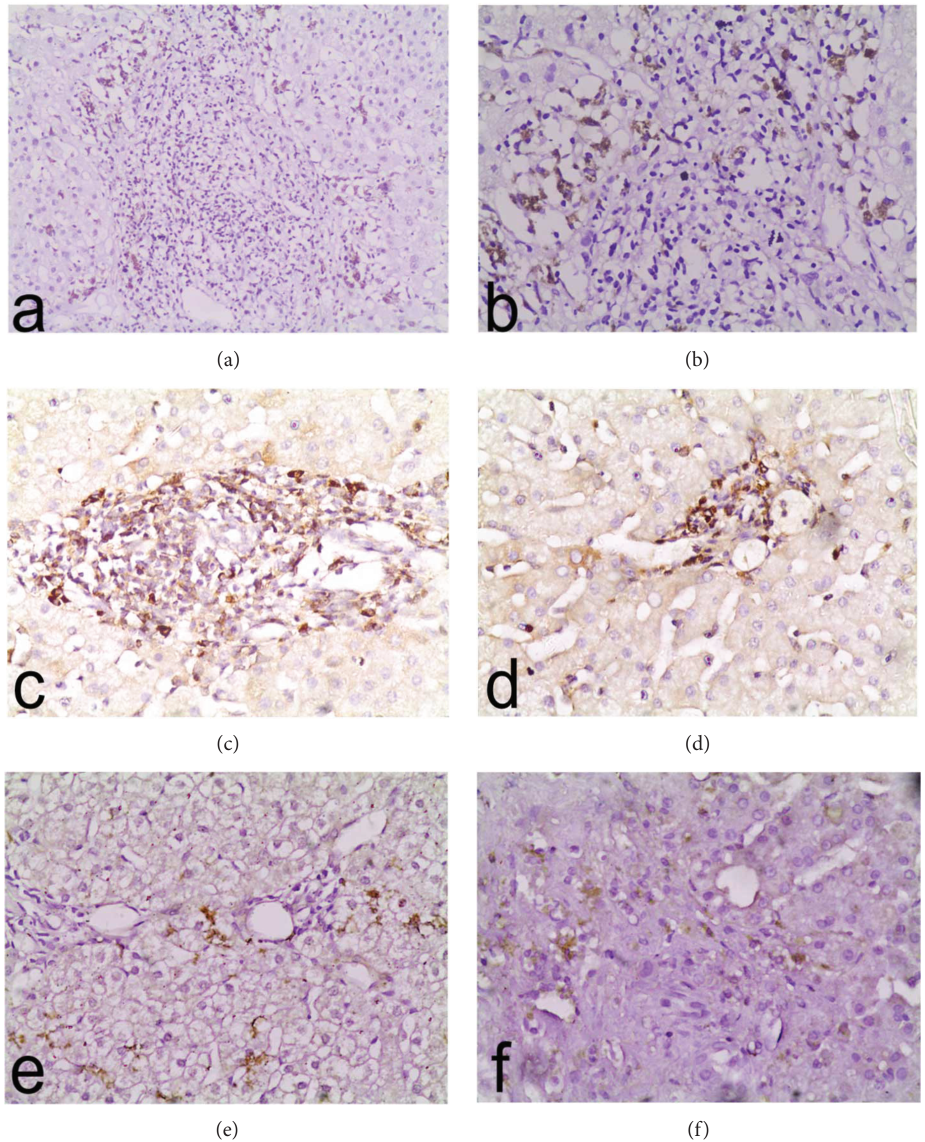
4. Discussion

Recurrent hepatitis C is characterized by the presence of lobular inflammation, apoptotic bodies, spotty necrosis, and lobular disarray, with portal lymphocyte predominance, while, in acute cellular rejection mixed portal/periportal inflammation composed of lymphocytes, plasma cells, and eosinophils, lymphocytic cholangitis and endothelialitis were observed. However, the histological diagnosis of HCV infection in the transplant setting may be altered, putting in mind that detectable serum HCV ribonucleic acid (RNA) after LT, even at a high level, does not necessarily indicate the presence of histologic recurrent hepatitis C [3]. Immunosuppression may alter the histological appearance of viral hepatitis post-transplant especially during the first few months; hence, classic histopathologic features of hepatitis C may be absent or modified. Moreover, ACR, ischemic injury, biliary obstruction, cytomegalovirus infection, or drug toxicity may superimpose chronic hepatitis [3]. Therefore, it is a challenge for the hepatopathologist to differentiate these overlapping microscopic features in some cases.

The present study aimed to determine the usefulness of macrophages' origin in the differential diagnosis of acute rejection and recurrent HCV after LT.

Macrophages have an important role in both recurrent hepatitis C and ACR posttransplantation. About 80% of all body macrophages reside in the liver and are furthermore patrolled by blood monocytes [22]. The circulating blood monocytes can principally infiltrate the liver and give rise to monocyte-derived macrophages, but this is characteristic to liver injury [23]. Liver macrophages have a wide range of functional heterogeneity; they may be pathogenic or even beneficial and they have been classified either into "proinflammatory" M1 or "immunoregulatory" M2 macrophages. Macrophages play a key role in acute and chronic liver inflammation and regression of liver disease. Upon injury to the liver, macrophages often perform immediate multiple functions including cytokine and chemokine secretion, leukocyte adhesion, phagocytosis, angiogenesis control, and extracellular matrix remodelling [24]. Although Kupffer cells can protect the transplanted liver, rejection of allografts was found also promoted by macrophages due to their antigen-presenting and cytokine-releasing function [24]. In cases of ACR, selective targeting and destruction of donor parenchymal cells occur through complement activation and the resultant membrane attack complex is responsible for lysis of the donor cells. Another pathway for the destruction of donor parenchymal cells in ACR is via antibody-dependent cellular cytotoxicity, involving other immune cell mediators, such as NK cells, macrophages (CD68-positive cells), and neutrophils [25].

CD68 has been proposed as an indicator for Kupffer cells [26] and is used to distinguish Kupffer cells from monocyte-derived macrophages [23, 27]. However, no single marker is currently able to definitely discriminate these populations. CD68 is a specific marker for the various cells of the macrophage lineage, including monocytes, Kupffer cells, histiocytes, giant cells, and osteoclasts. In this study, CD68 was expressed in all cases of recurrent hepatitis C and ACR, indicating the presence of macrophage infiltration of portal tracts in both groups. There was no significant difference in the number of macrophages highlighted by CD68 between cases



(a) (b) (c) (d) (e) (f)

Figure 1: Immunohistochemistry of liver tissue postliver transplantation in recurrent hepatitis C (a, c, and e) and acute cellular rejection (b, d, and f) showing immunoreaction for CD68 (a and b), CD11b (c and d), and CXCR3 (e and f). Original magnification ×200.

Table 4: Comparison between recurrent chronic hepatitis C (CHC) and acute cellular rejection (ACR) regarding the studied markers (CD68, CD11b, and CXCR3) in absence of HCC.

Marker (mean ± SD)	Recurrent CHC	ACR	t-test	P value
CD68	33.5 ± 17.3	36.7 ± 11.7	−0.59	0.56
CD11b	26.5 ± 15.8	18.6 ± 15.5	1.40	0.17
CXCR3	7.6 ± 9.7	18.0 ± 10.8	−2.85	0.008

Table 5: Comparison between recurrent chronic hepatitis C (CHC) and acute cellular rejection (ACR) regarding the studied markers (CD68, CD11b, and CXCR3) in presence of HCC.

Marker (mean ± SD)	Recurrent CHC	ACR	t-test	P value
CD68	30.0 ± 18.7	38.6 ± 15.7	−0.97	0.35
CD11b	26.6 ± 20.6	14.3 ± 5.3	1.53	0.15
CXCR3	3.3 ± 5.0	24.3 ± 11.3	−4.99	<0.001

of recurrent hepatitis C or ACR ($P = 0.21$). In accordance with this finding, CD68+ macrophages were found in the infiltrate of hepatic lobules in acute liver allograft rejection and the number of infiltrating cells correlated with the severity of the ACR in a previous report [28]. Also, the number of CD14+CD68+ Kupffer cells is increased in patients with viral hepatitis in another study [29]. In addition, CD68-positive monocytes were the main inflammatory cell-infiltrating renal graft in cases of ACR [30].

Monocyte-derived (freshly infiltrating) macrophages are characterized as CD11b$^+$ F4/80$^+$ cells by FACS in mice, whereas matured monocyte-derived and resident Kupffer

cells are CD11b^{lo} F4/80^{hi} [31]. The number of CD11b(+), F4/80(+), CD11c(−), and CD206(+) (M2) macrophages in the liver of HCV transgenic mice was notably increased compared to control mice. These M2 macrophages in the liver produced elevated levels of IL-6 and TNF-α. These results suggested that inflammatory cytokines produced by M2-like macrophages contribute to the induction of chronic liver inflammation in HCV transgenic mice [32]. In agreement with these results, the present study demonstrated that the CD11b expression was in favor of recurrent hepatitis C compared with ACR ($P = 0.03$).

A massive necrosis of hepatocytes can provoke a strong inflammatory immune response within the liver [33] leading to secretion of diverse proinflammatory chemokines and cytokines, including interferon (IFN)-γ by liver-resident and -infiltrating immune cells [34] which perpetuate liver cell damage. IFN-γ strongly activates the transcription of the chemokines CXCL9, CXCL10, and CXCL11 [35, 36]. CXCR3, one of the peripheral blood monocyte surface markers, is the receptor for CXCL9, CXCL10, and CXCL11 chemokines which is expressed on various cell subpopulations within the liver, including liver endothelial cells, stellate cells, T cells, NK cells, and NKT cells [37, 38]. The interaction between these three chemokines and their receptor mediates the recruitment of T, NK, and NKT cells into the liver and their attachment to endothelial cells [39–41].

Our results clearly show great differences in CXCR3 expression between both groups. CXCR3 expression was significantly higher in ACR than recurrent HCV.

In conclusion, CD68 was expressed in both recurrent HCV infection and ACR. A significantly stronger CD11b deposits in liver biopsies of patients' suffering from recurrent HCV was detected. On the other hand, CXCR3 was a marker and plays a considerable role in acute rejection following liver transplantation suggesting the involvement of humoral mechanisms in ACR. Using immunohistochemistry beside clinical, laboratory, and histopathological criteria in discrimination between recurrent HCV and ACR may improve the diagnostic ability, morbidity, and mortality of these patients.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

References

[1] N. A. Terrault, "Treatment of recurrent hepatitis C in liver transplant recipients," *Clinical Gastroenterology and Hepatology*, vol. 3, no. 10, Supplement 2, pp. S125–S131, 2005.

[2] R. B. Guerrero, K. P. Batts, L. J. Burgart et al., "Early detection of hepatitis C allograft reinfection after orthotopic liver transplantation: a molecular and histologic study," *Modern Pathology*, vol. 13, no. 3, pp. 229–237, 2000.

[3] A. Regev, E. Molina, R. Moura et al., "Reliability of histopathologic assessment for the differentiation of recurrent hepatitis C from acute rejection after liver transplantation," *Liver Transplantation*, vol. 10, no. 10, pp. 1233–1239, 2004.

[4] E. Osman, E. Gamal, A. Gad, E. Soliman, E. A. Mohammad, and A. Hatia, "Factors affecting recurrence of hepatitis C virus in adult living donor liver transplantation among Egyptian patients," *Macedonian Journal of Medical Sciences*, vol. 4, no. 1, pp. 64–69, 2011.

[5] A. J. Demetris, "Evolution of hepatitis C virus in liver allografts," *Liver Transplantation*, vol. 15, Supplement S2, pp. S35–S41, 2009.

[6] B. Roche and D. Samuel, "Risk factors for hepatitis C recurrence after liver transplantation," *Journal of Viral Hepatitis*, vol. 14, no. s1, pp. 89–96, 2007.

[7] H. Reis, J. Wohlschläger, S. Hagemann et al., "(Cleaved) CK18 serum and tissue expression levels differentiate acute HCV reinfection from acute rejection in liver allografts," *Liver International*, vol. 35, no. 3, pp. 905–913, 2015.

[8] S. J. Knechtle and J. Kwun, "Unique aspects of rejection and tolerance in liver transplantation," *Seminars in Liver Disease*, vol. 29, no. 1, pp. 91–101, 2009.

[9] A. Shaked, R. M. Ghobrial, R. M. Merion et al., "Incidence and severity of acute cellular rejection in recipients undergoing adult living donor or deceased donor liver transplantation," *American Journal of Transplantation*, vol. 9, no. 2, pp. 301–308, 2009.

[10] M. Schmeding, A. Dankof, V. Krenn et al., "C4d in acute rejection after liver transplantation—a valuable tool in differential diagnosis to hepatitis C recurrence," *American Journal of Transplantation*, vol. 6, no. 3, pp. 523–530, 2006.

[11] D. Joshi, M. Pinzani, I. Carey, and K. Agarwal, "Recurrent HCV after liver transplantation–mechanisms, assessment and therapy," *Nature Reviews Gastroenterology & Hepatology*, vol. 11, no. 12, pp. 710–721, 2014.

[12] J. R. Burton Jr and H. R. Rosen, "Acute rejection in HCV-infected liver transplant recipients: the great conundrum," *Liver Transplantation*, vol. 12, no. S2, Supplement S2, pp. S38–S47, 2006.

[13] C. Varol, A. Mildner, and S. Jung, "Macrophages: development and tissue specialization," *Annual Review of Immunology*, vol. 33, no. 1, pp. 643–675, 2015.

[14] F. Heymann and F. Tacke, "Immunology in the liver – from homeostasis to disease," *Nature Reviews Gastroenterology & Hepatology*, vol. 13, no. 2, pp. 88–110, 2016.

[15] Z. X. Liu, S. Govindarajan, and N. Kaplowitz, "Innate immune system plays a critical role in determining the progression and severity of acetaminophen hepatotoxicity," *Gastroenterology*, vol. 127, no. 6, pp. 1760–1774, 2004.

[16] J. A. Harris, S. Jain, Q. Ren, A. Zarineh, C. Liu, and S. Ibrahim, "CD163 versus CD68 in tumor associated macrophages of classical Hodgkin lymphoma," *Diagnostic Pathology*, vol. 7, no. 1, pp. 12–19, 2012.

[17] Z.-F. Yang, D. W.-Y. Ho, A. C.-Y. Chu, Y.-Q. Wang, and S.-T. Fan, "Linking inflammation to acute rejection in small-for-size liver allografts: the potential role of early macrophage activation," *American Journal of Transplantation*, vol. 4, no. 2, pp. 196–209, 2004.

[18] P. H. McGuinness, D. Painter, S. Davies, and G. W. McCaughan, "Increases in intrahepatic CD68 positive cells, MAC387 positive cells, and proinflammatory cytokines (particularly interleukin 18) in chronic hepatitis C infection," *Gut*, vol. 46, no. 2, pp. 260–269, 2000.

[19] J. Brownell and S. J. Polyak, "Molecular pathways: hepatitis C virus, CXCL10, and the inflammatory road to liver cancer," *Clinical Cancer Research*, vol. 19, no. 6, pp. 1347–1352, 2013.

[20] B. Li, W. Xu, L. Xu et al., "I-TAC is a dominant chemokine in controlling skin intragraft inflammation via recruiting

CXCR3$^+$ cells into the graft," *Cellular Immunology*, vol. 260, no. 2, pp. 83–91, 2010.

[21] D. Sirena, B. Lilienfeld, M. Eisenhut et al., "The human membrane cofactor CD46 is a receptor for species B adenovirus serotype 3," *Journal of Virology*, vol. 78, no. 9, pp. 4454–4462, 2004.

[22] A. G. Abdou, N. Y. Asaad, N. Ehsan, S. Younes, A. I. Gomaa, and W. Elgendy, "The diagnostic impact of C4d, CD68, and NF-κB expression in the differentiation between recurrent hepatitis C and acute cellular rejection after liver transplantation," *Applied Immunohistochemistry & Molecular Morphology*, vol. 24, no. 9, pp. 639–647, 2016.

[23] Y. Lavin, D. Winter, R. Blecher-Gonen et al., "Tissue-resident macrophage enhancer landscapes are shaped by the local microenvironment," *Cell*, vol. 159, no. 6, pp. 1312–1326, 2014.

[24] K. R. Karlmark, R. Weiskirchen, H. W. Zimmermann et al., "Hepatic recruitment of the inflammatory Gr1$^+$ monocyte subset upon liver injury promotes hepatic fibrosis," *Hepatology*, vol. 50, no. 1, pp. 261–274, 2009.

[25] F. Heymann, J. Peusquens, I. Ludwig-Portugall et al., "Liver inflammation abrogates immunological tolerance induced by Kupffer cells," *Hepatology*, vol. 62, no. 1, pp. 279–291, 2015.

[26] P. I. Terasaki and J. Cai, "Humoral theory of transplantation: further evidence," *Current Opinion in Immunology*, vol. 17, no. 5, pp. 541–545, 2005.

[27] C. G. Antoniades, A. Quaglia, L. S. Taams et al., "Source and characterization of hepatic macrophages in acetaminophen-induced acute liver failure in humans," *Hepatology*, vol. 56, no. 2, pp. 735–746, 2012.

[28] S. Epelman, K. J. Lavine, and G. J. Randolph, "Origin and functions of tissue macrophages," *Immunity*, vol. 41, no. 1, pp. 21–35, 2014.

[29] T. Sawada, A. Shimizu, K. Kubota, S. Fuchinoue, and S. Teraoka, "Lobular damage caused by cellular and humoral immunity in liver allograft rejection," *Clinical Transplantation*, vol. 19, no. 1, pp. 110–114, 2005.

[30] N. G. Sandler, C. Koh, A. Roque et al., "Host response to translocated microbial products predicts outcomes of patients with HBV or HCV infection," *Gastroenterology*, vol. 141, no. 4, pp. 1220–1230.e3, 2011.

[31] P. L. Zhang, S. K. Malek, T. M. Blasick et al., "C4d positivity is often associated with acute cellular rejection in renal transplant biopsies following Campath-1H (alemtuzumab) induction," *Annals of Clinical & Laboratory Science*, vol. 37, no. 2, pp. 121–126, 2007.

[32] T. Ohtsuki, K. Kimura, Y. Tokunaga et al., "M2 macrophages play critical roles in progression of inflammatory liver disease in hepatitis C virus transgenic mice," *Journal of Virology*, vol. 90, no. 1, pp. 300–307, 2015.

[33] Z. Wu, M. Han, T. Chen, W. Yan, and Q. Ning, "Acute liver failure: mechanisms of immune-mediated liver injury," *Liver International*, vol. 30, no. 6, pp. 782–794, 2010.

[34] A. Bendelac, P. B. Savage, and L. Teyton, "The biology of NKT cells," *Annual Review of Immunology*, vol. 25, no. 1, pp. 297–336, 2007.

[35] D. Rossi and A. Zlotnik, "The biology of chemokines and their receptors," *Annual Review of Immunology*, vol. 18, no. 1, pp. 217–242, 2000.

[36] D. Jiang, J. Liang, J. Hodge et al., "Regulation of pulmonary fibrosis by chemokine receptor CXCR3," *The Journal of Clinical Investigation*, vol. 114, no. 2, pp. 291–299, 2004.

[37] C. H. Kim, B. Johnston, and E. C. Butcher, "Trafficking machinery of NKT cells: shared and differential chemokine receptor expression among Vα24$^+$Vβ11$^+$ NKT cell subsets with distinct cytokine-producing capacity," *Blood*, vol. 100, no. 1, pp. 11–16, 2002.

[38] S. Lacotte, S. Brun, S. Muller, and H. Dumortier, "CXCR3, inflammation, and autoimmune diseases," *Annals of the New York Academy of Sciences*, vol. 1173, no. 1, pp. 310–317, 2009.

[39] A. Bonacchi, P. Romagnani, R. G. Romanelli et al., "Signal transduction by the chemokine receptor CXCR3: activation of Ras/ERK, Src, and phosphatidylinositol 3-kinase/Akt controls cell migration and proliferation in human vascular pericytes," *Journal of Biological Chemistry*, vol. 276, no. 13, pp. 9945–9954, 2001.

[40] B. Johnston, C. H. Kim, D. Soler, M. Emoto, and E. C. Butcher, "Differential chemokine responses and homing patterns of murine TCR$\alpha\beta$ NKT cell subsets," *The Journal of Immunology*, vol. 171, no. 6, pp. 2960–2969, 2003.

[41] S. M. Curbishley, B. Eksteen, R. P. Gladue, P. Lalor, and D. H. Adams, "CXCR3 activation promotes lymphocyte transendothelial migration across human hepatic endothelium under fluid flow," *The American Journal of Pathology*, vol. 167, no. 3, pp. 887–899, 2005.

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Research Article

Gene Expression Profiling in Behcet's Disease Indicates an Autoimmune Component in the Pathogenesis of the Disease and Opens New Avenues for Targeted Therapy

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Behçet disease (BD) is a chronic inflammatory multisystem disease characterized by oral and genital ulcers, uveitis, and skin lesions. Disease etiopathogenesis is still unclear. We aim to elucidate some aspects of BD pathogenesis and to identify specific gene signatures in peripheral blood cells (PBCs) of patients with active disease using novel gene expression and network analysis. 179 genes were modulated in 10 PBCs of BD patients when compared to 10 healthy donors. Among differentially expressed genes the top enriched gene function was immune response, characterized by upregulation of Th17-related genes and type I interferon- (IFN-) inducible genes. Th17 polarization was confirmed by FACS analysis. The transcriptome identified gene classes (vascular damage, blood coagulation, and inflammation) involved in the pathogenesis of the typical features of BD. Following network analysis, the resulting interactome showed 5 highly connected regions (clusters) enriched in T and B cell activation pathways and 2 clusters enriched in type I IFN, JAK/STAT, and TLR signaling pathways, all implicated in autoimmune diseases. We report here the first combined analysis of the transcriptome and interactome in PBCs of BD patients in the active stage of disease. This approach generates useful insights in disease pathogenesis and suggests an autoimmune component in the origin of BD.

1. Introduction

Behçet disease (BD) is a chronic multisystem disease mainly characterized by mucous-cutaneous lesions such as oral and genital ulcers, erythema nodosum-like lesions, and papulopustular lesions, and by uveitis. Moreover, manifestations of vascular, articular, neurologic, urogenital, gastrointestinal, pulmonary, and cardiac involvement may occur.

BD was first described by Hulusi Behçet in 1937 as a trisymptom complex represented by recurrent aphthous stomatitis, genital ulcers, and uveitis. The diagnosis of the disease is still based on clinical criteria since universally accepted pathognomonic laboratory tests are lacking. An international study group on Behcet's disease has recently revised the criteria for classification/diagnosis of BD [1].

There are sporadic cases of BD all around the world, but it is most frequently seen along the ancient Silk Route, with a prevalence of 14–20/100,000 inhabitants. According to epidemiological studies, the disease is most prevalent in countries located between 30 and 45° north latitude through the Mediterranean Basin, the Middle East and Far East regions such as China and Japan [2].

The interaction between a complex genetic background and both innate and adaptive immune systems leads to the clinical features of the disease. The presence of familiar cases in 10% of the patients, the particular geographic distribution

and the high frequency of HLA-B51, a split antigen of HLA-B5, among a wide range of ethnic populations favours the role of genetic factors in the pathogenesis of the disease, but it remains poorly understood [3]. Non-HLA genes also contribute to the development of BD [3]. Genome-wide association studies have shown that polymorphisms in genes encoding for cytokines, activator factors, and chemokines are associated with increased BD susceptibility. Among cytokines, IL-10 polymorphisms cause a reduction of the serum level of IL-10, an inhibitory cytokine that regulates innate and adaptive immune responses; on the other hand, IL-23 receptor polymorphism, which reduces its ability to respond to IL-23 stimulation, is associated with protection from BD [3–5]. Recent data also reported associations with CCR1, STAT4, and KLRC4 encoding a chemokine receptor, a transcription factor implicated in IL-12 and IL-23 signaling and a natural killer receptor, respectively [6, 7]. Moreover, susceptibility genes implicating the innate immune response to microbial exposure have recently been identified by Immunochip analysis [8].

Increased Th1, $CD4^+$, and $CD8^+$ T cell, $\gamma\delta^+$ T cell, and neutrophil activities were found both in the serum and in inflamed tissues of BD patients, which suggests that innate and adaptive immunities are involved together in the pathogenesis of BD [2, 9]. Similar to other autoimmune disorders, BD shows Th1-type cytokine profiles. IL-2- and interferon-(INF-) γ-producing T cells were increased in patients with active BD, while IL-4-producing T cells were lower than in controls [10]. Recent findings have shown that Th17 may play an important role in the pathogenesis of the disease [2, 11]. This hypothesis is supported by the observation of high IL-21 and IL-17 levels in sera of patients affected by BD with neurologic involvement [12, 13]. Another study showed that Th17/Th1 ratio in peripheral blood of patients with BD was higher than those of healthy controls, whereas the Th1/Th2 and Th17/Th2 ratios were similar among the two groups. Patients with uveitis or folliculitis had higher Th17/Th1 ratio compared with patients without these manifestations [14, 15]. Further investigation is required in order to better understand the role of the immune system in BD and whether the polarization towards a Th1/Th17 pathway may play a critical role in BD pathogenesis.

In this study, we used a gene array strategy to identify transcriptional profiles of PBCs obtained from patients with active BD. Using this approach, we think we have been able to shed a new light on some aspects of the disease pathogenesis by dissecting different aspects of this complex pathology in order to better clarify the role of the immune system in BD.

2. Patients and Methods

2.1. Patients. We studied a cohort of 51 patients (16 males and 35 females, mean age: 37 ± 11 years) affected by BD, attending the Unit of Autoimmune Diseases at the University Hospital in Verona, Italy.

All patients fulfilled the International Criteria for Behçet Disease (ICBD): oral aphthosis, genital ulcers, and ocular lesions were each given 2 points, whereas 1 point was assigned to each of skin lesions, vascular manifestations, and neurological manifestations. A patient scoring 4 points or above was classified as having BD [16, 17].

At enrollment, none of the patients had active infections or was affected by malignancies.

A group of 10 subjects with BD was selected within the entire cohort of BD patients and utilized for the gene array study. The clinical features of the patients are reported in Table 1 that also includes a description of the BD patients selected for the gene array study.

A written informed consent was obtained from all the participants of the study. The study was approved by local Ethical Committee of the Azienda Ospedaliera Universitaria of Verona, Verona, Italy. All investigations have been conducted according to the principles expressed in the Helsinki declaration.

2.2. Gene Array. Blood sample collection was prepared using PAXgene Blood RNA tubes (PreAnalytiX, Hombrechtikon, Switzerland), and total RNA was extracted by following the manufacturer's instructions. cRNA preparation, sample hybridization, and scanning were performed as recommended by the Affymetrix (Affymetrix, Santa Clara, CA, USA) supplied protocols and by the Cogentech Affymetrix microarray unit (Campus IFOM IEO, Milan, Italy) using Human Genome U133A 2.0 (HG-U133A 2.0) GeneChip (Affymetrix). For gene expression profile analysis, we followed the methods of Dolcino et al. [18]. Trancripts with an expression level at least 2.0 fold different in the test sample versus control sample ($p \le 0.01$) were functionally classified according to the Gene Ontology (GO) annotations and submitted to the pathway analysis using the PANTHER expression analysis tools (http://pantherdb.org/) [19]. The enrichment of all pathways associated with the differentially expressed genes compared to the distribution of genes represented on the Affymetrix HG-U133A microarray was analyzed, and p values ≤ 0.05, calculated by the binomial statistical test, were considered as significant enrichment.

2.3. Protein-Protein Interaction (PPI) Network Construction and Network Clustering. The search tool for the retrieval of interacting genes (STRING version 1.0; http://string-db.org/) is an online database which includes experimental as well as predicted interaction information and comprises >1100 completely sequenced organisms [20]. DEGs were directly mapped to the STRING database for acquiring significant protein-protein interaction (PPI) pairs from a range of sources, including data from experimental studies and data retrieved by text mining and homology searches [21]. PPI pairs with the combined score of ≥0.7 were retained for the construction of the PPI network.

The graph-based Markov clustering algorithm (MCL) allows the visualization of high-flow regions (clusters/modules) separated by boundaries with no flow, containing gene products that are expected to be involved in the same (or similar) biological processes [22].

In order to detect highly connected subgraphs (areas), the MCL algorithm was applied to the protein interactome graph.

Table 1: Clinical features of the patients with BD included in the study.

Patients		51 (100%)
Sex	Male	16 (31%)
	Female	35 (68%)
Clinical features	Aphthous stomatitis	51 (100%)
	Genital ulcers	34 (66%)
	Erythema nodosum-like lesions	7 (13%)
	Papulopustular lesion	37 (72%)
	Uveitis	5 (9%)
	Epididymitis	3 (5%)
	Neurological symptoms	8 (14%)
	Vasculitis	6 (12%)
	Joints manifestations	43 (84%)
	Gastrointestinal involvement	3 (5%)
	Association with HLA-B51	32 (62%)
Patients utilised for gene array study		10 (100%)
Sex	Male	6
	Female	4
Clinical features	Aphthous stomatitis	10 (100%)
	Genital ulcers	4 (40%)
	Erythema nodosum-like lesions	1 (10%)
	Papulopustular lesion	8 (80%)
	Uveitis	1 (10%)
	Epididymitis	0
	Neurological symptoms	1 (10%)
	Vasculitis	3 (30%)
	Joint manifestation	8 (80%)
	Gastrointestinal involvement	0
	Association with HLA-B51	7 (70%)

Cytoscape software [22] was used to visualize all the constructed networks.

2.4. PBMCs Isolation. PBMCs were obtained from 30 healthy donors and 30 patients affected by BD through a density-gradient centrifugation on Lymphoprep (Nycomed Pharma, Oslo, NO) at 800 ×g. Cells were washed twice with PBS and counted using acridine orange (Thermo Fisher Scientific, Waltham, MA, USA), considering only viable cells for FACS analyses.

2.5. FACS Analysis. Cell samples were treated by following the methods of Dolcino et al. [18]. Cells were stimulated over night with Dynabeads Human T-Activator CD3/CD28 (Life Technologies, Carlsbad, CA, USA). The detection of IL-17 production was analyzed using the IL-17 Secretion Assay (Miltenyi Biotec, Bergisch Gladbach), following the manufacturer's instruction as described in the methods of Dolcino et al. [18].

2.6. Real-Time RT-PCR. Total RNA was isolated from PBC using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. PCR was performed by following the methods of Dolcino et al. [18]. Predesigned, gene-specific primers and probe sets for each gene (CCL2, CXCL2, ICAM1, and IL-8) were obtained from Assay-on-Demand Gene Expression Products (Applied Biosystems).

2.7. Detection of Soluble Mediators in Sera of BD Patients and Healthy Controls. Serum levels of TNF alpha, IL-8, CXCL1, CCL2, CCL3, and CCL20 were detected using commercially available ELISA kits (Quantikine, R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions in 51 BD patients when compared to the 30 normal healthy donors.

2.8. Statistical Analysis. Statistical testing was performed using SPSS Statistics 2 software (IBM, United States). Data obtained from the analysis of the soluble mediators and from the analysis of IL-17-positive $CD4^+$ T cells in PBMCs were analyzed using the Student's unpaired t-test.

3. Results

3.1. Gene Array Analysis. In order to identify specific gene signatures typically associated with BD, we compared the gene expression profiles of 10 PBC samples obtained from 10 individual BD patients with 10 PBC samples obtained from healthy age- and sex-matched donors.

We found that 179 modulated genes complied with the Bonferroni-corrected p value criterion ($p \leq 0.01$) and the fold change criterion (FC≥2), showing robust and statistically significant variation between healthy controls and BD PBC. In particular, 160 and 19 transcripts resulted to be up- and downregulated, respectively.

Figure 1(a) is a hierarchical cluster diagram representing the signal intensity of DEGs across samples; the heat map shows a different gene expression profile between BD patients and healthy donors that clearly separates the two sets of specimens.

Figure 1(b) shows a functional classification of all DEGs according to the Gene Ontology (GO) terms.

The Gene Ontology analysis showed that the vast majority of the regulated transcripts can be ascribed to biological processes that may play a role in BD, including inflammation, immune response, apoptosis, blood coagulation, vascular damage, and cell proliferation. Table 2 shows a detailed selection of DEGs within the abovementioned processes. The table also includes GenBank accession numbers and fold changes. The complete list of modulated genes can be found in Supplementary Table 1.

Interestingly, regulated transcripts are distributed in gene categories that control different biological processes. However, the functional classes which show the highest enrichment in modulated genes are immune response (71/179) and inflammation (55/179).

Among genes ascribed to the immune response, twenty Th17-lymphocyte-related genes were upregulated including interleukin 6 signal transducer, IL6ST, chemokine (C-C motif) ligand 20, CCL20, suppressor of cytokine signaling 3, SOCS3, chemokine (C-X-C motif) ligand 1, CXCL1, chemokine (C-X-C motif) ligand 2, CXCL2, chemokine (C-X-C motif) ligand 3, CXCL3, inducible T cell costimulator, ICOS, intercellular adhesion molecule 1, ICAM1, interleukin 8, IL-8, interleukin 1 beta, and IL-1B (see also Table 2). Some genes involved in B cell activity (CD83 molecule, CD72 molecule, Fc receptor-like 2, FCRL2, and SAM domain, SH3 domain and nuclear localization signals 1 (SAMSN1)) are modulated in patients' samples, indicating a concomitant activation of this lymphocyte cell subset in BD.

Several upregulated genes play a role in innate immunity and are expressed in neutrophils (i.e., defensin, alpha 1, DEFA1, Fc fragment of IgA, receptor, and FCAR), dendritic cells (i.e., Dab, mitogen-responsive phosphoprotein, and homolog 2 DAB2), and in macrophages (adaptor-related protein complex 2, mu 1 subunit, and AP2M1).

In agreement with the typical presence of a marked inflammatory response in BD, we also observed overexpression of several proinflammatory transcripts. The upregulated genes comprise IL-8, IL-1B, CXCL2, CXCL1, CXCL3, interleukin 1, alpha (IL-1A), tumor necrosis factor (TNF), and oxidized low-density lipoprotein (lectin-like) receptor 1 (OLR1/LOX1).

Remarkably, in these two functional classes, we observed that a large number of genes are involved in well-known signaling networks that have been associated with autoimmune diseases.

These signal cascades include: (1) the interferon-alpha (IFN-A) pathway also named "type I interferon signature" [23], (2) the Toll-like receptor (TLR) signaling network, and (3) the JAK/STAT signaling pathway.

In particular, 9 type I interferon-inducible genes (IFIG) were upregulated (Table 2), thus showing the presence of an IFN type I signature, typically present in autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Crohn's disease, and Sjogren syndrome (SS) [24–30].

Twelve DEGs belong to the TLR signaling cascade (Table 2) which is thought to play a role in the onset of several autoimmune diseases and has been also implicated in the pathogenesis of BD [31–34].

Eight upregulated genes belong to the JAK/STAT signaling pathway, and interestingly, an increased JAK/STAT signaling has been associated with almost every autoimmune disease [35].

Moreover, activation of the JAK/STAT signaling pathway has been observed in monocytes and $CD4^+$ T cells of patients with BD [36].

Several genes involved in apoptosis and/or in apoptosis regulation were modulated in BD samples including myeloid cell leukemia sequence 1 (BCL2-related), MCL-1, BCL2-like 11, BCL2L11, immediate early response 3, IER3 and ZFP36 ring finger protein-like 2, and ZFP36L2.

Cell proliferation was also deregulated, and we found modulation of several transcripts including BTG family, member 2, BTG2, epiregulin, EREG, proline-rich coiled-coil 2C (PRRC2), phosphatase, tensin homolog pseudogene 1 (PTENP1), and amyloid beta (A4) precursor-like protein 2 (APLP2).

Endothelial dysfunction and altered coagulation are typical features of BD vasculitis, and consistently with these aspects of the disease, several genes involved in vascular damage are modulated in BD specimens, including thrombospondin 1, THBS1; protein S alpha, PROS1; plasminogen activator, urokinase receptor, PLAU-UPAR; thrombomodulin, THBD; and vascular endothelial growth factor A, VEGFA.

The 179 DEGs were then submitted to a pathway analysis using the PANTHER expression analysis tool and functionally annotated according to canonical pathways. Eight canonical pathways were found to be significantly overrepresented among the differentially expressed genes, and inflammation was the most enriched pathways, followed by interleukin signaling, Toll-like receptor signaling, blood coagulation, T cell activation, apoptosis, angiogenesis, and the B cell activation pathway (Figure 2).

The modulation of some genes showed by gene array analysis was validated by Q-PCR (Figure 3).

3.2. PPI Network Analysis. The gene expression profiling of BD PBC was then complemented by the study of functional interactions between DEGs' protein products.

To this aim, an interaction network was constructed upon the 179 DEGs, using the STRING data mining tool for retrieving well-documented connections between proteins. The obtained protein-protein interaction (PPI)

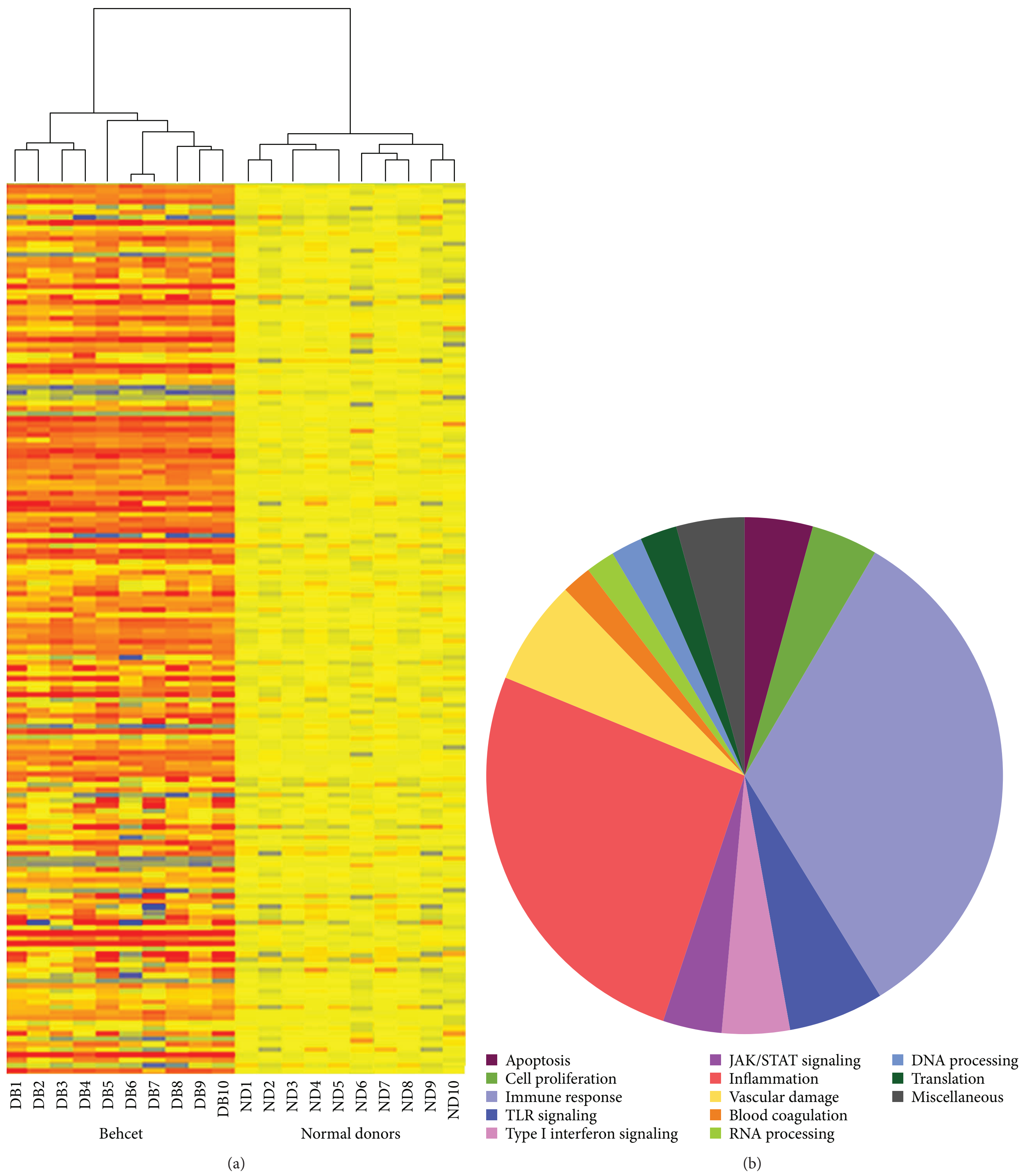


Figure 1: Modulated genes in PBCs of 10 BD patients and their functional classification. Heat map of significantly modulated genes (a). Each row represents a gene, each column shows the expression of selected genes in each individual sample. Blue-violet indicates genes that are expressed at lower levels when compared with the mean value of the control subjects, orange-red indicates genes that are expressed at higher levels when compared to the control means, and yellow indicates genes whose expression levels are similar to the control mean. Panel (b) shows the functional categorization of BD modulated genes according to GO terms. In the legend, the gene classes are listed in a clock-wise order starting at the "12 o'clock" position.

Table 2: Annotated genes differentially expressed in BD PBC versus healthy controls grouped according to their function.

Probe set ID	Gene title	Gene symbol	FC	p value	Representative public ID
Adaptive immune response					
T cell response					
204794_at	Dual specificity phosphatase 2	DUSP2	2.12	0.001	NM_004418
216248_s_at	Nuclear receptor subfamily 4, group A, member 2	NR4A2	5.54	0.012	NM_006186
211861_x_at	CD28 molecule	**CD28**	2.00	0.014	AF222343
203547_at	CD4 molecule	**CD4**	2.02	0.016	BT019811
217394_at	T cell receptor alpha variable 13-1	TRAV13-1	2.53	0.001	AE000521
210439_at	Inducible T cell costimulator	**ICOS**	2.26	0.006	AB023135
221331_x_at	Cytotoxic T-lymphocyte-associated protein 4	CTLA4	2.13	0.003	NM_005214
211085_s_at	Serine/threonine kinase 4	STK4/ MST1	2.14	0.005	Z25430
205456_at	CD3e molecule, epsilon (CD3-TCR complex)	**CD3E**	2.11	0.004	NM_000733
208602_x_at	CD6 molecule	CD6	2.47	<0.001	NM_006725
211302_s_at	Phosphodiesterase 4B, cAMP specific	PDE4B	3.17	0.004	L20966
218880_at	FOS-like antigen 2	FOSL2/ FRA2	3.17	0.005	NM_005253
206360_s_at	Suppressor of cytokine signaling 3	**SOCS3**	2.01	0.001	NM_003955
212079_s_at	Lysine- (K-) specific methyltransferase 2A	KMT2A/ MLL1	2.57	0.004	NM_001197104
209722_s_at	Serpin peptidase inhibitor, clade B (ovalbumin), member 9	SERPINB9	2.03	0.001	NM_004155
B cell response					
201694_s_at	Early growth response 1	EGR1	3.86	0.002	NM_001964
207655_s_at	B cell linker	BLNK	−2.10	<0.001	NM_013314
215925_s_at	CD72 molecule	CD72	−2.09	0.015	NM_001782
220330_s_at	SAM domain, SH3 domain, and nuclear localization signals 1	SAMSN1	2.03	0.006	NM_022136
204440_at	CD83 molecule	CD83	3.47		NM_004233
221239_s_at	Fc receptor-like 2	FCRL2	−2.36	0.001	NM_030764
213810_s_at	Akirin 2	AKIRIN2	3.12	<0.001	NM_018064
T/B cell response					
216901_s_at	IKAROS family zinc finger 1 (Ikaros)	IKZF1/ IKAROS	6.08	<0.001	NM_006060
221092_at	IKAROS family zinc finger 3 (Aiolos)	IKZF3/ AIOLOS	3.11	0.006	NM_012481
212249_at	Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	PIK3R1	3.19	<0.001	JX133164
Innate immune response					
201278_at	Dab, mitogen-responsive phosphoprotein, homolog 2	DAB2	−2.17	0.006	NM_032552
205033_s_at	Defensin, alpha 1	DEFA1	3.80	0.007	NM_004084
205468_s_at	Interferon regulatory factor 5	IRF5	2.01	<0.001	EF064718
M97935_5_at	Signal transducer and activator of transcription 1, 91 kDa	STAT1	2.26	0.001	GU211347
217199_s_at	Signal transducer and activator of transcription 2, 113 kDa	STAT2	2.02	0.002	S81491
217502_at	Interferon-induced protein with Tetratricopeptide repeats 2	IFIT2	2.00	<0.001	NM_001547
201211_s_at	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	DDX3X	2.25	<0.001	NM_001356
211307_s_at	Fc fragment of IgA, receptor for	FCAR/ CD89	5.04	0.004	U43677
212105_s_at	DEAH (Asp-Glu-Ala-His) box helicase 9	DHX9	3.82	<0.001	NM_001357
200613_at	Adaptor-related protein complex 2, mu 1 subunit	AP2M1	2.17	<0.001	NM_004068

Table 2: Continued.

Probe set ID	Gene title	Gene symbol	FC	p value	Representative public ID
NK cell response					
215339_at	Natural killer-tumor recognition sequence	NKTR	2.15	0.001	NM_005385
211242_x_at	Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 4	KIR2DL4	2.00	0.004	AF276292
216552_x_at	Killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 4	KIR2DS4	2.08	0.002	NM_001281972
209722_s_at	Serpin peptidase inhibitor, clade B (ovalbumin), member 9	SERPINB9	2.03	0.001	NM_004155
Adaptive/innate immune response					
204863_s_at	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	**IL6ST**	4.44	0.005	AB102799
211192_s_at	CD84 molecule	CD84	2.32	0.002	AF054818
213810_s_at	Akirin 2	AKIRIN2	3.12	0.001	AW007137
209722_s_at	Serpin peptidase inhibitor, clade B (ovalbumin), member 9	SERPINB9	2.03	0.001	NM_004155
221491_x_at	Major histocompatibility complex, class II, DR beta 1	HLA-DRB1	2.19	<0.001	U65585
213494_s_at	YY1 transcription factor	**YY1**	2.00	0.006	NM_003403
Toll-like receptors signaling					
205067_at	Interleukin 1, beta	IL-1B	6.32	0.001	NM_000576
206676_at	Carcinoembryonic antigen-related cell adhesion molecule 8	CEACAM8	5.07	0.003	M33326
204924_at	Toll-like receptor 2	TLR2	2.00	0.015	NM_003264
221060_s_at	Toll-like receptor 4	**TLR4**	2.10	0.001	NM_003266
211027_s_at	Inhibitor of kappa light polypeptide gene enhancer in B cells, kinase beta	IKBKB/ IKKb	2.33	<0.001	AY663108
213281_at	Jun protooncogene	JUN/AP1	4.51	0.011	NM_002228
206035_at	V-rel reticuloendotheliosis viral oncogene homolog	REL/c-REL	2.56	0.005	NM_002908
217738_at	Nicotinamide phosphoribosyltransferase	NAMPT	2.64	0.008	NM_005746
216450_x_at	Heat shock protein 90 kDa beta (Grp94), member 1	HSP90B1/ GP96	3.24	<0.001	NM_003299
214370_at	S100 calcium binding protein A8	S100A8	3.85	<0.001	NM_002964
211016_x_at	Heat shock 70 kDa protein 4	HSPA4	2.25	<0.001	NM_002154
211622_s_at	ADP-ribosylation factor 3	ARF3	2.12	<0.001	M33384
Type I interferon signaling					
205468_s_at	Interferon regulatory factor 5	IRF5	2.01	<0.001	EF064718
M97935_5_at	Signal transducer and activator of transcription 1, 91 kDa	STAT1	2.26	0.001	GU211347
217199_s_at	Signal transducer and activator of transcription 2, 113 kDa	STAT2	2.02	0.002	S81491
216598_s_at	Chemokine (C-C motif) ligand 2	CCL2	2.00	0.002	S69738
210001_s_at	Suppressor of cytokine signaling 1	SOCS1	2.16	0.012	AB005043
207433_at	Interleukin 10	IL-10	2.16	<0.001	NM_000572
210512_s_at	Vascular endothelial growth factor A	VEGFA	2.01	0.003	AF022375
217502_at	Interferon-induced protein with tetratricopeptide repeats 2	IFIT2	2.00	<0.001	NM_001547
201211_s_at	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	DDX3X	2.25	<0.001	NM_001356
JAK/STAT signaling					
M97935_5_at	Signal transducer and activator of transcription 1, 91 kda	STAT1	2.26	0.001	GU211347
217199_s_at	Signal transducer and activator of transcription 2, 113 kda	STAT2	2.02	0.002	S81491
204863_s_at	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	**IL6ST**	4.44	0.005	AB102799
207433_at	Interleukin 10	IL-10	2.16	<0.001	NM_000572

Table 2: Continued.

Probe set ID	Gene title	Gene symbol	FC	p value	Representative public ID
217489_s_at	Interleukin 6 receptor	**IL6R**	2.03	0.011	S72848
212249_at	Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	PIK3R1	3.19	<0.001	JX133164
210001_s_at	Suppressor of cytokine signaling 1	SOCS1	2.16	0.012	AB005043
206360_s_at	Suppressor of cytokine signaling 3	SOCS3	2.01	0.001	NM_003955
Inflammatory response					
207113_s_at	Tumor necrosis factor	**TNF**	2.00	0.008	NM_000594
211506_s_at	Interleukin 8	**IL-8**	10.44	0.013	AF043337
207433_at	Interleukin 10	**IL-10**	2.16	<0.001	NM_000572
205067_at	Interleukin 1, beta	**IL-1B**	6.32	0.001	NM_000576
217489_s_at	Interleukin 6 receptor	IL6R	2.03	0.011	S72848
209774_x_at	Chemokine (C-X-C motif) ligand 2	**CXCL2**	5.75	0.007	M57731
201939_at	Polo-like kinase 2	PLK2	6.20	0.012	NM_006622
204470_at	Chemokine (C-X-C motif) ligand 1	**CXCL1**	4.64	0.003	NM_001511
207850_at	Chemokine (C-X-C motif) ligand 3	**CXCL3**	3.53	0.015	NM_002090
210118_s_at	Interleukin 1, alpha	IL-1A	3.93	0.001	M15329
203751_x_at	Jun D protooncogene	JUND	3.39	0.008	NM_005354
216598_s_at	Chemokine (C-C motif) ligand 2	**CCL2**	2.00	0.002	S69738
205476_at	Chemokine (C-C motif) ligand 20	**CCL20**	2.23	0.011	NM_004591
205114_s_at	Chemokine (C-C motif) ligand 3	CCL3	2.21	0.007	NM_002983
210001_s_at	Suppressor of cytokine signaling 1	**SOCS1**	2.16	0.012	AB005043
212190_at	Serpin peptidase inhibitor, clade e, member 2	SERPINE2	−2.14	0.003	NM_006216
211919_s_at	Chemokine (C-X-C motif) receptor 4	CXCR4	2.06	0.008	AF348491
205099_s_at	Chemokine (C-C motif) receptor 1	CCR1	2.14	0.002	NM_001295
207075_at	NLR family, pyrin domain containing 3	NLRP3	2.15	0.011	NM_004895
203591_s_at	Colony-stimulating factor 3 receptor	CSF3R	1.89	0.012	NM_000760
215485_s_at	Intercellular adhesion molecule 1	**ICAM1**	2.04	0.015	NM_000201
209701_at	Endoplasmic reticulum aminopeptidase 1	ERAP1	2.44	<0.001	NM_016442
216243_s_at	Interleukin 1 receptor antagonist	IL-1RN	2.26	0.008	NM_173842
202643_s_at	Tumor necrosis factor, alpha-induced protein 3	TNFAIP3	2.15	0.012	NM_001270508
201044_x_at	Dual specificity phosphatase 1	DUSP1	4.71	0.013	NM_004417
210004_at	Oxidized low-density lipoprotein (lectin-like) receptor 1	OLR1/ LOX1	4.13	0.002	AF035776
214370_at	S100 calcium binding protein A8	S100A8	3.85	<0.001	AW238654
213281_at	Jun protooncogene	JUN/AP1	4.51	0.011	NM_002228
216450_x_at	Heat shock protein 90 kDa beta (Grp94), member 1	HSP90B1/ GP96	3.24	<0.001	NM_003299
Vascular damage					
Blood coagulation					
204614_at	Serpin peptidase inhibitor, clade B (ovalbumin), member 2	SERPINB2	3.96	0.013	NM_002575
202833_s_at	Serpin peptidase inhibitor, clade A, member 1	SERPINA1	2.25	<0.001	NM_000295
207808_s_at	Protein S alpha	PROS1	−2.16	0.002	NM_000313
201110_s_at	Thrombospondin 1	THBS1	5.08	0.001	NM_003246
204713_s_at	Coagulation factor V (proaccelerin, labile factor)	F5	2.53	0.001	NM_000130
203294_s_at	Lectin, mannose binding, 1	LMAN1	2.50	<0.001	U09716
213258_at	Tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)	TFPI	−2.53	0.001	BF511231
203650_at	Protein C receptor, endothelial	PROCR	−2.67	0.001	NM_006404

Table 2: Continued.

Probe set ID	Gene title	Gene symbol	FC	p value	Representative public ID
Angiogenesis					
211924_s_at	Plasminogen activator, urokinase receptor	PLAUR/ UPAR	3.42	0.007	NM_002659
207329_at	Matrix metallopeptidase 8 (neutrophil collagenase)	MMP8	2.42	0.012	NM_002424
210512_s_at	Vascular endothelial growth factor A	VEGFA	2.01	0.003	AF022375
209959_at	Nuclear receptor subfamily 4, group A, member 3	NR4A3/ NOR1	5.69	<0.001	U12767
208751_at	N-Ethylmaleimide-sensitive factor attachment protein, alpha	NAPA	2.56	0.001	XM_011527436
Vasculitis					
203887_s_at	Thrombomodulin	THBD	2.00	0.015	NM_000361
206157_at	Pentraxin 3, long	PTX3	2.22	0.001	NM_002852
218880_at	FOS-like antigen 2	FOSL2/ FRA2	3.17	0.005	NM_005253
Apoptosis					
M97935_5_at	Signal transducer and activator of transcription 1, 91 kDa	STAT1	2.26	0.001	GU211347
200796_s_at	Myeloid cell leukemia sequence 1 (BCL2-related)	MCL1	8.02	<0.001	AF118124
200664_s_at	DnaJ (Hsp40) homolog, subfamily B, member 1	DNAJB1/ HSP40	2.20	0.010	NM_006145
208536_s_at	BCL2-like 11 (apoptosis facilitator)	BCL2L11	2.38	0.001	NM_006538
213606_s_at	Rho GDP dissociation inhibitor (GDI) alpha	ARHGDIA	2.30	<0.001	NM_001185077
219228_at	Zinc finger protein 331	ZNF331/ RITA	2.50	0.004	NM_018555
209722_s_at	Serpin peptidase inhibitor, clade b (ovalbumin), member 9	SERPINB9	2.03	0.001	NM_004155
201631_s_at	Immediate early response 3	IER3	2.85	0.001	NM_003897
201367_s_at	ZFP36 ring finger protein-like 2	ZFP36L2	9.07	<0.001	NM_006887
Cell proliferation					
201235_s_at	BTG family, member 2	BTG2	4.53	<0.001	U72649
205767_at	Epiregulin	EREG	3.51	0.013	NM_001432
214052_x_at	Proline-rich coiled-coil 2C	PRRC2C/ XTP2	3.11	<0.001	NM_015172
208701_at	Amyloid beta (A4) precursor-like protein 2	APLP2	2.64	<0.001	NM_001642
217494_s_at	Phosphatase and tensin homolog pseudogene 1	PTENP1	3.11	0.001	AF040103

Bold characters indicate TH17-related genes.

network comprised 172 genes (nodes) and 2583 pairs of interactions (edges) (see Supplementary Figure 1).

When we performed a topological analysis of the PPI network using the Cytoscape software, we found that the number of interactions in which the products of the 71 "immune response genes" (see Supplementary Table 1) were involved, accounted for the vast majority (70%) of connections present in the whole network (1819/2583).

Given the high connectivity (i.e., number of connections) of the immune response gene products, we decided to perform an additional network analysis that focused on these gene products, thinking that they could be more informative.

We found that 55 proteins were linked into a complex network accounting for 307 pairs of interactions. Figure 4(a) shows a graphical representation of the PPI network.

A clustering analysis was then carried out to detect clusters (modules) of proteins to which most of the interactions converged ("high flow areas") using the MCL algorithm, and we identified eight clusters that collectively accounted for 40 nodes and 242 edges (Figure 4(b)).

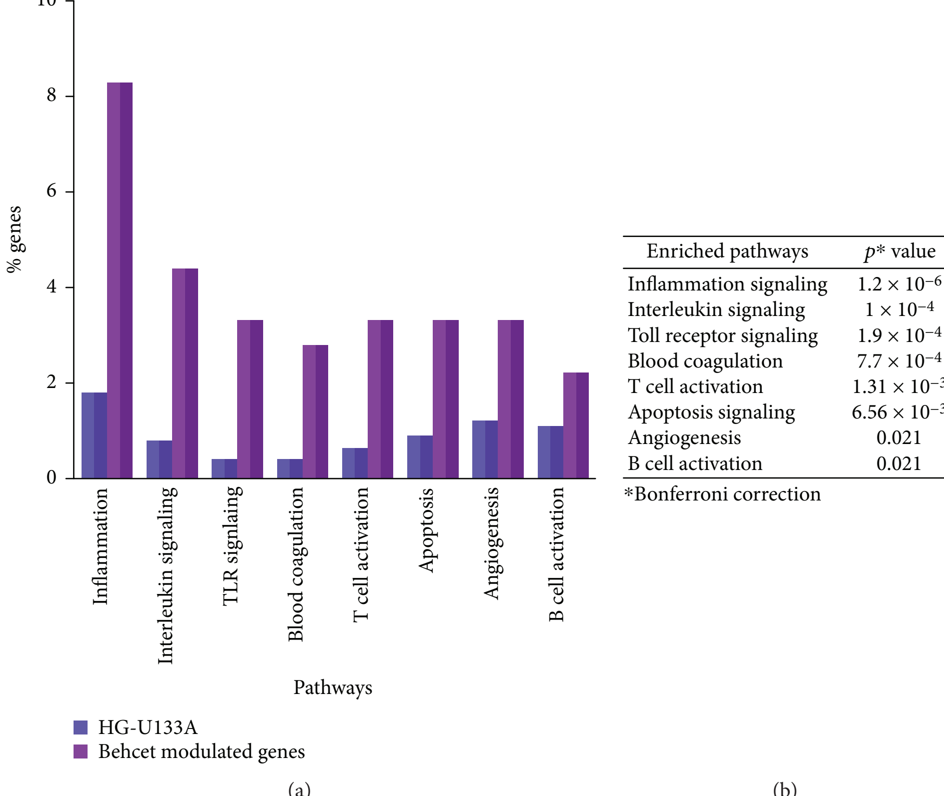
We next performed a functional enrichment analysis to identify association of genes, in each cluster, with different "GO terms" and pathways.

The significantly enriched categories for each cluster are shown in Figure 4(c).

Interestingly, five out of eight clusters (CL1, CL5, CL6, CL7, and CL8) were representative of the adaptive immune response.

In particular, three clusters (CL1, CL5, and CL6) showed a statistically significant enrichment in "T cell-related" gene categories and included several genes typically associated with T cell-mediated immune responses such as: CD3E, CD4, CD6, CD28, CTLA4, and DUSP2.

The most enriched GO biological processes (GO-BP) in these clusters were: "T cell differentiation" and "T cell



Enriched pathways	p* value
Inflammation signaling	1.2×10^{-6}
Interleukin signaling	1×10^{-4}
Toll receptor signaling	1.9×10^{-4}
Blood coagulation	7.7×10^{-4}
T cell activation	1.31×10^{-3}
Apoptosis signaling	6.56×10^{-3}
Angiogenesis	0.021
B cell activation	0.021

*Bonferroni correction

Figure 2: Pathways enrichment of BD modulated genes. (a) Graphical representation of genes ascribed to the enriched pathways, expressed in y-axis as percentage of all genes represented on the Affymetrix Human gene chip U133A 2.0 (dark purple bars) or as the percentage of genes in the dataset of Behcet's modulated genes (light purple bars); x-axis: enriched pathways. (b) p values associated with the significantly enriched pathways. Pathways with p values < 0.05 versus the distribution of all genes on the microarray chip, after a Bonferroni correction, were considered as significantly enriched.

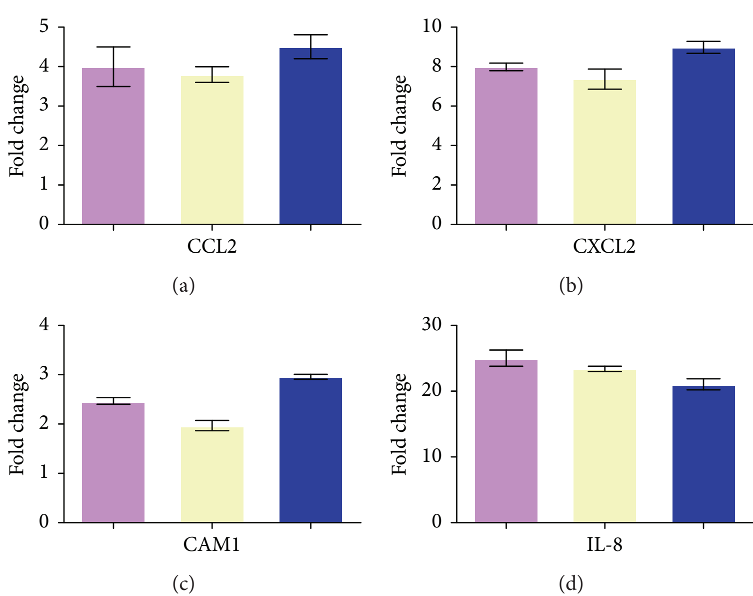
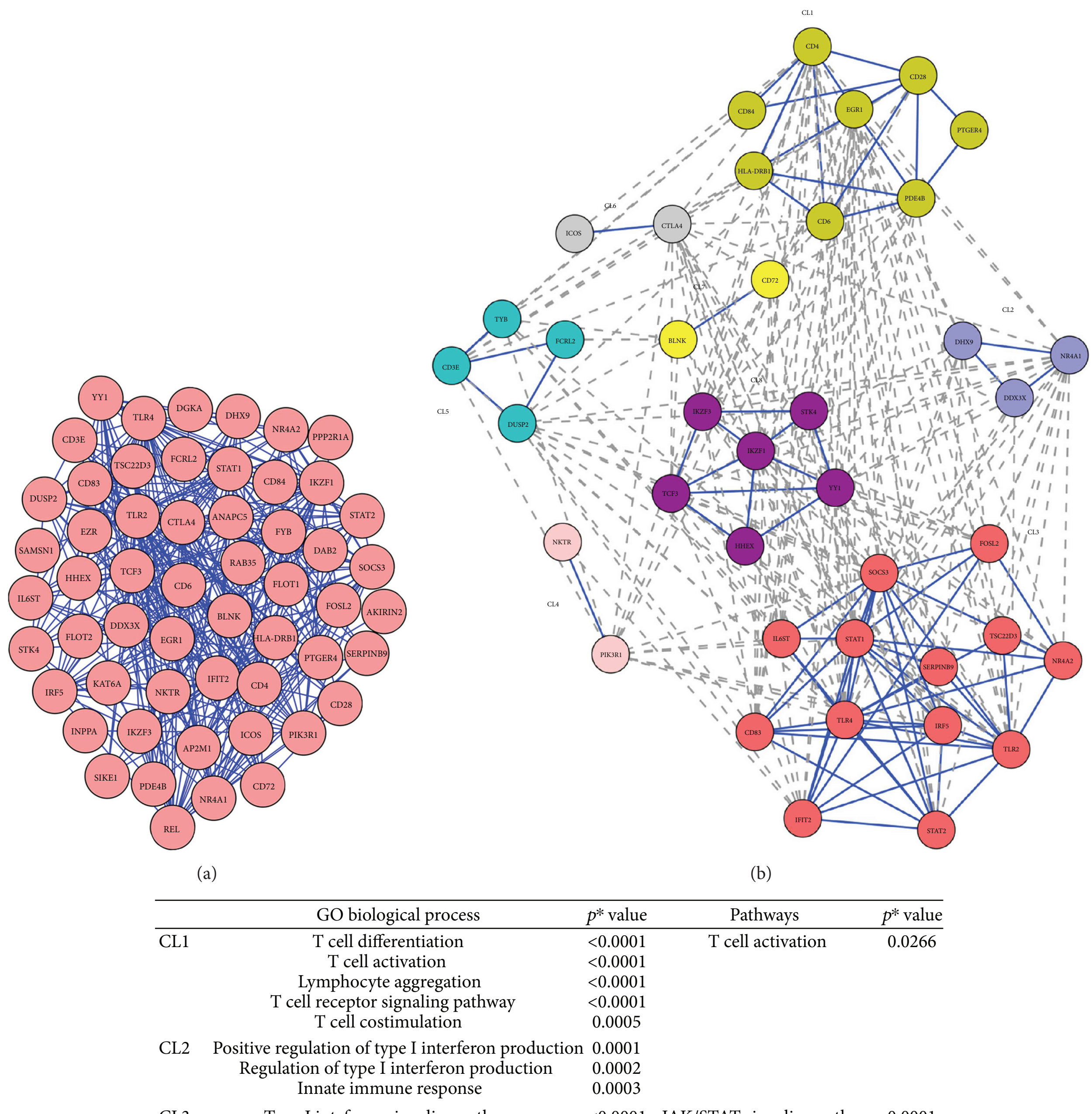


Figure 3: Real-time RT-PCR of some modulated genes confirms the results of gene array analysis. Genes selected for validation were CCL2, CXCL2, ICAM1, and IL-8. All the transcripts were increased in BD samples when compared to healthy donors. Relative expression levels were calculated for each sample after normalization against the housekeeping genes 18s rRNA, beta-actin, and GAPDH. Experiments have been conducted in triplicates. Housekeeping genes: violet bar: 18s rRNA; yellow bar: beta-actin; and blue bar: GAPDH.

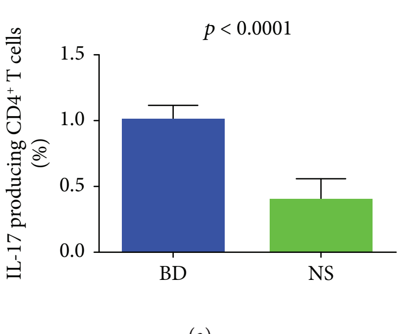


	GO biological process	p^* value	Pathways	p^* value
CL1	T cell differentiation	<0.0001	T cell activation	0.0266
	T cell activation	<0.0001		
	Lymphocyte aggregation	<0.0001		
	T cell receptor signaling pathway	<0.0001		
	T cell costimulation	0.0005		
CL2	Positive regulation of type I interferon production	0.0001		
	Regulation of type I interferon production	0.0002		
	Innate immune response	0.0003		
CL3	Type I inteferon signaling pathway	<0.0001	JAK/STAT signaling pathway	0.0001
	Cellular response to type I interferon	<0.0001	TLR signaling pathway	0.0001
CL5	T cell receptor sinaling pathway	0.0003	T cell activation	0.0246
	Regulation of alpha-beta T cell proliferation	0.0074		
	Positive regulation of alpha-beta T cell activation	0.0151		
CL6	T cell costimulation	<0.0001		
	Positive regulation of T cell activation	0.0002		
CL7	B cell diffrentation	0.0132	B cell activation	0.0103
	B cell activation	0.0193		
CL8	B cell activation	<0.0001		
	B cell diffrentiation	<0.0001		
	Regualtion of B cell activation	<0.0001		

*Bonferroni corrected

(c)

Figure 4: Network analysis of modulated genes in BD. Panel (a): PPI network of genes involved in immune response; panel (b): clusters extracted from the PPI network; panel (c): biological processes and pathways enriched in the eight modules.



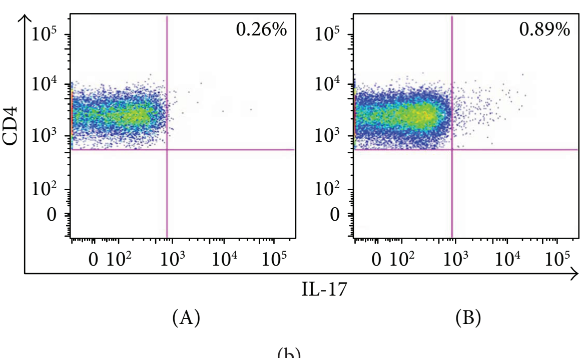


Figure 5: Flow cytometric analysis of $CD4^+$ T cells releasing IL-17 in patients with BD. Panel (a) displays the mean percentage of $CD4^+$ T cells releasing IL-17 of 30 healthy donors and 30 BD patients. PBMCs were stimulated over night with anti-CD3/CD28-coated beads. Panel (b) shows a representative experiment ((A) normal subjects (NS); (B) BD patients).

activation" (CL1), "T cell receptor signaling pathway" (CL5), "T cell costimulation", and "positive regulation of T cell activation" (CL6). The most enriched pathway was the "T cell activation pathway" (CL1 and CL5).

Two clusters (CL7 and CL8) included DEGs typically associated with B cell functions (i.e., CD72). These clusters were significantly enriched in the GO-BP "B cell differentiation" (CL7) and "B cell activation" (CL7, CL8). Moreover, the B cell activation pathway was the top enriched pathway in cluster 7.

Several DEGs involved in the innate immune response (i.e., HDX9, DDX3X, SOCS3, STAT1, IRF5, IFIT2, and STAT2) were present in clusters CL2 and CL3. Interestingly, they were significantly enriched in functions of "positive regulation of type I interferon production" (CL2) and "type I interferon signaling pathway" (CL3), further confirming the presence of a type I interferon signature, typically associated with several autoimmune diseases. Moreover, genes in cluster 3 were significantly involved with the JAK/STAT signaling pathway ($p = 0.0001$) and the TLR signaling pathway ($p = 0.0001$), both implicated with the development of autoimmune diseases [31, 35]. Noteworthy, seven Th17-related proteins (CD28, CD4, ICOS, CD3E, YY1, TLR4, and IL6ST) were represented in the abovementioned clusters (CL1, CL3, CL5, CL6, and CL8). Finally, no significant GO-BP or pathway was identified in cluster CL4.

3.3. Frequency of IL-17-Positive $CD4^+$ T Cells in PBMCs from Patients with BD. We assessed by flow cytometry the intracellular expression of the IL-17 cytokine, in PBMCs from 30 BD patients and from 30 healthy control subjects. We found a higher amount of IL-17-producing $CD4^+$ T cells among the PBMCs of patients with BD compared with healthy controls.

The mean values obtained in 30 BD PBMC were 1% ±0.12 versus 0.4% ±0.16 ($p < 0.0001$) (Figure 5).

3.4. Detection of Soluble Mediators in BD Sera. The gene expression analysis was complemented by the detection of some of the corresponding soluble mediators in the sera of patients with BD. We chose to assess the levels of TNF alpha, IL-8, CXCL1, CCL2, CCL3, and CCL20. Figure 6 shows the concentration of these molecules in the sera of the 51 BD patients. All these molecules showed increased serum levels in BD patients when compared to the 30 normal healthy donors.

4. Discussion

In this paper, we report a comprehensive study of BD gene expression profiling where for the first time, a conventional global gene expression analysis was combined to a gene network analysis of functional interactions between DEGs. We believe that this integrated approach is likely to generate insights in the complex molecular pathways that control the different clinical features of BD.

The first contribution of our study is a detailed investigation of DEGs in PBCs of BD patients in the attempt to clarify some aspects of BD pathogenesis.

Indeed, the majority of DEGs analyzed is involved in biological processes closely connected to the key features of the disease.

BD is a recurrent inflammatory disease with a multisystem involvement, affecting the vasculature, mucocutaneous tissues, eyes, joints, gastrointestinal tract, and brain.

Consistently with the strong inflammatory response typical of BD, DEGs indicate upregulation of a large number of proinflammatory molecules, including TNF, IL-1, IL-8, IL-10, CXCL1, CCL2, CCL3, and ICAM1, which can be detected at increased concentration in sera or plasma of BD patients when compared to healthy controls. [37–42].

Elevated serum levels of IL-8 are detectable in the active phase of BD and indicate the presence of vascular involvement [37], whereas high serum levels of CXCL1 correlate with BD disease activity [39]. Since flares of disease are characterized by neutrophil infiltration around blood vessels following increased chemotaxis of neutrophils [43], it is not surprising to observe upregulation of CSF3R/GCSFR, which controls neutrophil functions.

Consistently with the gene array data, serum levels of the proinflammatory mediators TNF alpha, IL-8, CXCL1, CCL2, CCL3, and CCL20 were significantly higher in our cohort of 51 BD patients when compared to healthy subjects.

The main histopathological finding in BD is a widespread vasculitis of blood vessels, arteries, and veins characterized by

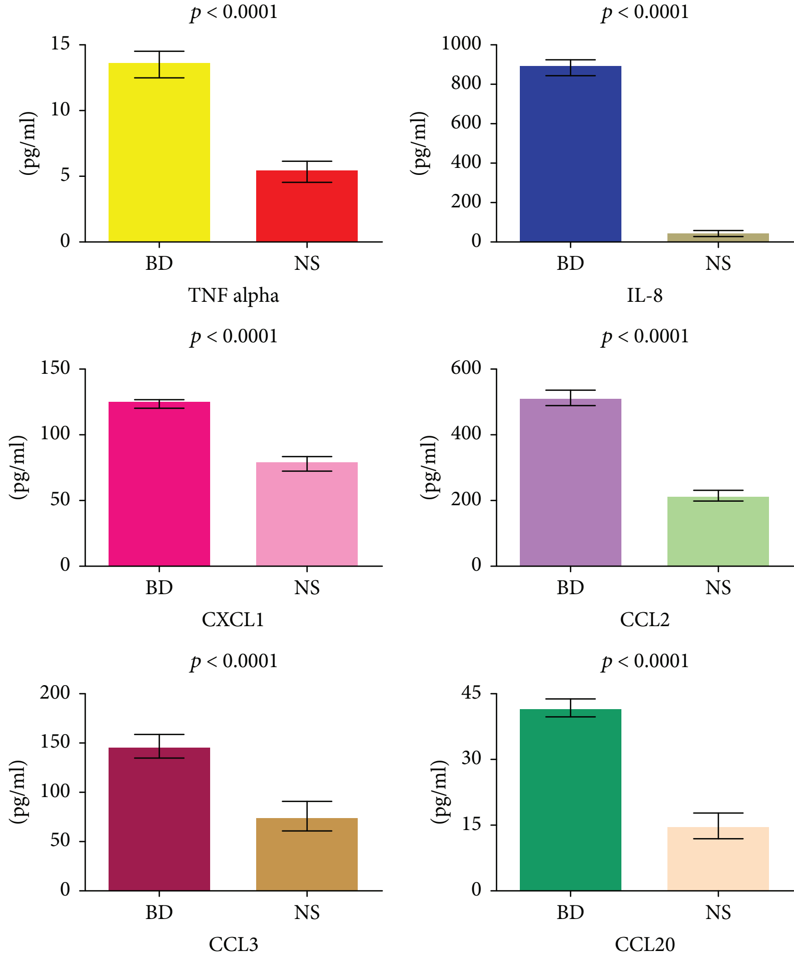


Figure 6: Serum levels of selected soluble mediators in BD patients and in normal subjects. The histograms represent the mean of the results obtained in 30 normal subjects (NS) and in 51 BD patients. p values were calculated using the Student's unpaired t-test.

myointimal proliferation, fibrosis, and thrombus formation leading to tissue ischemia [44]. Occlusion of the vascular lumen creates a hypoxic milieu that effectively can induce new vessel formation. Angiogenesis can further stimulate inflammation since new born endothelial cells release chemoattractive mediators for leukocytes and express adhesion molecules [44]. Several DEGs play a role in angiogenesis, and the highest level of induction was observed for NOR1 (also named NR4A3), a gene expressed in developing neointima that promotes endothelial survival and proliferation, acting as a transcription factor in vascular development [45]. DEGs also showed upregulation of NAPA, known to induce VE-cadherin localization at endothelial junctions and regulate barrier function [46]. Interestingly, soluble VE-cadherins may be increased in the sera of BD patients [47]. Another upregulated transcript was the gene encoding for UPAR, expressed in several cell types including monocytes, neutrophils, activated T lymphocytes, macrophages, and endothelial cells. Indeed, high levels of the soluble form of UPAR have been detected in the plasma of BD patients [48].

Other genes, typically associated with the vasculitic process, were overexpressed in our array including THBD and PTX3. THBD can be detected at higher levels in sera of BD patients compared with healthy controls, and it is associated with the skin pathergy test, considered as a useful test for BD diagnosis. PTX3, an acute-phase reactant produced at sites of active vasculitis, is an indicator of active small vessel vasculitis [49].

Defects in blood coagulation and fibrinolysis have been described in patients with BD with or without thrombosis, and accordingly, we found downregulation of genes encoding for proteins that have an anticoagulant effect (i.e., TFPI, PROS1, and PROCR/EPCR) and upregulation of transcripts which promote the coagulation process (including THBS1, F5, and LMAN1).

Several DEGs indicate an altered apoptotic process with up- or downregulation of several apoptosis-related genes. Endothelial cell apoptosis which plays a pivotal role in vascular damage and autoantibodies which are able to induce endothelial cell apoptosis have been reported in BD [50]. In BD, an altered apoptosis has been described also in other cell subsets, that is, neutrophils and lymphocytes. Indeed, neutrophil apoptosis is reduced in the remission phase of uveitis and is restored in the active phase [51], whereas T lymphocytes are resistant to Fas-mediated apoptosis in BD with

active uveitis [52]. On the contrary, an excessive expression of FasL on skin-infiltrating lymphocytes and the presence of apoptotic cells in the skin lesions have been also reported [53], suggesting that lymphocytes expressing increased levels of FasL may have a role in the development of BD skin lesions. Among genes that control cell proliferation, we observed overexpression of the gene EREG1, which plays an autocrine role in the proliferation of corneal epithelial cells [54], and APLP2 gene, involved in corneal epithelial wound healing [55]. In this regard, it is worthwhile mentioning that keratitis can be one of the ocular manifestations of BD.

Several aspects of BD are typical of an immune-mediated disease, but whether BD is an autoimmune or an autoinflammatory disease is still debated. A great number of DEGs (71/179) are involved in the immune response, and the majority of these genes can be ascribed to the adaptive immune response. In particular, DEGs indicate a T cell response with a prevailing upregulation of many TH17-related genes.

In this regard, it is worthwhile mentioning that Th17 cells have been associated with the pathogenesis of several autoimmune diseases including psoriasis, RA, and SLE [56–58]. Noteworthy, the involvement of this T cell subset in the pathogenesis of BD has been suggested, since Th17-related cytokines are considerably increased in BD and peripheral blood Th17/Th1 ratio is significantly higher in patients with active BD compared to healthy controls [11].

To further validate our data on overexpression of the Th17 pathway in our cohort of patients, we analyzed the presence of IL-17-producing $CD4^+$ T cells and found a significantly increased percentage of these cells in PBCs of patients with BD when compared with healthy donors.

Among DEGs regulating B cell responses, we observed overexpression of SAMSN1, a transcript induced upon B cell activation [59], and EGR1, involved in the differentiation program of B cells into plasma cells, whereas the inhibitory receptor CD72 that downmodulates B cell receptor (BCR) signaling was downregulated. All together, these data indicate the activation of the B cell immune response and (auto)-antibody production suggesting a possible role of these cells in BD pathogenesis.

Other genes associated with the adaptive immune response include ICOS, SOCS3, and HLA-DRB1. Interestingly, a high expression of ICOS on $CD4^+$ T cells has been described in BD patients with active uveitis, suggesting a role in the pathogenesis of uveitis, possibly through upregulation of IFN-g, IL-17, and TNF [60].

An increased expression of SOCS3, a regulator of the JAK/STAT pathway of cytokine induction, has been observed in all patients with BD irrespective of disease activity [61], and polymorphisms of HLA-DRB1 alleles have been associated with BD [62].

In addition, it is worthwhile mentioning that DEGs of the adaptive immune response include transcripts already associated with the development of autoimmune diseases, including CTLA4, MST1, CD6, and the abovementioned SOCS3 [63–66].

We observed that several upregulated genes, including IRF5, IFIT2, DDX3X, STAT1, and STAT2 participate to type I interferon and JAK/STAT signaling pathways.

As already mentioned, type I interferon signaling is associated with autoimmune diseases including SLE, RA, Sjogren's syndrome, and Crohn's disease [24–30].

In this regard, the copresence of type I IFN signaling and Th17-related genes suggests an autoimmune component in the origin of BD, since a synergy between IFN and Th17 pathways is commonly involved in autoimmunity [24–30, 67–69].

Moreover, the JAK/STAT signaling is activated in BD [36] and this pathway has been associated with the development of systemic autoimmune diseases such as SLE and RA [70].

Our dataset indicates also the overexpression of several genes belonging to the TLR pathway. Growing body of evidence suggests the association between TLRs and autoimmunity. Indeed, the expression of TLRs in B cells is required for the synthesis of most of the SLE-associated autoantibodies [71]. Moreover, in RA, extracellular ligands can enhance the production of the proinflammatory mediators IL6 and IL-17 in human synoviocytes and in PBCs [72]. In addition, in systemic sclerosis activation of TLR4 on the surface of fibroblasts contributes to the upregulation of profibrotic chemokines [73]. Finally, stimulation of TLR2 induces the production of IL-23 and IL-17 cytokines from the PBCs of patients affected by Sjogren's syndrome [74].

TLR2 and TLR4 have been shown to be overexpressed in PBCs from patients with eye involvement [72] and in buccal mucosal biopsies and in PBCs obtained from patients with flare of the disease [32]. Noteworthy, both TLR2 and TLR4 were upregulated in our array, in accordance with the above reported data [32, 72].

Pathway analysis may help to elucidate the pathogenesis of complex or multifactorial diseases, such as BD, that are often caused by a mixture of abnormalities of correlated transcripts or biological pathways [75]. To this aim, we mapped our DEGs onto canonical pathways to identify signaling cascades which were overrepresented in our dataset.

Interestingly, pathway enrichment analysis revealed that inflammation, IL, TLR, blood coagulation, T cell activation, apoptosis, angiogenesis, and B cell activation signaling pathways were the most enriched in BD transcriptoma, further confirming their crucial role in the disease pathogenesis.

Thanks to our global analysis we have identified modulated genes involved in biological processes that could recapitulate most of the typical features of BD. Indeed, the majority of DEGs were involved in immune response and inflammation; moreover, we observed the activation of pathways (i.e., JAK/STAT and TLRs) and the presence of signatures (i.e., type I interferon and TH17 cell) typically associated with an autoimmune response, thus suggesting an autoimmune component in the origin of BD.

We carried our analysis in order to highlight key DEGs functionally collaborating in networks that could be involved in the disease onset and progression.

Indeed, in the second part of our study, instead of looking at single component of biological processes, we aim to study the interactions among the protein products of DEGs by a network analysis.

A network representation is an intriguing way to study the complex dynamic of disease-associated molecular interactions, and in this perspective, disorders can be considered

in view of disturbances of molecular networks [76]. Interestingly, we observed that the protein products of genes ascribed to the immune response showed the highest degree of connectivity in the whole network of DEGs products, thus indicating a preeminent role of this gene category in driving the global gene expression profiles in BD pathogenesis. Then, we focused our attention on the PPI network specifically obtained from the immune response gene products, since it has been described that deregulation of genes, encoding for highly interactive proteins, interferes with physiological processes and that molecules involved in diseases development show a high attitude to interact with each other [77]. The clustering analysis of this sub-network helped us to further prioritize deregulated gene products that were placed in "highest connectivity areas" (clusters) of the network, where the hubs of biological process regulation are usually positioned [78].

In most of the clusters, we found an enrichment in molecular pathways of B and T cell-mediated adaptive immune response, thus suggesting a leading role of the adaptive immunity in the pathogenesis of BD. Moreover, DEGs in these classes included genes associated with the Th17 cell response.

Interestingly, we also observed that the molecules present in the few clusters enriched in innate immune response were involved in molecular signalings known to play a role in autoimmune diseases including JAK/STAT, TLRs, and type I interferon signaling.

These findings support that the disease may be sustained by an autoimmune process and are not in contrast with the hypothesis of an autoinflammatory component in the origin of BD.

The network analysis emphasizes the crucial role played by the molecular pathways emerged from our first global gene expression study in BD pathogenesis. Indeed, the molecules that participate to these signaling pathways are concentrated in areas (clusters) of the whole network that display the highest density of connection between genes, thus indicating their prominent role in the disease.

Through this analysis, we believe that we could identify pathogenically meaningful interactions that would have been hidden in the whole native dataset and that may be strongly associated with BD. Moreover, we provide evidence, at least at a level of gene expression, that BD may have an autoimmune origin.

Finally, we believe that our data can provide a deeper insight into BD pathogenesis, highlighting crucial molecular pathways including IL-17, IL-6, and JAK/STAT pathways that may be targeted by biological drugs and by novel therapeutical strategies.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Antonio Puccetti, Claudio Lunardi, and Marzia Dolcino conceived and designed the experiments. Piera Filomena Fiore, Andrea Pelosi, and Giuseppe Argentino performed the experiments. Marzia Dolcino and Andrea Pelosi analyzed the data. Elisa Tinazzi, Giuseppe Patuzzo, and Francesca Moretta selected the patients and contributed reagents. Marzia Dolcino wrote the paper with inputs from Claudio Lunardi and Antonio Puccetti. Antonio Puccetti, Piera Filomena Fiore, Andrea Pelosi, Claudio Lunardi, and Marzia Dolcino contributed equally to this paper.

Supplementary Materials

Supplementary 1. Supplementary Table 1: annotated genes differentially expressed in BD PBCs versus healthy controls grouped according to their function.

Supplementary 2. Supplementary Figure 1: PPI network of modulated genes in BD PBCs.

References

[1] International Team for the Revision of the International Criteria for Behçet's Disease (ITR-ICBD), F. Davatchi, S. Assaad-Khalil et al., "The International Criteria for Behçet's Disease (ICBD): a collaborative study of 27 countries on the sensitivity and specificity of the new criteria," *Journal of the European Academy of Dermatology and Venereology*, vol. 28, no. 3, pp. 338–347, 2014.

[2] E. Alpsoy, "Behçet's disease: a comprehensive review with a focus on epidemiology, etiology and clinical features, and management of mucocutaneous lesions," *The Journal of Dermatology*, vol. 43, no. 6, pp. 620–632, 2016.

[3] L. T. Morton, D. Situnayake, and G. R. Wallace, "Genetics of Behçet's disease," *Current Opinion in Rheumatology*, vol. 28, no. 1, pp. 39–44, 2016.

[4] N. Mizuki, A. Meguro, M. Ota et al., "Genome-wide association studies identify *IL23R-IL12RB2* and *IL10* as Behçet's disease susceptibility loci," *Nature Genetics*, vol. 42, no. 8, pp. 703–706, 2010.

[5] E. F. Remmers, F. Cosan, Y. Kirino et al., "Genome-wide association study identifies variants in the MHC class I, *IL10*, and *IL23R-IL12RB2* regions associated with Behçet's disease," *Nature Genetics*, vol. 42, no. 8, pp. 698–702, 2010.

[6] I. Fresko, "15th International Congress on Behçet's Disease," *Clinical and Experimental Rheumatology*, vol. 30, 3 Supplement 72, pp. S118–S128, 2012.

[7] M. Takeuchi, D. L. Kastner, and E. F. Remmers, "The immunogenetics of Behçet's disease: a comprehensive review," *Journal of Autoimmunity*, vol. 64, pp. 137–148, 2015.

[8] M. Takeuchi, N. Mizuki, A. Meguro et al., "Dense genotyping of immune-related loci implicates host responses to microbial exposure in Behçet's disease susceptibility," *Nature Genetics*, vol. 49, no. 3, pp. 438–443, 2017.

[9] H. Direskeneli, H. Fujita, and C. A. Akdis, "Regulation of T_H17 and regulatory T cells in patients with Behçet disease," *The Journal of Allergy and Clinical Immunology*, vol. 128, no. 3, pp. 665-666, 2011.

[10] N. Sugi-Ikai, M. Nakazawa, S. Nakamura, S. Ohno, and M. Minami, "Increased frequencies of interleukin-2- and interferon-gamma-producing T cells in patients with active Behçet's disease," *Investigative Ophthalmology & Visual Science*, vol. 39, no. 6, pp. 996–1004, 1998.

[11] K. Hamzaoui, "Th17 cells in Behçet's disease: a new immunoregulatory axis," *Clinical and Experimental Rheumatology*, vol. 29, 4 Supplement 67, pp. S71–S76, 2011.

[12] G. Geri, B. Terrier, M. Rosenzwajg et al., "Critical role of IL-21 in modulating T_H17 and regulatory T cells in Behçet disease," *The Journal of Allergy and Clinical Immunology*, vol. 128, no. 3, pp. 655–664, 2011.

[13] S. Y. Na, M. J. Park, S. Park, and E. S. Lee, "Up-regulation of Th17 and related cytokines in Behçet's disease corresponding to disease activity," *Clinical and Experimental Rheumatology*, vol. 31, 3 Supplement 77, pp. S32–S40, 2013.

[14] J. Kim, J. A. Park, E. Y. Lee, Y. J. Lee, Y. W. Song, and E. B. Lee, "Imbalance of Th17 to Th1 cells in Behçet's disease," *Clinical and Experimental Rheumatology*, vol. 28, 4 Supplement 60, pp. S16–S19, 2010.

[15] M. C. Mat, A. Sevim, I. Fresko, and Y. Tuzun, "Behçet's disease as a systemic disease," *Clinics in Dermatology*, vol. 32, no. 3, pp. 435–442, 2014.

[16] H. Yazici and Y. Yazici, "Criteria for Behçet's disease with reflections on all disease criteria," *Journal of Autoimmunity*, vol. 48-49, pp. 104–107, 2014.

[17] F. Davatchi, C. Chams-Davatchi, H. Shams et al., "Behcet's disease: epidemiology, clinical manifestations, and diagnosis," *Expert Review of Clinical Immunology*, vol. 13, no. 1, pp. 57–65, 2017.

[18] M. Dolcino, A. Ottria, A. Barbieri et al., "Gene expression profiling in peripheral blood cells and synovial membranes of patients with psoriatic arthritis," *PLoS One*, vol. 10, no. 6, article e0128262, 2015.

[19] H. Mi and P. Thomas, "Panther pathway: an ontology-based pathway database coupled with data analysis tools," *Methods in Molecular Biology*, vol. 563, pp. 123–140, 2009.

[20] A. Franceschini, D. Szklarczyk, S. Frankild et al., "String v9.1: protein-protein interaction networks, with increased coverage and integration," *Nucleic Acids Research*, vol. 41, no. D1, pp. D808–D815, 2013.

[21] L. J. Jensen, M. Kuhn, M. Stark et al., "STRING 8—a global view on proteins and their functional interactions in 630 organisms," *Nucleic Acids Research*, vol. 37, Supplement 1, pp. D412–D416, 2009.

[22] G. D. Bader and C. W. Hogue, "An automated method for finding molecular complexes in large protein interaction networks," *BMC Bioinformatics*, vol. 4, no. 1, p. 2, 2003.

[23] L. Ronnblom and M. L. Eloranta, "The interferon signature in autoimmune diseases," *Current Opinion in Rheumatology*, vol. 25, no. 2, pp. 248–253, 2013.

[24] R. A. Gordon, G. Grigoriev, A. Lee, G. D. Kalliolias, and L. B. Ivashkiv, "The interferon signature and *STAT1* expression in rheumatoid arthritis synovial fluid macrophages are induced by tumor necrosis factor α and counter-regulated by the synovial fluid microenvironment," *Arthritis & Rheumatism*, vol. 64, no. 10, pp. 3119–3128, 2012.

[25] A. Nzeusseu Toukap, C. Galant, I. Theate et al., "Identification of distinct gene expression profiles in the synovium of patients with systemic lupus erythematosus," *Arthritis & Rheumatism*, vol. 56, no. 5, pp. 1579–1588, 2007.

[26] R. M. Thurlings, M. Boumans, J. Tekstra et al., "Relationship between the type I interferon signature and the response to rituximab in rheumatoid arthritis patients," *Arthritis & Rheumatism*, vol. 62, no. 12, pp. 3607–3614, 2010.

[27] H. G. Raterman, S. Vosslamber, S. de Ridder et al., "The interferon type I signature towards prediction of non-response to rituximab in rheumatoid arthritis patients," *Arthritis Research & Therapy*, vol. 14, no. 2, p. R95, 2012.

[28] F. Moschella, G. F. Torelli, M. Valentini et al., "Cyclophosphamide induces a type I interferon-associated sterile inflammatory response signature in cancer patients' blood cells: implications for cancer chemoimmunotherapy," *Clinical Cancer Research*, vol. 19, no. 15, pp. 4249–4261, 2013.

[29] N. I. Maria, Z. Brkic, M. Waris et al., "MxA as a clinically applicable biomarker for identifying systemic interferon type I in primary Sjögren's syndrome," *Annals of the Rheumatic Diseases*, vol. 73, no. 6, pp. 1052–1059, 2014.

[30] G. Caignard, M. Lucas-Hourani, K. P. Dhondt et al., "The V protein of Tioman virus is incapable of blocking type I interferon signaling in human cells," *PLoS One*, vol. 8, no. 1, article e53881, 2013.

[31] E. Gianchecchi and A. Fierabracci, "Gene/environment interactions in the pathogenesis of autoimmunity: new insights on the role of Toll-like receptors," *Autoimmunity Reviews*, vol. 14, no. 11, pp. 971–983, 2015.

[32] N. Seoudi, L. A. Bergmeier, E. Hagi-Pavli, D. Bibby, M. A. Curtis, and F. Fortune, "The role of TLR2 and 4 in Behçet's disease pathogenesis," *Innate Immunity*, vol. 20, no. 4, pp. 412–422, 2014.

[33] X. Liu, C. Wang, Z. Ye, A. Kijlstra, and P. Yang, "Higher expression of Toll-like receptors 2, 3, 4, and 8 in ocular Behcet's disease," *Investigative Ophthalmology & Visual Science*, vol. 54, no. 9, pp. 6012–6017, 2013.

[34] J. Fang, L. Chen, J. Tang et al., "Association between copy number variations of *TLR7* and ocular Behçet's disease in a Chinese Han population," *Investigative Ophthalmology & Visual Science*, vol. 56, no. 3, pp. 1517–1523, 2015.

[35] K. Hirahara, D. Schwartz, M. Gadina, Y. Kanno, and J. J. O'Shea, "Targeting cytokine signaling in autoimmunity: back to the future and beyond," *Current Opinion in Immunology*, vol. 43, pp. 89–97, 2016.

[36] A. Tulunay, M. G. Dozmorov, F. Ture-Ozdemir et al., "Activation of the JAK/STAT pathway in Behcet's disease," *Genes & Immunity*, vol. 16, no. 2, p. 176, 2015.

[37] S. P. K. Durmazlar, G. B. Ulkar, F. Eskioglu, S. Tatlican, A. Mert, and A. Akgul, "Significance of serum interleukin-8 levels in patients with Behcet's disease: high levels may indicate vascular involvement," *International Journal of Dermatology*, vol. 48, no. 3, pp. 259–264, 2009.

[38] B. Turan, H. Gallati, H. Erdi, A. Gürler, B. A. Michel, and P. M. Villiger, "Systemic levels of the T cell regulatory cytokines IL-10 and IL-12 in Bechçet's disease; soluble TNFR-75 as a biological marker of disease activity," *The Journal of Rheumatology*, vol. 24, no. 1, pp. 128–132, 1997.

[39] Y. Kato and T. Yamamoto, "Serum levels of GRO-α are elevated in association with disease activity in patients with Behçet's disease," *International Journal of Dermatology*, vol. 51, no. 3, pp. 286–289, 2012.

[40] H. T. E. Ozer, E. Erken, R. Gunesacar, and O. Kara, "Serum RANTES, MIP-1α, and MCP-1 levels in Behçet's disease," *Rheumatology International*, vol. 25, no. 6, pp. 487-488, 2005.

[41] K. Saglam, I. M. Yilmaz, A. Saglam, M. Ulgey, F. Bulucu, and Y. Baykal, "Levels of circulating intercellular adhesion molecule-1 in patients with Behçet's disease," *Rheumatology International*, vol. 21, no. 4, pp. 146–148, 2002.

[42] N. Düzgün, E. Ayaşlioğlu, H. Tutkak, and O. T. Aydintuğ, "Cytokine inhibitors: soluble tumor necrosis factor receptor 1 and interleukin-1 receptor antagonist in Behçet's disease," *Rheumatology International*, vol. 25, no. 1, pp. 1–5, 2005.

[43] F. S. Neves and F. Spiller, "Possible mechanisms of neutrophil activation in Behçet's disease," *International Immunopharmacology*, vol. 17, no. 4, pp. 1206–1210, 2013.

[44] N. Maruotti, F. P. Cantatore, B. Nico, A. Vacca, and D. Ribatti, "Angiogenesis in vasculitides," *Clinical and Experimental Rheumatology*, vol. 26, no. 3, pp. 476–483, 2008.

[45] Y. Zhao and D. Bruemmer, "NR4A orphan nuclear receptors: transcriptional regulators of gene expression in metabolism and vascular biology," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 30, no. 8, pp. 1535–1541, 2010.

[46] A. V. Andreeva, M. A. Kutuzov, R. Vaiskunaite et al., "Gα_{12} interaction with αSNAP induces VE-cadherin localization at endothelial junctions and regulates barrier function," *Journal of Biological Chemistry*, vol. 280, no. 34, pp. 30376–30383, 2005.

[47] T. Chen, Z. P. Guo, N. Cao, S. Qin, M. M. Li, and R. Z. Jia, "Increased serum levels of soluble vascular endothelial-cadherin in patients with systemic vasculitis," *Rheumatology International*, vol. 34, no. 8, pp. 1139–1143, 2014.

[48] G. Saylam Kurtipek, R. Kesli, F. Tuncez Akyurek, F. Akyurek, A. Ataseven, and Y. Terzi, "Plasma-soluble urokinase plasminogen activator receptor (suPAR) levels in Behçet's disease and correlation with disease activity," *International Journal of Rheumatic Diseases*, vol. 21, no. 4, pp. 866–870, 2018.

[49] F. Fazzini, G. Peri, A. Doni et al., "PTX3 in small-vessel vasculitides: an independent indicator of disease activity produced at sites of inflammation," *Arthritis & Rheumatism*, vol. 44, no. 12, pp. 2841–2850, 2001.

[50] P. Margutti, P. Matarrese, F. Conti et al., "Autoantibodies to the C-terminal subunit of RLIP76 induce oxidative stress and endothelial cell apoptosis in immune-mediated vascular diseases and atherosclerosis," *Blood*, vol. 111, no. 9, pp. 4559–4570, 2008.

[51] K. Fujimori, K. Oh-i, M. Takeuchi et al., "Circulating neutrophils in Behçet disease is resistant for apoptotic cell death in the remission phase of uveitis," *Graefe's Archive for Clinical and Experimental Ophthalmology*, vol. 246, no. 2, pp. 285–290, 2008.

[52] P. Yang, L. Chen, H. Zhou et al., "Resistance of lymphocytes to Fas-mediated apoptosis in Behçet's disease and Vogt-Koyangi-Harada syndrome," *Ocular Immunology and Inflammation*, vol. 10, no. 1, pp. 47–52, 2002.

[53] S. Wakisaka, Y. Takeba, S. Mihara et al., "Aberrant Fas ligand expression in lymphocytes in patients with Behçet's disease," *International Archives of Allergy and Immunology*, vol. 129, no. 2, pp. 175–180, 2002.

[54] S. Morita, Y. Shirakata, A. Shiraishi et al., "Human corneal epithelial cell proliferation by epiregulin and its cross-induction by other EGF family members," *Molecular Vision*, vol. 13, pp. 2119–2128, 2007.

[55] J. Guo, G. Thinakaran, Y. Guo, S. S. Sisodia, and F. X. Yu, "A role for amyloid precursor-like protein 2 in corneal epithelial wound healing," *Investigative Ophthalmology & Visual Science*, vol. 39, no. 2, pp. 292–300, 1998.

[56] N. Rother and J. van der Vlag, "Disturbed T cell signaling and altered Th17 and regulatory T cell subsets in the pathogenesis of systemic lupus erythematosus," *Frontiers in Immunology*, vol. 6, p. 610, 2015.

[57] E. Lubberts, "The IL-23–IL-17 axis in inflammatory arthritis," *Nature Reviews Rheumatology*, vol. 11, no. 10, p. 562, 2015.

[58] M. Diani, G. Altomare, and E. Reali, "T helper cell subsets in clinical manifestations of psoriasis," *Journal of Immunology Research*, vol. 2016, Article ID 7692024, 7 pages, 2016.

[59] M. von Holleben, A. Gohla, K. P. Janssen, B. M. Iritani, and S. Beer-Hammer, "Immunoinhibitory adapter protein Src homology domain 3 lymphocyte protein 2 (SLy2) regulates actin dynamics and B cell spreading," *The Journal of Biological Chemistry*, vol. 286, no. 15, pp. 13489–13501, 2011.

[60] Y. Usui, M. Takeuchi, N. Yamakawa et al., "Expression and function of inducible costimulator on peripheral blood $CD4^+$ T cells in Behçet's patients with uveitis: a new activity marker?," *Investigative Ophthalmology & Visual Science*, vol. 51, no. 10, pp. 5099–5104, 2010.

[61] M. Hamedi, L. A. Bergmeier, E. Hagi-Pavli, S. R. Vartoukian, and F. Fortune, "Differential expression of suppressor of cytokine signalling proteins in Behçet's disease," *Scandinavian Journal of Immunology*, vol. 80, no. 5, pp. 369–376, 2014.

[62] Y. B. Shang, et al.N. Zhai, J. P. Li et al., "Study on association between polymorphism of HLA-DRB1 alleles and Behçet's disease," *Journal of the European Academy of Dermatology and Venereology*, vol. 23, no. 12, pp. 1419–1422, 2009.

[63] D. Saverino, R. Simone, M. Bagnasco, and G. Pesce, "The soluble CTLA-4 receptor and its role in autoimmune diseases: an update," *Autoimmunity Highlights*, vol. 1, no. 2, pp. 73–81, 2010.

[64] K. V. Salojin, B. D. Hamman, W. C. Chang et al., "Genetic deletion of Mst1 alters T cell function and protects against autoimmunity," *PLoS One*, vol. 9, no. 5, article e98151, 2014.

[65] M. Pinto and A. M. Carmo, "CD6 as a therapeutic target in autoimmune diseases: successes and challenges," *BioDrugs*, vol. 27, no. 3, pp. 191–202, 2013.

[66] Y. Liang, W. D. Xu, H. Peng, H. F. Pan, and D. Q. Ye, "SOCS signaling in autoimmune diseases: molecular mechanisms and therapeutic implications," *European Journal of Immunology*, vol. 44, no. 5, pp. 1265–1275, 2014.

[67] A. Ambrosi, A. Espinosa, and M. Wahren-Herlenius, "IL-17: a new actor in IFN-driven systemic autoimmune diseases," *European Journal of Immunology*, vol. 42, no. 9, pp. 2274–2284, 2012.

[68] R. C. Axtell, B. A. de Jong, K. Boniface et al., "T helper type 1 and 17 cells determine efficacy of interferon-β in multiple sclerosis and experimental encephalomyelitis," *Nature Medicine*, vol. 16, no. 4, pp. 406–412, 2010.

[69] Z. Brkic, O. B. Corneth, C. G. van Helden-Meeuwsen et al., "T-helper 17 cell cytokines and interferon type I: partners in crime in systemic lupus erythematosus?," *Arthritis Research & Therapy*, vol. 16, no. 2, p. R62, 2014.

[70] J. J. O'Shea and R. Plenge, "JAK and STAT signaling molecules in immunoregulation and immune-mediated disease," *Immunity*, vol. 36, no. 4, pp. 542–550, 2012.

[71] D. H. Kono, R. Baccala, and A. N. Theofilopoulos, "TLRs and interferons: a central paradigm in autoimmunity," *Current Opinion in Immunology*, vol. 25, no. 6, pp. 720–727, 2013.

[72] Y. Liu, H. Yin, M. Zhao, and Q. Lu, "TLR2 and TLR4 in autoimmune diseases: a comprehensive review," *Clinical Reviews in Allergy & Immunology*, vol. 47, no. 2, pp. 136–147, 2014.

[73] S. Fineschi, L. Goffin, R. Rezzonico et al., "Antifibroblast antibodies in systemic sclerosis induce fibroblasts to produce profibrotic chemokines, with partial exploitation of Toll-like receptor 4," *Arthritis & Rheumatism*, vol. 58, no. 12, pp. 3913–3923, 2008.

[74] S. K. Kwok, M. L. Cho, Y. M. Her et al., "TLR2 ligation induces the production of IL-23/IL-17 via IL-6, STAT3 and NF-κB pathway in patients with primary Sjogren's syndrome," *Arthritis Research & Therapy*, vol. 14, no. 2, p. R64, 2012.

[75] L. Li, H. Yu, Y. Jiang et al., "Genetic variations of NLR family genes in Behcet's disease," *Scientific Reports*, vol. 6, no. 1, article 20098, 2016.

[76] A. del Sol, R. Balling, L. Hood, and D. Galas, "Diseases as network perturbations," *Current Opinion in Biotechnology*, vol. 21, no. 4, pp. 566–571, 2010.

[77] A. L. Barabasi, N. Gulbahce, and J. Loscalzo, "Network medicine: a network-based approach to human disease," *Nature Reviews Genetics*, vol. 12, no. 1, pp. 56–68, 2011.

[78] A. Polo, A. Crispo, P. Cerino et al., "Environment and bladder cancer: molecular analysis by interaction networks," *Oncotarget*, vol. 8, no. 39, pp. 65240–65252, 2017.

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Research Article

Preterm Neonates with Respiratory Distress Syndrome: Ventilator-Induced Lung Injury and Oxidative Stress

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Ventilator-induced lung injury is well recognized, and appropriate arterial saturation target is unknown, so gentle modes of ventilation and minimizing oxidative stress have been well studied. Our objective was to analyze any association between the oxygen levels at blood sampling and plasma levels of the interleukins IL-6, IL-1β, IL-10, and IL-8 and TNF-α in preterm newborns under mechanical ventilation (MV) in their first two days. *Methods*. Prospective cohort including neonates with severe respiratory distress. Blood samples were collected right before and 2 hours after invasive MV. For analysis purposes, newborns were separated according to oxygen requirement: low oxygen (≤30%) and high oxygen (>30%) groups. Interleukins were measured using a commercially available kit. *Results*. 20 neonates (gestational age 32.2 ± 3 weeks) were evaluated. Median O_2 saturation levels pre-MV were not different in both oxygen groups. In the high oxygen group, IL-6, IL-8, and TNF-α plasma levels increased significantly after two hours under MV. *Conclusions*. Despite the small sample studied, data showed that there is a relationship between VILI, proinflammatory cytokines, and oxygen-induced lung injury, but a study considering oxidative marker measurements is needed. It seems that less oxygen may keep safer saturation targets playing a less harmful role.

1. Introduction

It is well recognized that in preterm neonates, the need for invasive mechanical ventilation (MV) is associated with the so-called ventilator-induced lung injury (VILI); thus, lung protection strategies have been intensely studied in the past 20 years. Research has focused on gentle modes of ventilation as well as in reducing oxidative stress.

Superoxide, hydrogen peroxide, and perhydroxyl radicals cause oxygen-induced lung injury, and the premature infant is notably susceptible to free radical-induced injury because antioxidant systems develop late during the gestation. Excessive hyperoxia can lead to lung inflammation, diffuse alveolar injury, progressive pulmonary damage, and death [1].

The suitable arterial saturation target in preterm neonates is unknown, but exposing them to a high concentration of oxygen is related to increased risks of retinopathy of prematurity and bronchopulmonary dysplasia (BPD). Recently published large multicenter trials have studied this issue [2].

In this pilot study, our objective was to analyze any association between the oxygen levels at blood sampling and plasma levels of interleukin- (IL-) 6, IL-1β, IL-10, IL-8, and tumor necrosis factor- (TNF-) α in preterm infants under MV in their first two days of life.

2. Methods

This prospective observational study included preterm infants ranging from 28 to 35 weeks of gestational age (GA) submitted to intubation and MV in the first 48 hours of life who were admitted to the newborn section of Hospital de Clínicas de Porto Alegre (HCPA), a tertiary referral medical center located in Southern Brazil. The Ethics Committee of

HCPA approved the study, and informed consent was obtained from the patients' guardians prior to enrollment.

The exclusion criteria were congenital infections, congenital malformations, proven sepsis or meningitis, need for intubation in the delivery room, and use of prophylactic surfactant prior to enrollment in the study.

Clinical data included gender, birth weight, GA, Score for Neonatal Acute Physiology Perinatal Extension II (SNAPPE-II) regarding the first 12 hours of life, the type of delivery, and antenatal factors such as the presence of amniorrhexis, preeclampsia, steroid use, and/or chorioamnionitis. The patients were separated into two groups according to oxygen need during their first two days of life: low oxygen (LO), when in MV and requiring ≤30% oxygen, and high oxygen (HO), when in MV and requiring >30% oxygen. The patients received more or less oxygen by the Neonatal Intensive Care Unit (NICU) staff based on their symptoms and signs at the initiation of the treatment, and this was not controlled by the research team.

The neonates were followed from birth to tracheal intubation and the onset of MV; blood samples were collected according to the NICU routine for arterial blood gas analysis, and an additional 500 μL aliquot was obtained for later cytokine analysis. Another sample was collected after two hours of invasive respiratory support. Plasma was frozen at −80°C for laboratory analysis, and the measurement of cytokines was performed using a commercially available multiplex kit (MILLIPLEX® Millipore Corporation). Readings were performed with Luminex 100 technology.

3. Statistical Analysis

The Mann–Whitney *U* test compared cytokine levels in both oxygen groups. Wilcoxon's signed-rank test compared the pre- and post-MV interleukin levels. Spearman's test was applied to verify any correlation between oxygen levels and cytokine levels in both MV moments. All analyses were carried out with Statistical Package for Social Sciences (SPSS), version 20.0 (Seattle, USA), and a significance level was established at $p < 0.05$.

4. Results

Twenty preterm infants were included: 9 (45%) were males, 7 (35%) were small for gestational age, 16 (80%) were delivered by C-section, and 7 (37%) were delivered by preeclamptic mothers. Only 7 mothers (35%) received a full antenatal steroid course. The mean birth weight was 1921.5 ± 743 grams, and the mean GA was 32.2 ± 3weeks. The median SNAPPE-II score at 12 hours of life was 19 (7–29). The sample collection time for cytokine measurement pre-MV was 9 (3–48) hours of life. Median arterial saturation levels were ≥90% in both oxygen groups and in both moments.

IL-6, IL-8, and TNF-α median plasma levels significantly increased after 2 hours of MV in the high oxygen group (Figure 1). Comparing both oxygen groups, IL-6 levels were higher in the HO group after MV, and IL-10 levels before MV were higher in the HO group than in the LO group (Table 1).

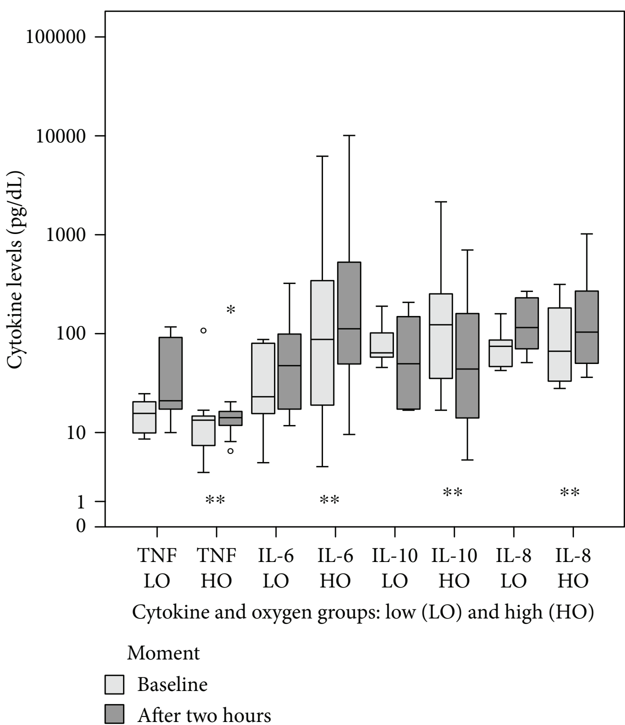

Figure 1: Cytokine levels immediately after the onset of MV and after 2 hours—Wilcoxon's test; *outlier, $^{**}p < 0.05$.

The oxygen levels pre-MV and interleukin levels were compared in both moments. There was a correlation between O_2 and IL-6 levels collected after two hours of MV ($r = 0,48$, $p = 0.03$) and a trend of correlation between O_2 and IL-6 levels collected pre-MV ($r = 0.4$, $p = 0.08$), but there were no correlations regarding the other interleukins or TNF-α.

5. Discussion

In this pilot study, we demonstrated that even a short period under invasive mechanical ventilation associated with higher oxygen levels may lead to lung inflammation: higher IL-6, IL-8, and TNF-α median levels and lower IL-10 median levels were found. This suggests a relationship between VILI and oxygen-induced lung injury.

During hyperoxia, the rupture of the airway epithelium barrier may increase pulmonary permeability, release of inflammatory mediators, and fluid congestion [1], with significant cell infiltration into the immature lung. These cells produce reactive oxygen species prompting pulmonary and airway remodeling, and it has been demonstrated that preterm neonates have elevated levels of proinflammatory mediators in tracheal aspirates and bronchoalveolar lavage fluid [3]. Indeed, all these modifications may occur due to MV per se and not because of high oxygen exposure, although an interaction between both risk factors occurs.

We demonstrated a statistically significant increase in IL-6, IL-8, and TNF-α after two hours of invasive mechanical ventilation. In a previous study, which included full-term newborns and late preterm newborns, similar IL-6

TABLE 1: Comparing cytokine levels regarding the FiO2 group immediately before the onset of MV and after 2 hours.

	IL-6	IL-8	IL-10	TNF-α	IL-1β
Pre-MV					
FiO2 ≤ 30%	23 (8–71)	56 (34–134)	51 (23–60)	17 (10.5–19)	11 (5.6–23.5)
FiO2> 30%	68 (15–94.5)	80 (32–176)	122 (52–236)	12 (7–13.5)	8 (7–11)
P^1	0.38	0.68	0.05	0.14	0.38
After 2 h					
FiO2 ≤ 30%	21.5 (9–81)	74.5 (56–142)	52 (45.5–169)	17 (10–20)	10 (5–24)
FiO2> 30%	110 (50–490)	179 (51–272)	29 (13–155)	12 (7–13.5)	8.5 (7.5–13)
P^1	0.05	0.55	0.43	0.61	0.61

[1]Mann–Whitney *U* test.

levels were obtained after two hours of MV [4], suggesting that lower gestational ages may play a role in IL-6 release. In fact, IL-6 was 6.4 times higher in preterm newborns as compared to full-term newborns at birth, which suggests that inflammatory stress occurs even prior to ventilation, resulting from preterm birth and/or labor [5]. The findings of Leviton et al. [6] also support this theory of a fetal inflammatory response, which is stronger at lower gestational ages.

IL-10 levels pre-MV were higher in the HO group than in the LO group; nevertheless, after MV, these levels had decreased. It is known that the release of anti-inflammatory IL-10 occurs only after the increase of IL-8, notably in immature infants below 30 weeks of GA [7], leaving them more vulnerable to intense proinflammatory response [8]. Pre-MV IL-10 higher levels, however, would suggest an early and efficient response to respiratory distress that required high oxygen, which was reduced after adding MV.

Randomized controlled masked trials enrolling almost 5000 very preterm babies have shown that, once patients are stable, keeping higher oxygen saturation targets (91–95%) until 36 weeks' postmenstrual age showed no differences in the combined outcome of mortality or severe disability compared to lower targets (85–89%); however, mortality before 18 to 24 months was higher in the lower-target group. Recent meta-analyses pooled these results and confirmed that the ideal SpO2 range for extremely low birth weight infants remains unclear [2]. In our sample, the SpO2 range was higher than the recommended in both oxygen groups, but our gestational age range was also higher, involving newborns who were still unstable. Besides that, as extremely preterm newborns are more likely to develop systemic inflammation than other preterm categories, they were not included in this pilot study also to avoid impact on baseline cytokine levels [9].

A trial comparing higher (90–100%) versus lower (21–30%) initial FiO_2 in the delivery room showed that newborn infants receiving a higher O_2 load had also higher concentrations of oxidative stress biomarkers and were more predisposed to develop bronchopulmonary dysplasia [10]. A meta-analysis [11] comparing high (>50%) and low (<50%) initial FiO_2 in patients < 32 weeks of GA found no differences regarding mortality and/or morbidity (from BPD, severe intraventricular hemorrhage, necrotizing enterocolitis, or retinopathy). Conversely, other meta-analyses [12] which included 10 recently published studies showed that mortality was higher in preterm newborns with a high initial FiO_2 (0.60–1.0) but did not find any differences regarding BPD and intraventricular hemorrhage. The authors therefore concluded that very preterm infants should be initially ventilated with lower FiO_2 (0.21–0.30) and that they should be titrated according to the neonate's response.

We described a positive correlation between baseline O_2 levels and IL-6 levels after two hours of MV ($r = 0.48$, $p = 0.03$) and also a trend of correlation regarding pre-MV IL-6 levels ($r = 0.4$, $p = 0.08$). It seems that these preterm infants who needed more oxygen pre-MV presented higher IL-6 levels in both moments, suggesting that they were more injured and sicker at baseline. It is postulated that oxidative stress promotes the expression of cytokines and the inflammatory process during respiratory distress syndrome [13], which is in agreement with our idea of sicker newborns requiring higher oxygen therefore releasing more cytokines. Also, it is well known that proinflammatory mediators such as IL-6 may be increased due to fetal exposure to maternal inflammatory mediators [14]. Thus, it is accepted that previous lung damage makes them more likely to have oxygen-induced injury, leading to inflammation that is not limited to the lung [15].

Despite the small sample studied, data showed that there is a relationship between VILI, proinflammatory cytokines, and oxygen-induced lung injury, but a larger study considering oxidative marker measurements should be carried out. Mechanical ventilation alone, oxygen, or both could have increased inflammatory mediators in this sample, so the causal relationship between them still remains unclear. In view of the trials and meta-analysis described and regarding the pathophysiologic aspects of oxidative stress, one should recommend low oxygen levels enough to keep a safe saturation target, at the most extreme preterm infants.

Data Availability

No data is available elsewhere regarding this manuscript.

Disclosure

This manuscript was previously published as an abstract in the Journal of Critical Care, 2017, regarding XIII World Congress of Intensive and Critical Care Medicine.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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References

[1] V. Bhandari, "Molecular mechanisms of hyperoxia-induced acute lung injury," *Frontiers in Bioscience*, no. 13, pp. 6653–6661, 2008.

[2] V. Manja, O. D. Saugstad, and S. Lakshminrusimha, "Oxygen saturation targets in preterm infants and outcomes at 18-24 months: a systematic review," *Pediatrics*, vol. 139, no. 1, article e20161609, 2017.

[3] E. R. Vogel, R. D. Britt Jr., M. C. Trinidad et al., "Perinatal oxygen in the developing lung," *Canadian Journal of Physiology and Pharmacology*, vol. 93, no. 2, pp. 119–127, 2015.

[4] B. Bohrer, R. C. Silveira, E. C. Neto, and R. S. Procianoy, "Mechanical ventilation of newborns infant changes in plasma pro- and anti-inflammatory cytokines," *The Journal of Pediatrics*, vol. 156, no. 1, pp. 16–19, 2010.

[5] C. Chiesa, F. Signore, M. Assumma et al., "Serial measurements of C-reactive protein and interleukin-6 in the immediate postnatal period: reference intervals and analysis of maternal and perinatal confounders," *Clinical Chemistry*, vol. 47, no. 6, pp. 1016–1022, 2001.

[6] A. Leviton, R. Fichorova, Y. Yamamoto et al., "Inflammation-related proteins in the blood of extremely low gestational age newborns. The contribution of inflammation to the appearance of developmental regulation," *Cytokine*, vol. 53, no. 1, pp. 66–73, 2011.

[7] M. W. Beresford and N. J. Shaw, "Detectable IL-8 and IL-10 in bronchoalveolar lavage fluid from preterm infants ventilated for respiratory distress syndrome," *Pediatric Research*, vol. 52, no. 6, pp. 973–978, 2002.

[8] B. Jónsson, Y. H. Li, G. Noack, A. Brauner, and K. Tullus, "Downregulatory cytokines in tracheobronchial aspirate fluid from infants with chronic lung disease of prematurity," *Acta Paediatrica*, vol. 89, no. 11, pp. 1375–1380, 2000.

[9] R. L. Goldenberg, J. F. Culhane, J. D. Iams, and R. Romero, "Epidemiology and causes of preterm birth," *The Lancet*, vol. 371, no. 9606, pp. 75–84, 2008.

[10] M. Vento, M. Moro, R. Escrig et al., "Preterm resuscitation with low oxygen causes less oxidative stress, inflammation, and chronic lung disease," *Pediatrics*, vol. 124, no. 3, pp. e439–e449, 2009.

[11] J. V. E. Brown, T. Moe-Byrne, M. Harden, and W. McGuire, "Lower versus higher oxygen concentration for delivery room stabilisation of preterm neonates: systematic review," *PLoS One*, vol. 7, no. 12, article e52033, 2012.

[12] O. D. Saugstad, D. Aune, M. Aguar, V. Kapadia, N. Finer, and M. Vento, "Systematic review and meta-analysis of optimal initial fraction of oxygen levels in the delivery room at ≤32 weeks," *Acta Paediatrica*, vol. 103, no. 7, pp. 744–751, 2014.

[13] E. Gitto, R. J. Reiter, S. P. Cordaro et al., "Oxidative and inflammatory parameters in respiratory distress syndrome of preterm newborns: beneficial effects of melatonin," *American Journal of Perinatology*, vol. 21, no. 4, pp. 209–216, 2004.

[14] S. Aversa, L. Marseglia, S. Manti et al., "Ventilation strategies for preventing oxidative stress-induced injury in preterm infants with respiratory disease: an update," *Paediatric Respiratory Reviews*, vol. 17, pp. 71–79, 2016.

[15] C. Poggi and C. Dani, "Antioxidant strategies and respiratory disease of the preterm newborn: an update," *Oxidative Medicine and Cellular Longevity*, vol. 2014, no. 36, Article ID 721043, 10 pages, 2014.

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Review Article

Herbal Compounds Play a Role in Neuroprotection through the Inhibition of Microglial Activation

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Since microglia possess both neuroprotective and neurotoxic potential, they play a crucial role in the central nervous system (CNS). Excessive microglial activation induces inflammation-mediated neuronal damage and degeneration. At present, numerous herbal compounds are able to suppress neurotoxicity via inhibiting microglial activation. Therefore, many researchers focus on pharmacological inhibitors of microglial activation to ameliorate neurodegenerative disorders. Further work should concentrate on the exploration of new herbal compounds, which characteristically inhibit microglial neurotoxicity, rather than modulating neuroprotection alone. In this review, we summarize these herbal compounds, which in the past several years have been shown to exert potential neuroprotective activity by inhibiting microglial activation. The therapeutic targets and pharmacological mechanisms of these compounds have also been discussed.

1. Introduction

A large amount of evidence has demonstrated that neuroinflammation plays a significant role in both acute and chronic neurodegenerative disorders including Parkinson's disease, Alzheimer's disease, multiple sclerosis, stroke, and traumatic brain injury (TBI) [1–3]. They are all related to microglial activation and are accompanied by high expression of proinflammatory mediators. Neuroinflammation is a defense mechanism with the purpose of preventing the CNS from being infected and damaged. Microglia, the resident macrophages of the CNS, act as the primary effector cells in mediating neuroinflammation, the activation of which is characteristic of several inflammatory and neurodegenerative disorders [4–7]. Microglia support the normal function of neurons and monitor the health of neurons in homeostasis, the resting state. Therefore, microglia display beneficial effects in normal conditions. Once brain injury or infection occurs, microglia turn into the activated state and secrete a series of proinflammatory and neurotoxic mediators, such as interleukin-1 beta (IL-1β), nitric oxide (NO), tumor necrosis factor alpha (TNF-α), and reactive oxygen species (ROS), which not only regulate neuronal function and synaptic transmission but also give rise to neuronal oxidative stress and degeneration associated with deficits in a variety of cognitive and memory tasks [8–10]. As mentioned above, activated microglia could cause and regulate the neuroinflammatory reaction by impairing neurons with a mass of proinflammatory mediators that may result in neuronal death in the end.

Thus, protecting neurons through suppressing microglial activation and neuroinflammation may be considered as a potential therapeutic method for improving neurodegenerative disorders. A number of studies proved that natural plants and their active ingredients could ameliorate neurodegenerative progression by suppressing microglial activation and

neuronal damage [11, 12]. This article discusses the conclusions about natural compounds, which based on recent studies could prevent neurons from damage via inhibiting microglial activation and neuroinflammation.

2. Herbal Compounds

2.1. Resveratrol. Resveratrol is a polyphenolic phytochemical that is extracted from plants including grape, peanut, and berry with pharmacological effects on multiple pathological phenomena [13, 14]. Research has shown that resveratrol has bioactivity-containing antioxidative, anti-inflammatory, and anticancer properties and neuroprotection [15–17].

Myeloperoxidase (MPO) plays a very important role in the host defense system against many pathogens. Research has demonstrated that both overactivation and deficiency of MPO result in a pathological state in the brain. However, resveratrol remarkably decreases MPO levels and NO production, which obviously suppressed neuroinflammatory responses including phagocytosis and ROS production in rotenone-triggered microglia. Resveratrol could alleviate the rotenone-induced impaired responses of primary mixed glia from $MPO^{-/-}$ mice. In neuron-glia cocultures, the impairment of neurons could be relieved by resveratrol. The results displayed that resveratrol affected the microglial response to rotenone via modulating MPO and thus prevented neurons from rotenone-induced injury. As mentioned above, the regulation of MPO levels in microglia by resveratrol provide its neuroprotective ability [18]. Resveratrol remarkably improved trigeminal allodynia dose dependently and reduced the high levels of calcitonin gene-related peptide and c-Fos expression in the spinal trigeminal nucleus. In addition, resveratrol inhibited chronic constriction injury-provoked astrocyte and microglial activation and decreased the levels of proinflammatory mediators in the spinal trigeminal nucleus. Moreover, the effect of resveratrol on pain relief was partially regulated via suppressing the phosphorylation of mitogen-activated protein kinases (MAPKs) through the activation of adenosine monophosphate-activated protein kinase [19]. A study demonstrated that in BV2 microglial cell lines, resveratrol could inhibit NLR family pyrin domain containing 3 (NLRP3) activation and IL-1β cleavage caused by ATP. In summary, resveratrol could alleviate the deficit of spatial memory in mice with sepsis-associated encephalopathy by suppressing the NLRP3/IL-1β axis in microglia [20]. Resveratrol could not only reduce nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-induced level of ROS generation but also alleviate the transposition of the subunit of NADPH oxidase to the cytomembrane caused by lipopolysaccharide (LPS). Furthermore, the effects of resveratrol on neuroprotection were also relevant to the suppression of the activation of MAPKs and nuclear factor-kappa B (NF-κB) signaling pathways in microglia. The study explicitly revealed that resveratrol could prevent dopaminergic neurons from being damaged by LPS, which depended on time and concentration by inhibiting microglial activation and the expression of proinflammatory mediators [21].

The other research has demonstrated that resveratrol treatment in rat with subarachnoid hemorrhage (SAH) could obviously decrease the expression of Toll-like receptor 4 (TLR4), high-mobility group box1 protein, myeloid differentiation factor (MyD88), and NF-κB. In addition, resveratrol remarkably inhibited microglial activation and proinflammatory mediators, which gave rise to the alleviation of neuronal apoptosis, cerebral edema, and behavior deficits at 24 h after SAH [22]. In conclusion, resveratrol is capable of exerting neuroprotection via suppressing microglial activation through the blockage of related pathways, such as the TLR4/MAPK and NF-κB pathways.

2.2. Gastrodin. Gastrodin is the primary bioactive component derived from the traditional Chinese herb *Gastrodia elata* Blume root and has been widely used as an anticonvulsant, analgesic, anti-inflammatory, antioxidative, and sedative agent [23, 24].

The research indicated that gastrodin remarkably decreased the levels of proinflammatory mediators such as cyclooxygenase-2 (COX-2), TNF-α, inducible nitric oxide synthase (iNOS), and IL-1β via blocking the activation of the NF-κB and MAPK pathways in microglia induced by LPS [25]. Li et al. revealed that gastrodin could protect dopaminergic neurons via obviously suppressing microglial activation and the level of IL-1β, COX-2, and iNOS in the substantia nigra of rotenone-induced rats with Parkinson's disease [26]. The other research also reported that gastrodin was capable of significantly improving chronic inflammatory pain and the accompanying anxiety-like behaviors in mice induced by complete Freund's adjuvant (CFA). Furthermore, gastrodin treatment could downregulate the increasing expression of glutamate receptor 1, N-methyl-d-aspartate receptor subunit 2A, N-methyl-d-aspartate receptor subunit 2B, and Ca^{2+}/calmodulin-dependent protein kinase II-alpha by reducing microglial activation and proinflammatory mediators such as TNF-α and IL-6 in the anterior cingulate cortex of mice with CFA injection [24].

Recently, there was also significant evidence indicating that gastrodin elicited strong neuroprotective effects against loss of retinal ganglion cells in an acute glaucoma rat via inhibiting phosphorylated p38 MAPK and the production of proinflammatory mediators in activated retinal microglia. The results demonstrated that gastrodin possessed a potential therapeutic effect on acute glaucoma and other retinal neurodegenerative diseases by suppressing microglial activation [27]. In conclusion, gastrodin is a new drug that could protect neurons through inhibiting microglial activation.

2.3. Trans-Cinnamaldehyde. Trans-cinnamaldehyde (TCA) is a main component isolated from the stem bark of *Cinnamomum cassia*, which has been reported to have anti-inflammatory, antioxidative, antibacterial, antifungal, and antiapoptotic properties in a large amount of in vitro and in vivo models [28–30].

A study revealed that TCA could decrease viability loss and apoptosis in neuronal PC12 cells induced by oxygen and glucose deprivation/reperfusion. The effect indicated that TCA could reduce the production of NO. Additionally, using LY294002, the inhibitor of phosphoinositide 3-kinase

(PI3K), could abolish the neuroprotection of TCA, demonstrating that the neuroprotection of TCA can be induced via provoking the PI3K pathway [31]. Other studies confirmed that TCA could inhibit LPS-induced inflammation in BV2 cells and reduce the infarction area and neurological deficit score in injured cerebral tissue of mice induced with ischemia/reperfusion. Furthermore, TCA could obviously alleviate neuronal damage by decreasing the levels of iNOS and COX-2 expression through blocking the NF-κB pathway in injured cerebral tissue of mice induced with ischemia/reperfusion. Therefore, TCA may recede neuroinflammation by suppressing microglial activation and play a key role in neuroprotection [32]. Recent research also showed that TCA could promote the degradation of iNOS mRNA in LPS-induced microglia, thus reducing NO production. Additionally, TCA could not only significantly decrease the expression of iNOS and phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2) in the hippocampus but also evidently alleviate memory deficits and synaptic plasticity damage in LPS-induced mice. They concluded that TCA improved neuronal damage by suppressing microglial activation via degrading the stability of iNOS mRNA [33]. In addition, TCA decreased the expression of iNOS and COX-2 in the LPS-stimulated BV2 cells and noticeably increased the number of tyrosine hydroxylase-positive dopaminergic neurons in the striatum and substantia nigra of mice with 6-hydroxydopamine challenge. These data indicated that TCA has a function of neuroprotection on dopaminergic neurons, which is associated with the suppression of neuroinflammatory responses induced by microglial activation [34]. As mentioned above, TCA has the potential to prevent neuronal damage via inhibiting microglial activation.

2.4. Salvianolic Acid B. Salvianolic acid B (Sal B) is the main active ingredient as a water-soluble component of *Salvia miltiorrhiza* roots (Danshen). The studies revealed that Sal B possessed anticancer activity [35, 36]. Other researches confirmed the therapeutic potential of Sal B on hepatic protection, cardiovascular protection, and neuroprotection [37, 38].

In recent research, Sal B could suppress neutrophil infiltration and microglial activation after TBI. Salvianolic acid B could not only reduce the productions of proinflammatory mediators such as TNF-α and IL-1β but could also upregulate the levels of anti-inflammatory mediators such as IL-10 and transforming growth factor beta 1. These results demonstrated that the neuroprotective role of Sal B on the TBI model may be related to its anti-inflammatory effects [38]. Research has shown that Sal B could reduce the mRNA levels of iNOS, TNF-α, and IL-1β in LPS-stimulated microglia by decreasing NF-κB activation. Moreover, Sal B could prevent neuronal damage via the inhibition of microglial activation in a coculture system including microglia and neurons [39]. A study demonstrated that Sal B treatment remarkably lessened the infarction volume and neuroinflammation in the middle cerebral artery occlusion rat model. The TLR4/NF-κB pathway could be significantly suppressed by Sal B treatment in the ischemic hemisphere via inhibiting the activation of microglia. Meanwhile, the secretion of IL-1β and IL-6 could be decreased by Sal B. This study confirmed that Sal B could significantly alleviate brain damage following cerebral ischemia by inhibiting inflammation in activated microglia [40]. In conclusion, Sal B is a potential herbal compound to improve neuronal damage through inhibiting microglial activation and neuroinflammation.

2.5. Tanshinone. Tanshinone is one of the constituents extracted from *Salvia miltiorrhiza* roots, containing tanshinone I and tanshinone IIA. Tanshinone I is one of the critical active ingredients and exhibits many bioactivities, including antioxidative and anti-inflammatory activities in several laboratorial models [41–43]. Research displayed that tanshinone I could destroy the biomembrane reactor in vitro and decrease the bacterial content in vivo [44]. Tanshinone I could protect mitochondria via the nuclear factor erythroid 2-related factor 2-dependent mechanism in SH-SY5Y cells induced by paraquat [45].

Further studies revealed that tanshinone I could significantly decrease the production of several proinflammatory mediators including TNF-α, NO, IL-1β, and IL-6 and also distinctly inhibit NF-κB activation in activated M1 microglia stimulated by LPS. Furthermore, tanshinone I had the ability to improve motor function, normalize striatal neurotransmitters, and protect dopaminergic neurons in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine- (MPTP-) intoxicated mice. The animal studies also revealed that tanshinone I might reduce the increase in TNF-α and IL-10 concentrations through modulating microglial activation in MPTP-intoxicated mice. Therefore, tanshinone I has the potential to protect nigrostriatal dopaminergic neurons by decreasing the level of proinflammatory mediators through the inhibition of NF-κB activation in microglia [46].

Tanshinone IIA is also an active constituent of *Salvia miltiorrhiza* and has been widely used for many years in Asia to treat various diseases for its observable organ protective activities [47, 48]. Research showed that MPTP could not only damage nigrostriatal dopaminergic neurons but also induce microglial activation. Western blot and immunohistochemistry revealed that MPTP could increase the expression of NADPH oxidase and iNOS in substantia nigra pars compacta. In addition, the impairment of nigrostriatal dopaminergic neurons and the high expression of NADPH oxidase and iNOS could be reversed by tanshinone IIA treatment. Thus, tanshinone IIA could protect nigrostriatal dopaminergic neurons through suppressing microglial activation and reducing the expression of NADPH oxidase and iNOS in the model of Parkinson's disease [49]. As mentioned above, tanshinones are likely to protect neurons via suppressing microglial activation and reducing neuroinflammation and oxidative stress.

2.6. Oxymatrine. Oxymatrine is a major active ingredient isolated from *Sophora flavescens* Ait (kushen), which has been used in China for thousands of years. It has been reported that oxymatrine could exhibit anticancer, antiapoptotic, and neuroprotective effects [50–52].

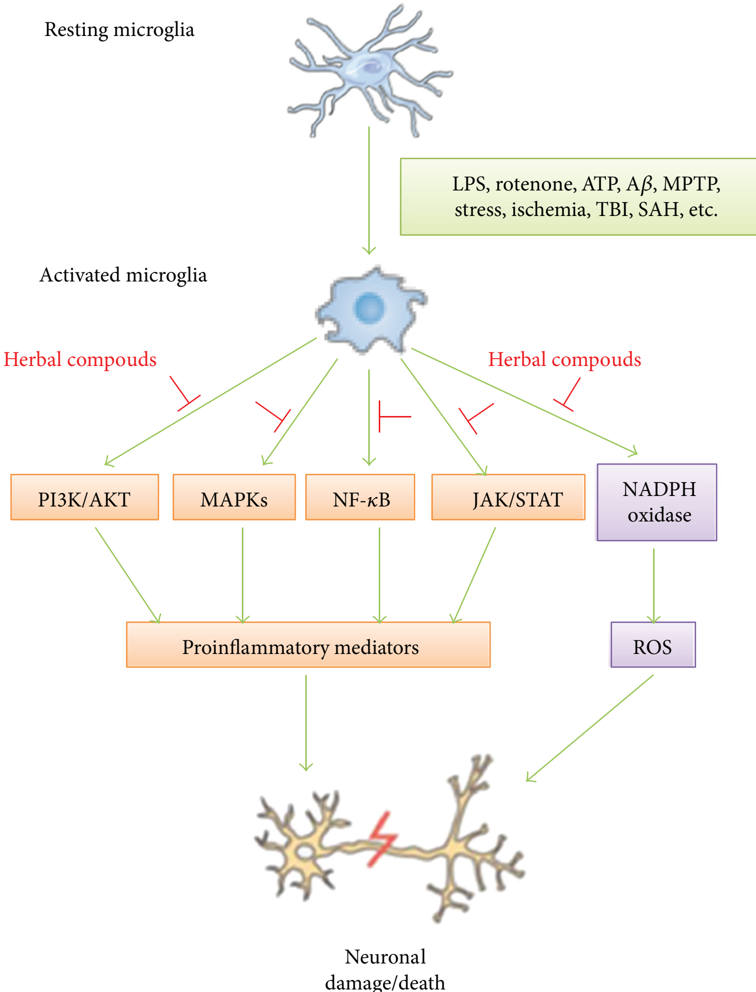

Figure 1: Schematic diagram represents prevention of neuronal damage/death by herbal compounds via inhibiting microglial activation through the blockage of related signaling pathway. LPS: lipopolysaccharide; Aβ: amyloid beta; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; TBI: traumatic brain injury; SAH: subarachnoid hemorrhage; PI3k/AKT: phosphoinositide 3-kinase/protein kinase B; MAPKs: mitogen-activated protein kinases; NF-κB: nuclear factor-kappa B; JAK/STAT: Janus kinase/signal transducers and activators of transcription; NADPH oxidase: nicotinamide adenine dinucleotide phosphate oxidase; ROS: reactive oxygen species.

Oxymatrine could not only reduce the secretion of heat shock protein 60 (HSP60) in BV2 cells stimulated by LPS but also decrease the expression of heat shock factor 1, which is the transcription factor of HSP60. In addition, oxymatrine could alleviate the expression of MyD88, caspase-3, NF-κB, IL-6, iNOS, TNF-α, and IL-1β in LPS-stimulated BV2 cells. From the results mentioned above, oxymatrine plays a key role in protecting neurons by blocking microglial activation and HSP60/TLR-4/MyD88/NF-κB pathways. Therefore, oxymatrine, herbal compound, represents as a potent therapeutic agent against microglial activation for ameliorating neurodegenerative disorders [53].

2.7. Curcumin. Curcumin is a primary ingredient of turmeric, and studies have shown its anti-inflammatory and antioxidative effects, and it observably alleviates CFA-induced pain hypersensitivity [54].

Studies revealed that curcumin could decrease amyloid beta 42- (Aβ42-) induced expression of IL-1β, IL-6, and TNF-α in microglia, depending on its concentration. Moreover, curcumin showed an effect of inhibiting the levels of phosphor-ERK1/2 and p38 in Aβ42-activated microglia. These results demonstrated that curcumin alleviated proinflammatory mediators released by microglia via inhibiting ERK1/2 and p38 signaling pathways [55]. Curcumin could dramatically ameliorate the phagocytic abilities of prostaglandin E_2- (PGE_2-) stimulated N9 cells. Further, curcumin could reverse the decreased effect of PGE_2 on Aβ42-induced microglial phagocytosis via inhibiting PGE_2 receptor subtype 2 and protein kinase A signaling pathways [56].

Curcumin inhibited inflammatory response and microglial activation by decreasing the upregulated fractalkine/CX3C chemokine receptor 1, thus protecting neuronal injury in the hippocampal dentate gyrus of fructose-fed mice [57]. Furthermore, the other result demonstrated that using curcumin nanoparticles may be a feasible way of enhancing neurological function in early brain injury of rat following SAH [58]. In conclusion, curcumin is a promising herbal compound to protect neuronal damage

in degenerative disorders via suppressing the inflammatory response in microglia.

2.8. Other Herbal Compounds. Isobavachalcone is the major constituent extracted from *Fructus psoraleae,* which presents versatile effects including antitumor [59, 60], antibacterial [61], and bone strengthening [62] effects. A study showed that isobavachalcone could recede the LPS-induced oxidative stress and inflammatory cytokine levels and that it possessed an effect of neuroprotection by inhibiting microglia-mediated inflammation [63]. Scutellarin, a member of flavone glucuronide, is considered the major active component of *Erigeron breviscapus* [64, 65]. Scutellarin was able to reduce the distribution of activated microglia and the levels of TNF-α, IL-1β, and iNOS in vivo. In vitro, it had the ability to prohibit the upregulated level of ROS, NO, and iNOS in LPS-induced BV2 cells [66]. Additionally, many studies demonstrated that scutellarin could regulate the activation of microglia and protection of neurons by the anti-inflammatory effect in primary microglia and BV2 cells [67]. Sophoraflavanone G decreased the cytotoxicity of conditioned medium prepared by activated BV2 cells induced by LPS to PC12 cells and increased cell viability. As mentioned above, sophoraflavanone G was able to suppress neuroinflammation via MAPKs, PI3K/protein kinase B, Janus kinase/signal transducers, and activators of transcription and nuclear factor erythroid 2-related factor 2/heme oxygenase-1 signaling pathways and might react as a potential constituent for various neuroinflammatory conditions [68]. A recent report revealed that four sesquiterpenoids isolated from *Tussilago farfara* also had neuroprotective effects by reducing the levels of NO, PGE_2, TNF-α, and ROS in the LPS-stimulated BV2 cell and PC12 cell coculture system through the blockage of the NF-κB pathway [69]. The other study indicated that treatment with baicalein, a flavonoid from *Scutellaria baicalensis* Georgi, exerted neuroprotective effects on dopaminergic neurons by decreasing TNF-α, NO, and superoxide productions in the neuron-glia coculture system with LPS stimulation and blocking morphological change of microglial activation [70]. In addition, Wogonin, another flavonoid from the root of *Scutellaria baicalensis* Georgi, also had a potent neuroprotection by suppressing microglial activation through the blockage NF-κB pathway in vivo and in vitro experiment [71]. These results suggest that a number of natural compounds have the potential to protect neurons via inhibiting microglial activation. Further, it is possible for these natural compounds to be used as therapeutics for neurodegenerative disorders with neuroinflammation.

3. Conclusions

An increasing amount of findings has demonstrated that microglial activation and neuroinflammation play a crucial role in the pathogenesis of neurodegenerative disorders. Recent researches revealed that there are many compounds isolated from natural plants that can delay the neuronal damaged and degenerative progression by inhibiting microglial activation, so they have attracted considerable attention as pharmacological intervention against neurodegenerative discords with neuroinflammatory condition. As illustrated in the summary diagram (Figure 1), these literatures provide the evidences that herbal compounds can protect neuronal damage characterized by neuroinflammatory and oxidative stress condition and they accomplish their role by suppressing microglial activation and proinflammatory and neurotoxic mediator expression via blocking the related signaling pathway in activated microglia. Moreover, the natural products and compounds are inexpensive, easily accessible, and safe. Therefore, they can be widely observed in laboratory researches. If these activities of herbal compounds that are found in laboratory research are beneficial to delaying the development of neurodegenerative disorders, then large and well-designed studies in clinic are required to confirm whether or not their activity is also possible in humans. Further study of the pharmacological mechanisms of natural herbal compounds on the inhibition of microglial activation and neuroinflammation could not only benefit the discovery of effective neuroprotective components but also help researchers to learn more about the pathological mechanisms of neurodegenerative disorders.

Conflicts of Interest

The authors declare no competing financial interests.

Authors' Contributions

Yan Fu, Jianmei Yang, and Xingyu Wang contributed equally to this work.

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References

[1] B. Grimmig, J. Morganti, K. Nash, and P. Bickford, "Immunomodulators as therapeutic agents in mitigating the progression of Parkinson's disease," *Brain Sciences*, vol. 6, no. 4, 2016.

[2] R. Hesse, A. Wahler, P. Gummert et al., "Decreased IL-8 levels in CSF and serum of AD patients and negative correlation of MMSE and IL-1β," *BMC Neurology*, vol. 16, no. 1, p. 185, 2016.

[3] E. Rissanen, J. Tuisku, J. Rokka et al., "In vivo detection of diffuse inflammation in secondary progressive multiple sclerosis using PET imaging and the radioligand 11C-PK11195," *Journal of Nuclear Medicine*, vol. 55, no. 6, pp. 939–944, 2014.

[4] A. Kumar, S. H. Chen, M. B. Kadiiska et al., "Inducible nitric oxide synthase is key to peroxynitrite-mediated, LPS-induced protein radical formation in murine microglial BV2 cells," *Free Radical Biology & Medicine*, vol. 73, pp. 51–59, 2014.

[5] K. Reemst, S. C. Noctor, P. J. Lucassen, and E. M. Hol, "The indispensable roles of microglia and astrocytes during

brain development," *Frontiers in Human Neuroscience*, vol. 10, 2016.

[6] L. Du, Y. Zhang, Y. Chen, J. Zhu, Y. Yang, and H. L. Zhang, "Role of microglia in neurological disorders and their potentials as a therapeutic target," *Molecular Neurobiology*, vol. 54, no. 10, pp. 7567–7584, 2017.

[7] V. Chhor, R. Moretti, T. Le Charpentier et al., "Role of microglia in a mouse model of paediatric traumatic brain injury," *Brain, Behavior, and Immunity*, vol. 63, pp. 197–209, 2017.

[8] O. Pascual, S. Ben Achour, P. Rostaing, A. Triller, and A. Bessis, "Microglia activation triggers astrocyte-mediated modulation of excitatory neurotransmission," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 4, pp. E197–E205, 2012.

[9] M. J. Surace and M. L. Block, "Targeting microglia-mediated neurotoxicity: the potential of NOX2 inhibitors," *Cellular and Molecular Life Sciences*, vol. 69, no. 14, pp. 2409–2427, 2012.

[10] E. J. Donzis and N. C. Tronson, "Modulation of learning and memory by cytokines: signaling mechanisms and long term consequences," *Neurobiology of Learning and Memory*, vol. 115, pp. 68–77, 2014.

[11] D. K. Choi, S. Koppula, and K. Suk, "Inhibitors of microglial neurotoxicity: focus on natural products," *Molecules*, vol. 16, no. 12, pp. 1021–1043, 2011.

[12] W. Liu, H. Ma, N. A. DaSilva et al., "Development of a neuroprotective potential algorithm for medicinal plants," *Neurochemistry International*, vol. 100, pp. 164–177, 2016.

[13] A. Malhotra, S. Bath, and F. Elbarbry, "An organ system approach to explore the antioxidative, anti-inflammatory, and cytoprotective actions of resveratrol," *Oxidative Medicine and Cellular Longevity*, vol. 2015, Article ID 803971, 15 pages, 2015.

[14] N. Singh, M. Agrawal, and S. Dore, "Neuroprotective properties and mechanisms of resveratrol in in vitro and in vivo experimental cerebral stroke models," *ACS Chemical Neuroscience*, vol. 4, no. 8, pp. 1151–1162, 2013.

[15] Y. Ido, A. Duranton, F. Lan, K. A. Weikel, L. Breton, and N. B. Ruderman, "Resveratrol prevents oxidative stress-induced senescence and proliferative dysfunction by activating the AMPK-FOXO3 cascade in cultured primary human keratinocytes," *PLoS One*, vol. 10, no. 2, article e0115341, 2015.

[16] K. Sekiguchi, S. Takehana, E. Shibuya et al., "Resveratrol attenuates inflammation-induced hyperexcitability of trigeminal spinal nucleus caudalis neurons associated with hyperalgesia in rats," *Molecular Pain*, vol. 12, article 174480691664308, 2016.

[17] Y.-T. Chin, S.-H. Yang, T.-C. Chang et al., "Mechanisms of dihydrotestosterone action on resveratrol-induced antiproliferation in breast cancer cells with different ERα status," *Oncotarget*, vol. 6, no. 34, pp. 35866–35879, 2015.

[18] C. Y. Chang, D.-K. Choi, D. K. Lee, Y. J. Hong, and E. J. Park, "Resveratrol confers protection against rotenone-induced neurotoxicity by modulating myeloperoxidase levels in glial cells," *PLoS One*, vol. 8, no. 4, article e60654, 2013.

[19] Y. J. Yang, L. Hu, Y. P. Xia et al., "Resveratrol suppresses glial activation and alleviates trigeminal neuralgia via activation of AMPK," *Journal of Neuroinflammation*, vol. 13, no. 1, p. 84, 2016.

[20] D. M. Sui, Q. Xie, W. J. Yi et al., "Resveratrol protects against sepsis-associated encephalopathy and inhibits the NLRP3/IL-1βaxis in microglia," *Mediators of Inflammation*, vol. 2016, Article ID 1045657, 10 pages, 2016.

[21] F. Zhang, J.-S. Shi, H. Zhou, B. Wilson, J.-S. Hong, and H.-M. Gao, "Resveratrol protects dopamine neurons against lipopolysaccharide-induced neurotoxicity through its anti-inflammatory actions," *Molecular Pharmacology*, vol. 78, no. 3, pp. 466–477, 2010.

[22] X. S. Zhang, W. Li, Q. Wu et al., "Resveratrol attenuates acute inflammatory injury in experimental subarachnoid hemorrhage in rats via inhibition of TLR4 pathway," *International Journal of Molecular Sciences*, vol. 17, no. 12, 2016.

[23] H. Kumar, I.-S. Kim, S. V. More, B.-W. Kim, Y.-Y. Bahk, and D.-K. Choi, "Gastrodin protects apoptotic dopaminergic neurons in a toxin-induced Parkinson's disease model," *Evidence-based Complementary and Alternative Medicine: Ecam*, vol. 2013, article 514095, 13 pages, 2013.

[24] T. Sun, J. Wang, X. Li et al., "Gastrodin relieved complete Freund's adjuvant-induced spontaneous pain by inhibiting inflammatory response," *International Immunopharmacology*, vol. 41, pp. 66–73, 2016.

[25] J.-N. Dai, Y. Zong, L.-M. Zhong et al., "Gastrodin inhibits expression of inducible NO synthase, cyclooxygenase-2 and proinflammatory cytokines in cultured LPS-stimulated microglia via MAPK pathways," *PLoS One*, vol. 6, no. 7, article e21891, 2011.

[26] C. Li, X. Chen, N. Zhang, Y. Song, and Y. Mu, "Gastrodin inhibits neuroinflammation in rotenone-induced Parkinson's disease model rats," *Neural Regeneration Research*, vol. 7, no. 5, pp. 325–331, 2012.

[27] J. W. Wang, Y. M. Liu, X. F. Zhao, and H. Zhang, "Gastrodin protects retinal ganglion cells through inhibiting microglial-mediated neuroinflammation in an acute ocular hypertension model," *International Journal of Ophthalmology*, vol. 10, no. 10, pp. 1483–1489, 2017.

[28] N.-T.-T. Trinh, E. Dumas, M. L. Thanh et al., "Effect of a Vietnamese Cinnamomum cassia essential oil and its major component trans-cinnamaldehyde on the cell viability, membrane integrity, membrane fluidity, and proton motive force of Listeria innocua," *Canadian Journal of Microbiology*, vol. 61, no. 4, pp. 263–271, 2015.

[29] K. Rajamani, Y.-C. Lin, T.-C. Wen et al., "The antisenescence effect of trans-cinnamaldehyde on adipose-derived stem cells," *Cell Transplantation*, vol. 24, no. 3, pp. 493–507, 2015.

[30] H. H. Andersen, P. Gazerani, and L. Arendt-Nielsen, "High-concentration L-menthol exhibits counter-irritancy to neurogenic inflammation, thermal and mechanical hyperalgesia caused by trans-cinnamaldehyde," *The Journal of Pain*, vol. 17, no. 8, pp. 919–929, 2016.

[31] X. Qi, R. Zhou, Y. Liu et al., "Trans-cinnamaldehyde protected PC12 cells against oxygen and glucose deprivation/reperfusion (OGD/R)-induced injury via anti-apoptosis and anti-oxidative stress," *Molecular and Cellular Biochemistry*, vol. 421, no. 1-2, pp. 67–74, 2016.

[32] Y. F. Chen, Y. W. Wang, W. S. Huang et al., "Trans-cinnamaldehyde, an essential oil in cinnamon powder, ameliorates cerebral ischemia-induced brain injury via inhibition of neuroinflammation through attenuation of iNOS, COX-2 expression and NFκ-B signaling pathway," *Neuromolecular Medicine*, vol. 18, no. 3, pp. 322–333, 2016.

[33] L. Zhang, Z. Zhang, Y. Fu et al., "Trans-cinnamaldehyde improves memory impairment by blocking microglial

activation through the destabilization of iNOS mRNA in mice challenged with lipopolysaccharide," *Neuropharmacology*, vol. 110, no. Part A, pp. 503–518, 2016.

[34] J.-H. Pyo, Y.-K. Jeong, S. Yeo et al., "Neuroprotective effect of trans-cinnamaldehyde on the 6-hydroxydopamine-induced dopaminergic injury," *Biological and Pharmaceutical Bulletin*, vol. 36, no. 12, pp. 1928–1935, 2013.

[35] Y. Zhao, Y. Guo, and X. Gu, "Salvianolic acid B, a potential chemopreventive agent, for head and neck squamous cell cancer," *Journal of Oncology*, vol. 2011, Article ID 534548, 8 pages, 2011.

[36] Z. S. Wang, P. Luo, S. H. Dai, Z. B. Liu, X. R. Zheng, and T. Chen, "Salvianolic acid B induces apoptosis in human glioma U87 cells through p38-mediated ROS generation," *Cellular and Molecular Neurobiology*, vol. 33, no. 7, pp. 921–928, 2013.

[37] S. C. Chen, Y. L. Lin, B. Huang, D. L. Wang, and J. J. Cheng, "Salvianolic acid B suppresses IFN-γ-induced JAK/STAT1 activation in endothelial cells," *Thrombosis Research*, vol. 128, no. 6, pp. 560–564, 2011.

[38] T. Chen, W. Liu, X. Chao et al., "Salvianolic acid B attenuates brain damage and inflammation after traumatic brain injury in mice," *Brain Research Bulletin*, vol. 84, no. 2, pp. 163–168, 2011.

[39] S. X. Wang, L. M. Hu, X. M. Gao, H. Guo, and G. W. Fan, "Anti-inflammatory activity of salvianolic acid B in microglia contributes to Its neuroprotective effect," *Neurochemical Research*, vol. 35, no. 7, pp. 1029–1037, 2010.

[40] P. Zhuang, Y. Wan, S. Geng et al., "Salvianolic acids for injection (SAFI) suppresses inflammatory responses in activated microglia to attenuate brain damage in focal cerebral ischemia," *Journal of Ethnopharmacology*, vol. 198, pp. 194–204, 2017.

[41] W. D. Lee, Y. J. Liang, and B. H. Chen, "Effects of tanshinone nanoemulsion and extract on inhibition of lung cancer cells A549," *Nanotechnology*, vol. 27, no. 49, article 495101, 2016.

[42] K.-W. Lee, S. Y. Jung, S.-M. Choi, and E. J. Yang, "Effects of ginsenoside Re on LPS-induced inflammatory mediators in BV2 microglial cells," *BMC Complementary and Alternative Medicine*, vol. 12, no. 1, 2012.

[43] J. C. Lee, J. H. Park, O. K. Park et al., "Neuroprotective effects of tanshinone I from Danshen extract in a mouse model of hypoxia-ischemia," *Anatomy & Cell Biology*, vol. 46, no. 3, pp. 183–190, 2013.

[44] D. Wang, C. Lu, F. Sun et al., "A tanshinone I derivative enhances the activities of antibiotics against Staphylococcus aureus in vitro and in vivo," *Research in Microbiology*, vol. 168, no. 1, pp. 46–54, 2017.

[45] M. R. de Oliveira, P. F. Schuck, and S. M. D. Bosco, "Tanshinone I induces mitochondrial protection through an Nrf2-dependent mechanism in paraquat-treated human neuroblastoma SH-SY5Y cells," *Molecular Neurobiology*, vol. 54, no. 6, pp. 4597–4608, 2017.

[46] S. Wang, H. Jing, H. Yang et al., "Tanshinone I selectively suppresses pro-inflammatory genes expression in activated microglia and prevents nigrostriatal dopaminergic neurodegeneration in a mouse model of Parkinson's disease," *Journal of Ethnopharmacology*, vol. 164, pp. 247–255, 2015.

[47] J. Zeng, Y. Xue, P. Shu et al., "Diterpenoids with immunosuppressive activities from Cinnamomum cassia," *Journal of Natural Products*, vol. 77, no. 8, pp. 1948–1954, 2014.

[48] L. Q. Jia, G. L. Yang, L. Ren et al., "Tanshinone IIA reduces apoptosis induced by hydrogen peroxide in the human endothelium-derived EA.hy926 cells," *Journal of Ethnopharmacology*, vol. 143, no. 1, pp. 100–108, 2012.

[49] B. Ren, Y. X. Zhang, H. X. Zhou et al., "Tanshinone IIA prevents the loss of nigrostriatal dopaminergic neurons by inhibiting NADPH oxidase and iNOS in the MPTP model of Parkinson's disease," *Journal of the Neurological Sciences*, vol. 348, no. 1-2, pp. 142–152, 2015.

[50] C. Wu, W. Huang, Y. Guo et al., "Oxymatrine inhibits the proliferation of prostate cancer cells in vitro and in vivo," *Molecular Medicine Reports*, vol. 11, no. 6, pp. 4129–4134, 2015.

[51] F. Liu, B. Wang, J. Wang et al., "Oxymatrine inhibits proliferation and migration while inducing apoptosis in human glioblastoma cells," *BioMed Research International*, vol. 2016, Article ID 1784161, 7 pages, 2016.

[52] M. Huang, Y. Y. Hu, X. Q. Dong, Q. P. Xu, W. H. Yu, and Z. Y. Zhang, "The protective role of oxymatrine on neuronal cell apoptosis in the hemorrhagic rat brain," *Journal of Ethnopharmacology*, vol. 143, no. 1, pp. 228–235, 2012.

[53] F. Ding, Y. Li, X. Hou, R. Zhang, S. Hu, and Y. Wang, "Oxymatrine inhibits microglia activation via HSP60-TLR4 signaling," *Biomedical Reports*, vol. 5, no. 5, pp. 623–628, 2016.

[54] J. J. Chen, L. Dai, L. X. Zhao, X. Zhu, S. Cao, and Y. J. Gao, "Intrathecal curcumin attenuates pain hypersensitivity and decreases spinal neuroinflammation in rat model of monoarthritis," *Scientific Reports*, vol. 5, no. 1, article 10278, 2015.

[55] X. Shi, Z. Zheng, J. Li et al., "Curcumin inhibits Aβ-induced microglial inflammatory responses in vitro: Involvement of ERK1/2 and p38 signaling pathways," *Neuroscience Letters*, vol. 594, pp. 105–110, 2015.

[56] G. L. He, Z. Luo, J. Yang, T. T. Shen, Y. Chen, and X. S. Yang, "Curcumin ameliorates the reduction effect of PGE2 on fibrillar β-amyloid peptide (1-42)-induced microglial phagocytosis through the inhibition of EP2-PKA signaling in N9 microglial cells," *PLoS One*, vol. 11, no. 1, article e0147721, 2016.

[57] M. X. Xu, R. Yu, L. F. Shao et al., "Up-regulated fractalkine (FKN) and its receptor CX3CR1 are involved in fructose-induced neuroinflammation: suppression by curcumin," *Brain, Behavior, and Immunity*, vol. 58, pp. 69–81, 2016.

[58] Z. Y. Zhang, M. Jiang, J. Fang et al., "Enhanced therapeutic potential of nano-curcumin against subarachnoid hemorrhage-induced blood-brain barrier disruption through inhibition of inflammatory response and oxidative stress," *Molecular Neurobiology*, vol. 54, no. 1, pp. 1–14, 2017.

[59] H. M. Wang, L. Zhang, J. Liu et al., "Synthesis and anti-cancer activity evaluation of novel prenylated and geranylated chalcone natural products and their analogs," *European Journal of Medicinal Chemistry*, vol. 92, pp. 439–448, 2015.

[60] Ismail, B. Kuthati, G. Thalari et al., "Synthesis of novel spiro [pyrazolo [4,3- d] pyrimidinones and spiro [benzo] [4, 5] thieno [2,3- d] pyrimidine-2,3′-indoline]-2′,4(3H)-diones and their evaluation for anticancer activity," *Bioorganic & Medicinal Chemistry Letters*, vol. 27, no. 6, pp. 1446–1450, 2017.

[61] P. Cui, X. Li, M. Zhu, B. Wang, J. Liu, and H. Chen, "Design, synthesis and antimicrobial activities of thiouracil derivatives containing triazolo-thiadiazole as SecA inhibitors," *European Journal of Medicinal Chemistry*, vol. 127, pp. 159–165, 2017.

[62] Y. G. Li, J. Hou, S. Y. Li et al., "Fructus Psoraleae contains natural compounds with potent inhibitory effects towards human carboxylesterase 2," *Fitoterapia*, vol. 101, pp. 99–106, 2015.

[63] H. Jing, S. Wang, M. Wang, W. Fu, C. Zhang, and D. Xu, "Isobavachalcone attenuates MPTP-induced Parkinson's disease in mice by inhibition of microglial activation through NF-κB pathway," *PLoS One*, vol. 12, no. 1, article e0169560, 2017.

[64] H. Tang, Y. Tang, N. Li et al., "Neuroprotective effects of scutellarin and scutellarein on repeatedly cerebral ischemia-reperfusion in rats," *Pharmacology, Biochemistry, and Behavior*, vol. 118, pp. 51–59, 2014.

[65] L. Qian, M. Shen, H. Tang et al., "Synthesis and protective effect of scutellarein on focal cerebral ischemia/reperfusion in rats," *Molecules*, vol. 17, no. 12, pp. 10667–10674, 2012.

[66] Y. Yuan, P. Rangarajan, E. Kan, Y. Wu, C. Wu, and E.-A. Ling, "Scutellarin regulates the Notch pathway and affects the migration and morphological transformation of activated microglia in experimentally induced cerebral ischemia in rats and in activated BV-2 microglia," *Journal of Neuroinflammation*, vol. 12, no. 1, p. 11, 2015.

[67] S. Wang, H. Wang, H. Guo, L. Kang, X. Gao, and L. Hu, "Neuroprotection of scutellarin is mediated by inhibition of microglial inflammatory activation," *Neuroscience*, vol. 185, pp. 150–160, 2011.

[68] C. Guo, L. Yang, C. X. Wan et al., "Anti-neuroinflammatory effect of Sophoraflavanone G from Sophora alopecuroides in LPS-activated BV2 microglia by MAPK, JAK/STAT and Nrf2/HO-1 signaling pathways," *Phytomedicine*, vol. 23, no. 13, pp. 1629–1637, 2016.

[69] H. J. Lim, G. Z. Dong, H. J. Lee, and J. H. Ryu, "In vitro neuroprotective activity of sesquiterpenoids from the flower buds of Tussilago farfara," *Journal of Enzyme Inhibition and Medicinal Chemistry*, vol. 30, no. 5, pp. 852–856, 2014.

[70] F. Q. Li, T. Wang, Z. Pei, B. Liu, and J. S. Hong, "Inhibition of microglial activation by the herbal flavonoid baicalein attenuates inflammation-mediated degeneration of dopaminergic neurons," *Journal of Neural Transmission*, vol. 112, no. 3, pp. 331–347, 2005.

[71] H. Lee, Y. O. Kim, H. Kim et al., "Flavonoid wogonin from medicinal herb is neuroprotective by inhibiting inflammatory activation of microglia," *FASEB Journal*, vol. 17, no. 13, pp. 1943-1944, 2003.

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Review Article

Linking Neuroinflammation and Neurodegeneration in Parkinson's Disease

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Neurodegenerative diseases such as Parkinson's disease (PD) and Alzheimer's disease (AD) impose a pressing burden on our developed and consequently aging society. Misfolded protein aggregates are a critical aspect of several neurodegenerative diseases. Nevertheless, several questions remain unanswered regarding the role of misfolded protein aggregates and the cause of neuronal cell death. Recently, it has been postulated that neuroinflammatory processes might play a crucial role in the pathogenesis of PD. Numerous postmortem, brain imaging, epidemiological, and animal studies have documented the involvement of the innate and adaptive immunity in neurodegeneration. Whether these inflammatory processes are directly involved in the etiology of PD or represent secondary consequences of nigrostriatal pathway injury is the subject of intensive research. Immune alterations in response to extracellular α-synuclein may play a critical role in modulating Parkinson's disease progression. In this review, we address the current concept of neuroinflammation and its involvement in PD-associated neurodegeneration.

1. Introduction

To the present day, neurodegenerative diseases such as Parkinson's disease (PD) and Alzheimer's disease (AD) impose a pressing burden on our developed and consequently aging society. Increasing life expectancy implicates that a large part of the population becomes susceptible to neurodegenerative disorders [1]. The currently available treatment options for PD are limited, mainly symptomatic and associated with decreased efficacy and unwanted side effects over time. Therapeutic strategies acting on the underlying pathogenesis of the disease in order to slow down or block the disease progression, as well as reliable and sensitive tests for early diagnosis, represent a large unmet medical need. PD has long been considered as a sporadic disorder with an age-related increase in incidence without a clear etiology. Over the last 20 years however, new genetic insights from monogenic Mendelian forms of PD and more recently from genome-wide association studies (GWAS) have strengthened the evidence that PD has a considerable genetic component [2]. In addition, environmental factors have been proposed as important risk factors or triggers for PD [3].

The neuropathological hallmarks of PD are the deposition of misfolded protein aggregates, predominantly composed of α-synuclein, in distinct brain regions and the progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc), subsequently leading to striatal dopamine depletion, which is responsible for the classical motor symptoms [4, 5]. Throughout the last decade, this classical view has been extended to other nonmotor-related brain regions. It has become clear that the disease progresses following a characteristic pattern of pathological changes throughout the brain leading to severe nonmotor symptoms such as olfactory dysfunction, sleep disturbance, cognitive impairment, and autonomic dysfunction [6]. Nevertheless, several questions remain unanswered regarding the cause of neuronal cell death and the role of misfolded protein aggregates in PD. Multiple cellular and molecular mechanisms that might contribute to neuronal cell death have been described including mitochondrial dysfunction, oxidative stress, excitotoxicity, and proteasomal dysfunction. In addition, it has been postulated that neuroinflammatory processes might play a crucial role in the pathogenesis of PD and other neurodegenerative disorders [7]. Numerous postmortem, brain imaging,

epidemiological, and animal studies have documented the involvement of the innate and adaptive immunity in neurodegeneration (schematic overview in Figure 1) [8–14]. Whether these inflammatory processes are directly involved in the etiology of PD or represent secondary consequences of nigrostriatal pathway injury is the subject of intensive research.

In this review, we address the current concept of neuroinflammation and its involvement in PD-associated neurodegeneration. We summarize our understanding of the involvement of innate and adaptive immunity in PD in patients as well as preclinical animal models. Lastly, we emphasize the current and potentially new therapeutic strategies targeting neuroinflammatory processes.

2. Evidence for Neuroinflammation in PD Patients

Typically, inflammation is a complex defense mechanism occurring in the body in response to perturbed homeostasis. The term "neuroinflammation" broadly defines the inflammatory processes occurring in the central nervous system (CNS) involving both the innate and adaptive immune system. This is a double-edged sword since the cells involved in neuroinflammatory responses can induce beneficial or harmful effects. Indeed, neuroinflammatory mechanisms contribute to both normal brain development and neuropathological events. Neuroinflammation has been repeatedly linked to neurodegeneration, including PD, over the last years, but whether the neuroinflammatory processes are a cause or a consequence of neuronal degeneration remains unanswered [7]. Two potential pathological mechanisms responsible for neuronal cell death exist in PD and most neurodegenerative disorders: "cell-autonomous" and "non-cell-autonomous" mechanisms. The cell-autonomous mechanism refers to an accumulation of intrinsic damage in the degenerating neurons resulting in their death. The second mechanism indicates an indirect degeneration of the affected neurons caused by pathological interactions with neighboring cells, such as resident glial cells (i.e., microglia and astrocytes) and/or infiltrating immune cells from the periphery (i.e., macrophages and lymphocytes).

Microglia are the principal innate immune cells in the brain and have fundamental roles in CNS homeostasis. They continuously scan their microenvironment and monitor ongoing synaptic activity (including synaptic pruning), clear apoptotic cells, and provide trophic support for neurons. They represent the first line of defense in the brain and react to pathological events through a cascade of inflammatory processes. In the presence of pathogens (pathogen-associated molecular patterns or PAMPS) or tissue damage (danger-associated molecular patterns or DAMPS), microglia induce complex immune responses by increasing the expression of toll-like receptors (TLRs) and several proinflammatory mediators, which consequently activate peripheral immune cells, with the aim of restoring tissue homeostasis [15, 16].

Earliest observations supporting the idea that neuroinflammatory processes are involved in PD date from postmortem studies 25 years ago. McGeer et al. reported the presence of human leukocyte antigen D-related- (HLA-DR-) positive reactive microglia in the SNpc of patients with PD [8]. Cytokines, chemokines, and other inflammatory mediators are known to trigger microglial activation, potentially contributing to nigrostriatal pathway injury. As dopaminergic neurons express a wide range of cytokine and chemokine receptors, it has been suggested that they are responsive to these inflammatory mediators which are derived from or which activate microglia. Accordingly, higher expression levels of the chemokine CXCL12 and its receptor CXCR4 were detected in the SN [17]. Elevated levels of the proinflammatory interleukins IL1β, IL2, IL6, and tumor necrosis factor α (TNFα) as well as the anti-inflammatory transforming growth factor β1 (TGFβ1) have also been detected in the striatum, and concentrations of TNFα, IL1β, interferon γ (IFNγ), nitric oxide synthase (NOS), and reactive oxygen species (ROS) were found to be increased in the SN of postmortem samples [18–21]. These findings further confirm the involvement of microglia in the initiation of both pro- and anti-inflammatory events pointing towards the existence of multiple phenotypes in PD and distinct functions during disease progression. Aside from postmortem data, numerous *in vivo* studies have been conducted on biological fluids including blood and cerebrospinal fluid (CSF) of PD patients. Plasma and serum analysis showed upregulation of proinflammatory cytokines such as IL1β, IL2, IL6, IFNγ, and TNFα as well as the anti-inflammatory cytokine IL10 [22, 23]. Increased IL6 plasma concentrations were linked to a higher risk to develop PD [24]. An elevation in the serum levels of macrophage migration inhibitory factor (MIF) was also observed [25]. In line with these findings, the same proinflammatory cytokines (i.e., IL1β, IL6, and TNFα) have been reported in CSF samples [20, 26]. CSF is a valuable source of information for the discovery of new neurodegenerative and neuroinflammatory biomarkers as it mirrors metabolic and pathological alterations in the CNS more accurately than other biological fluids. In addition, noninvasive positron emission tomography (PET) imaging studies using PK-11195, a ligand of the peripheral benzodiazepine receptor (PBR; also known as the mitochondrial 18 kDa translocator protein or TSPO), which is selectively expressed by activated microglia, further confirmed the occurrence of microglial activation in PD [9]. PD patients showed significantly increased levels of PK-11195 binding in the pons, basal ganglia, and cortical regions. However, Gerhard et al. failed to find a clear correlation between PK-11195 binding and disease progression during a longitudinal follow-up study, suggesting that microglia might get activated early in the disease process [9]. Newer highly specific PET ligands (e.g., DPA-714 and P2X7) have been developed and are currently being tested to further confirm these imaging data [27, 28]. Which stimuli are responsible for this activation is currently under intensive debate, but abnormal misfolded proteins like extracellular α-synuclein have been proposed as one of the major candidates.

Besides microglia, astrocytes have also been found to participate in the neuropathology of PD [29, 30]. Astrocytes are resident cells from the brain contributing to the development and plasticity of the CNS, providing energy to neurons and maintaining brain homeostasis. In healthy individuals, astrocytes are heterogeneously distributed within the

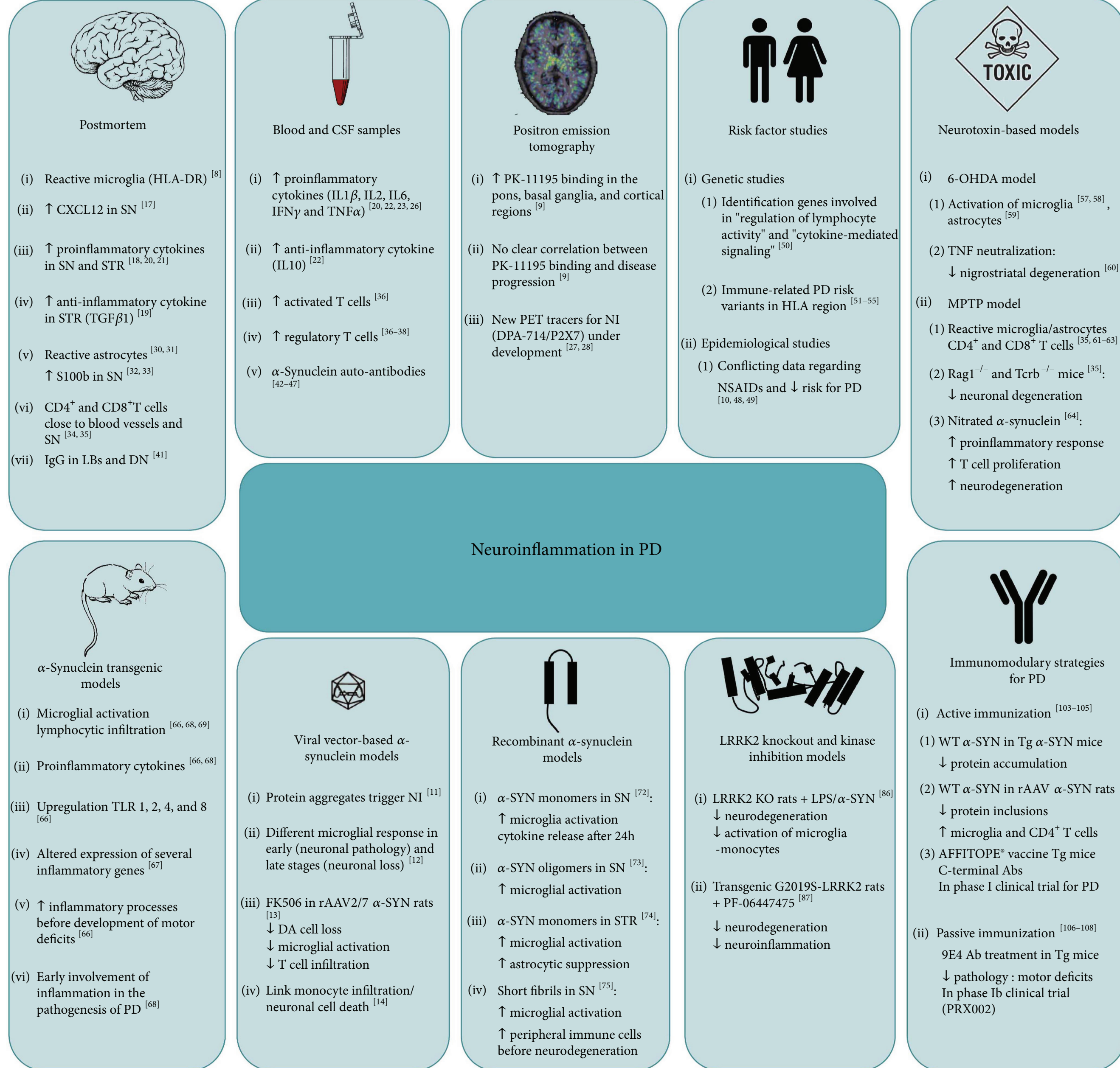

Figure 1: Overview of clinical and preclinical evidence linking neuroinflammation to neurodegeneration in Parkinson's disease. DN: dopaminergic neurons; TLR: toll-like receptor; STR: striatum; Tg: transgenic; PD: Parkinson's disease; KO: knockout; LBs: Lewy bodies; α-SYN: α-synuclein; NI: neuroinflammation.

mesencephalon, with a low astrocytic density in the SN. In PD, an elevation in the number of astrocytes in the SN as well as pathological changes in astrocytes following a specific distribution pattern has been reported postmortem [30, 31]. Furthermore, upregulation of calcium-binding protein S100b, which is primarily expressed by astrocytes and acts as a cytokine, has been shown in the SN of postmortem PD patients. S100b may increase the expression of inducible nitric oxide synthase (iNOS) which, in turn, may result in the activation of the proinflammatory enzyme cyclo-oxygenase-2 (COX-2) in microglia as well as an increased production of nitric oxide (NO) and superoxide radicals [32, 33]. These events may directly or indirectly cause neuronal cell death. The role of astrocytes in the neuropathology of PD is still not well understood, since it has been hypothesized that these glial cells may prevent and/or exacerbate nigrostriatal injury due to a perturbed balance.

Over the last decades, the adaptive immune system has been shown to be involved in PD through the presence of $CD4^+$ and $CD8^+$ T lymphocytes in the vicinity of blood vessels and near dopaminergic neurons in postmortem brain tissues [34, 35]. On the other hand, B cells and natural killer cells were not detected [35]. Decreased serum levels of naïve lymphocytes were observed in PD patients while the

numbers of activated T cells were increased, indicating that peripheral activation occurs in PD pathology [36]. Elevated numbers of regulatory T cells were also detected and exhibited an impaired ability to suppress the effector T cell function [36–38]. Recently, Sulzer and coworkers reported that well-defined α-synuclein peptides act as antigenic epitopes and drive $CD4^+$ and $CD8^+$ T cell responses in patients with PD [39]. The first antigenic region near the N-terminus, also referred to as the Y39 region, is strikingly close to the α-synuclein mutations causing PD (A30P, E46K, H50Q, G51D, and A53T) [40]. The second antigenic region encompasses S129 and requires S129 phosphorylation, a pathological form present in Lewy bodies. Further, they showed that T cells can respond to both α-synuclein epitopes arising from processing native α-synuclein present in the blood and fibrilized α-synuclein associated with PD. Orr et al. have described a role for humoral immunity in the pathogenesis of PD. They demonstrated a strong immunolabeling for IgG (immunoglobulin G) in Lewy bodies as well as one-third of the nigrostriatal dopaminergic neurons, implicating that IgG antibody coating labels these cells for degradation [41]. In the last years, the presence of auto-antibodies towards α-synuclein linking PD to humoral immunity has been investigated. These auto-antibodies are thought to be implicated in clearance pathways and tissue homeostasis, suggesting that they may play a crucial and protective role throughout disease progression [42]. Several studies based on the detection of α-synuclein auto-antibodies in biological fluids (i.e., blood, CSF, and saliva) using a wide range of assays, including electrochemistry, enzyme-linked immunosorbent assay (ELISA), immunoblotting, and surface plasmon resonance, have produced mixed results [42–47]. A plausible explanation could be interlaboratory variability regarding the assays and sample collecting procedures as well as the natural variation within patient cohorts. Therefore, additional studies overarching different patient groups with PD are indispensable.

Genetic and epidemiological studies have further implicated neuroinflammation as a risk factor in PD. Epidemiological studies based on the use of nonsteroidal anti-inflammatory drugs (NSAIDs) were conducted in order to investigate the involvement of neuroinflammation in the disease progression, with the underlying hypothesis that NSAIDs would decrease the risk of developing PD [10, 48, 49]. However, conflicting data were generated, possibly due to the fact that the NSAIDs were not clearly subdivided based on their working mechanism and pharmacokinetics. Therefore, more scrupulous studies regarding the possible correlation between the use of specific anti-inflammatory drugs and the risk of developing PD are required. More recently, pathway analysis-based GWAS have identified functional categories of genes involved in the “regulation of lymphocyte activity” and “cytokine-mediated signaling” providing support for a strong immune-related genetic susceptibility to PD [50]. The most obvious immune-related PD risk variants are located in the human leukocyte antigen (HLA) region (e.g., HLA-DRB1 and HLA-DRB5) [51–55]. HLA genes encode the major histocompatibility complex (MHC) proteins that are intimately involved in antigen presentation and immunity. This complex resides on the surface of antigen-presenting cells, including microglia, and promotes T cell activation. Altogether, these data point to the involvement of the innate immune system in PD. This is in line with findings from the AD field where GWAS have identified 20 well-validated genes harboring risk alleles (such as HLA-DRB1 and HLA-DRB5), of which about half are predominantly or only expressed in microglia [56].

3. Evidence from Neurotoxin-Based PD Models

Microglial activation has initially been observed in the SN and along the nigrostriatal tract in the classical 6-hydroxydopamine (6-OHDA) rat and mouse PD model [57, 58]. In addition, reactive astrocytes have been identified in the SN and the striatum of rats after exposure to 6-OHDA [59]. The 6-OHDA model is widely known to induce rather acute massive degeneration of dopaminergic neurons in the nigrostriatal pathway. McCoy et al. have shown that neutralization of the proinflammatory cytokine TNF led to reduced nigrostriatal degeneration in this model [60]. However, to investigate the involvement of peripheral immune and/or inflammatory components, proper controls are necessary as the blood-brain barrier (BBB) is transiently disrupted upon intracerebral injection of the neurotoxin. An alternative model that overcomes this issue is the systemic injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Reactive microglia and astrocytes have been detected in the MPTP-treated mouse and primate brain [61, 62]. Brochard et al. observed a selective nigrostriatal infiltration of $CD4^+$ and $CD8^+$ T cells in mice treated with MPTP, whereas no B lymphocytes were found [35]. It was postulated that the infiltration of T lymphocytes into the brain occurred after microglial activation but before astrogliosis. This model also provided evidence for the direct involvement of activated microglia in the processes leading to neuronal cell death as microglial activation precedes neuronal degeneration [35, 61, 63]. Furthermore, two immunodeficient mouse models lacking mature T lymphocytes (recombination activating gene $1^{-/-}$ ($Rag1^{-/-}$) and T cell receptor beta $chain^{-/-}$ ($Tcrb^{-/-}$) mice) displayed a remarkably reduced susceptibility to MPTP-induced dopaminergic neurodegeneration [35]. Mice reconstituted with functional naïve lymphocytes partially lost their MPTP-related resistance, and $CD4^+$ T cells were identified as the key players in this neurodegenerative process [35, 64]. Benner and coworkers hypothesized that protein oxidative modifications associated with PD (e.g., nitration of α-synuclein) might lead to novel antigenic epitopes able to initiate peripheral T cell responses that might consequently affect the nigrostriatal pathway [64]. Mice immunized with nitrated α-synuclein were shown to display marked proinflammatory responses and T cell proliferation. Adoptive transfer of T cells from mice immunized with nitrated α-synuclein to MPTP-treated mice led to a strong neuroinflammatory response as well as accelerated neuronal degeneration. These observations support the idea that $CD4^+$ T cells are crucially implicated in neuroinflammation and subsequent neurodegeneration in animal models of PD. On the other hand, $CD4^+CD25^+$ regulatory T cells were

shown to be neuroprotective through the suppression of microglia responses in the MPTP model [65].

4. Alpha-Synuclein: The Key Player in Neuroinflammation?

As the gene encoding for α-synuclein was the first to be unequivocally associated with a familial form of PD, α-synuclein transgenic mice have become one of the most studied genetic animal models for PD. However, since α-synuclein transgenic mice are in general hampered by a lack of dopaminergic cell loss and dopamine-dependent behavioral deficits, they may only provide partial evidence regarding the link between neuroinflammatory responses and genetic alterations. Watson and coworkers studied the temporospatial distribution of microglial activation and lymphocytic infiltration as well as the production of proinflammatory cytokines in mice overexpressing wild-type (WT) human α-synuclein under the neuron-specific Thy1-promoter [66]. Their study revealed an elevation in the number of $IbaI^+$ reactive microglia and increased levels of TNFα in the striatum and later in the SN of young Thy1-α-synuclein mice. Microglial activation was maintained until 14 months of age, but increased serum levels of TNFα could only be detected until 5 to 6 months. Increased expression of TLR 1, 4, and 8, which potentially mediate microglial reactivity, was found in the SN of 5 to 6 months old animals, while TLR2 expression was elevated in the SN at 14 months. Serum levels of $CD4^+$ and $CD8^+$ T cells were upregulated at 22 months in the Thy1-α-synuclein mice reflecting the later role of the adaptive immunity in PD pathology. These results point to the occurrence of inflammatory processes before the development of motor deficits. In another study, three different α-synuclein transgenic mouse lines (WT and double A53T and A30P mutant human α-synuclein under the control of the tyrosine hydroxylase (TH) promoter and A53T human α-synuclein under the prion promoter) were used to investigate the molecular changes induced by α-synuclein [67]. The authors reported an altered expression of several genes, including inflammatory genes, in the SN of WT α-synuclein transgenic mice at stages preceding neuronal degeneration. In the mutant α-synuclein transgenic mice, in an advanced stage of the pathology, new candidate genes were identified that may be involved in protein deposition and neuronal cell death. Su et al. further documented the early involvement of both α-synuclein and inflammation in the pathogenesis of PD using mice overexpressing WT human α-synuclein driven by the rat TH promoter [68]. They observed an increase in the number of $IbaI^+$ activated microglia in the SN as well as significantly elevated expression levels of TNFα. In line with these results, mice expressing the truncated form of human α-synuclein under the rat TH promoter also exhibited microglial activation in the SN [69].

Next to transgenic mouse models, recombinant adeno-associated viral vector- (rAAV-) based α-synuclein rodent and primate models, presenting various degrees of neuronal cell loss, have extended our view on neuroinflammatory events in PD pathogenesis. Theodore and coworkers noticed an increase in the numbers of $CD68^+$ microglia and an expanding infiltration of B and T cells in the SN of rAAV2 α-synuclein mice before the onset of mild neuronal cell loss 6 months post injection [11]. The number of microglia declined 12 weeks post injection, but a persistent B and T cell infiltration was observed. Expression of proinflammatory cytokines was elevated, while the anti-inflammatory markers arginase I, IL4, and IL13 remained at the same level. These observations revealed that overexpression of α-synuclein, in the absence of overt neurodegeneration, is sufficient to initiate neuroinflammation. Sanchez-Guajardo et al. investigated in depth the early (neuronal pathology) and late stages (neuronal pathology in association with neurodegeneration) of the disease in a rAAV2/5 α-synuclein rat model [12]. A rapid and transient elevation in the number of microglia was shown after induction of neuronal pathology, resulting in a long-lasting activation of $MHCII^+$ microglia. Neuronal pathology along with neuronal cell loss resulted in a delayed increase in the number of $CD68^+$ microglia displaying a morphology similar to peripheral macrophages. Infiltration of T lymphocytes appeared to increase in function of the neurodegeneration-related severity. The authors concluded that the microglial response alters depending on the presence or absence of neuronal cell death implicating that microglia may exert different functions during the disease progression. Our group has shown that treatment with the immunophilin ligand FK506 improved the survival of dopaminergic neurons in a dose-dependent manner in a rAAV2/7 α-synuclein rat model [13]. Moreover, we demonstrated that FK506 led to decreased numbers of microglia/macrophages and lowered lymphocytic infiltration. These findings emphasize the anti-inflammatory properties of FK506 in reducing neurodegeneration as well as the causal role of neuroinflammatory processes in the pathogenesis of PD. In a recent publication, Harms et al. reported the necessary recruitment of peripheral $CCR2^+$ monocytes to induce inflammation and subsequent neurodegeneration in a rAAV2 α-synuclein mouse model [14]. The role of the infiltrating monocytes in α-synuclein-mediated neuroinflammation and neurodegeneration was further investigated using the CCR2 knockout mouse model combined with α-synuclein overexpression. The authors found that the absence of infiltrating monocytes was neuroprotective compared to WT rAAV2 α-synuclein animals. These data suggest that the entry of peripheral monocytes into the brain might play a crucial role in α-synuclein-mediated neuronal cell death.

Evidence is emerging that α-synuclein can adopt distinct conformations or “strains” with remarkable differences in structural and phenotypic traits [70, 71]. Therefore, presentation of different α-synuclein assemblies (monomers, oligomers, or fibrils) to the innate immune system and the subsequent immune response might be a driving force in the disease, although different studies have not yet put forward one conformation as major determinant. Direct injection of recombinant monomeric α-synuclein in the mouse SN induced nigral microglial activation 24 hours post injection [72]. The proinflammatory cytokines IL1β, IL6, and TNFα, the proinflammatory enzyme COX-2, and the anti-inflammatory cytokine TGFβ were upregulated in the SN compared to controls. Accordingly, strong microglial

activation in the rat SN was found one week after injection of oligomeric (protofibrillar) α-synuclein [73]. Sznejder-Pachołek et al. have reported robust activation of microglia and elevated expression of IL1α, TNFα, and IFNγ in the striatum after striatal injection of monomeric α-synuclein. Also, an increase in striatal GFAP was shown four weeks post injection. These data further suggest that microglia and astrocytes are key players in α-synuclein-related neurotoxicity [74]. Very recently, Harms et al. have shown that short α-synuclein fibrils, but not monomeric α-synuclein, injected into the rat SN led to the formation of α-synuclein inclusions in dopaminergic neurons potentially spreading to the projection neurons in the striatum and rapidly induced microglial activation in the brain [75]. Axon loss was observed in the striatum along with the recruitment of monocytes two months post injection. Monocytes and macrophages initially displayed low MHCII expression in the striatum but, later, when α-synuclein inclusions were present in neighboring projection neurons, MHCII expression was strongly increased. These exciting results further support the hypothesis that recruitment of peripheral immune cells might happen before neurodegeneration.

Overall, these observations from animal models using distinct approaches implicate that the mechanisms leading to neuronal cell death involve nonneuronal cells (Figure 2). Molecular factors and mediators involved in these cellular interactions, including microglial activation as well as monocytic and lymphocytic infiltration, still need to be identified and characterized in order to find new targets for disease-modifying therapies.

5. LRRK2: α-Synuclein's Copilot in Neuroinflammation?

A second gene linked to autosomal dominant familial PD is the more recently discovered LRRK2 gene, encoding the leucine-rich repeat kinase 2 protein. Both LRRK2 and α-synuclein are key players in PD, but establishing a functional link between the two proteins has proven elusive. Interestingly, both proteins have been linked to microglial activation. Extracellular α-synuclein can directly initiate microglial activation and be phagocytosed by microglia, whereas LRRK2 has been reported to be involved in the intrinsic regulation of microglial activation as well as autophagolysosomal degradation [76, 77]. Genetic studies have also implicated LRRK2 in the development of autoimmune disorders including Crohn's disease and ulcerative colitis [78]. Microglia and monocytes display high LRRK2 expression levels, even higher than neurons, emphasizing its involvement in the innate immune system. This suggests that microglia and monocytes might be deeply involved in LRRK2-dependent processes and signaling (e.g., kinase activity and interactions with cofactors) [79, 80]. Indeed, several studies have shown that TLR2 and TLR4 stimulation results in the upregulation of LRRK2 expression and phosphorylation in primary microglia and monocytes [80, 81]. LRRK2 phosphorylation is currently thought to reflect the functional activity of the protein. Further, Russo et al. observed that LRRK2 deletion or pharmacological inhibition led to decreased production of proinflammatory mediators (i.e., IL1β and COX-2) and subsequent decline in the inflammatory response elicited by LPS or α-synuclein fibrils in microglial cells [82].

Over the last years, various studies using LRRK2 knockout or LRRK2 kinase inhibition in rodent models have been conducted to evaluate the effects of LRRK2 inactivation on neurodegeneration and neuroinflammation; however, conflicting data have been obtained. Lin et al. noticed a strong protection after genetic depletion of LRRK2 in a A53T α-synuclein CAMKII-promoter-driven transgenic mouse model [83]. In contrast, Herzig et al. found that high levels of both WT and G2019S-LRRK2 did not alter endogenous α-synuclein levels or exacerbate α-synucleinopathy in WT or A53T transgenic mice [84]. In line with these findings, Daher et al. reported that modulation of LRRK2 (i.e., deletion of LRRK2 or overexpression of human G2019S-LRRK2) in A53T α-synuclein transgenic mice had a minimal effect on α-synuclein-induced pathology in the mouse hindbrain, suggesting that these events are principally independent from LRRK2 expression [85]. Later, in line with the first study of Lin et al., it was shown that the LRRK2 knockout rat model was resistant to neuronal degeneration in association with a reduced activation of microglia and monocytes in response to LPS exposure or human WT α-synuclein overexpression [86]. While endogenous LRRK2 expression in the SN of WT rats is below detection level under physiological conditions, increased LRRK2 expression levels were found in the SN upon exposure to LPS or human WT α-synuclein overexpression. Based on these data, it has been suggested that knockdown of LRRK2 might be neuroprotective through the inhibition of chronically activated microglia as well as the recruitment of monocytes. These findings may provide valuable insights into the development of LRRK2-targeting therapeutic strategies. Indeed, LRRK2 kinase inhibitors are currently investigated with regard to their therapeutic potential as well as their efficacy and tolerability in α-synuclein models. PF-06447475, a LRRK2 kinase inhibitor, was administered for four weeks in both transgenic G2019S-LRRK2 and WT rats stereotactically injected with the rAAV2/1 vector expressing human WT α-synuclein [87]. G2019S-LRRK2 rats displayed aggravated degeneration of dopaminergic neurons and neuroinflammation upon α-synuclein overexpression, whereas treatment with PF-06447475 diminished neurodegeneration and neuroinflammation in these animals. Neuronal cell death was also found to be reduced in WT rAAV2/1 α-synuclein rats upon administration of PF-06447475. Further insights into the potential LRRK2-dependent regulation of microglial activation and neuroinflammation will provide valuable and crucial information.

6. Neuroinflammation beyond the CNS

The recently proposed prion-like behavior of α-synuclein may explain the observations made by Braak et al. implicating that the neuropathology of PD evolves in a patterned and sequential manner, with premotor symptoms preceding the presence of motor deficits [88, 89]. This pathological process is divided into six stages in PD initiating with α-synuclein-rich inclusions in the olfactory bulb and the dorsal

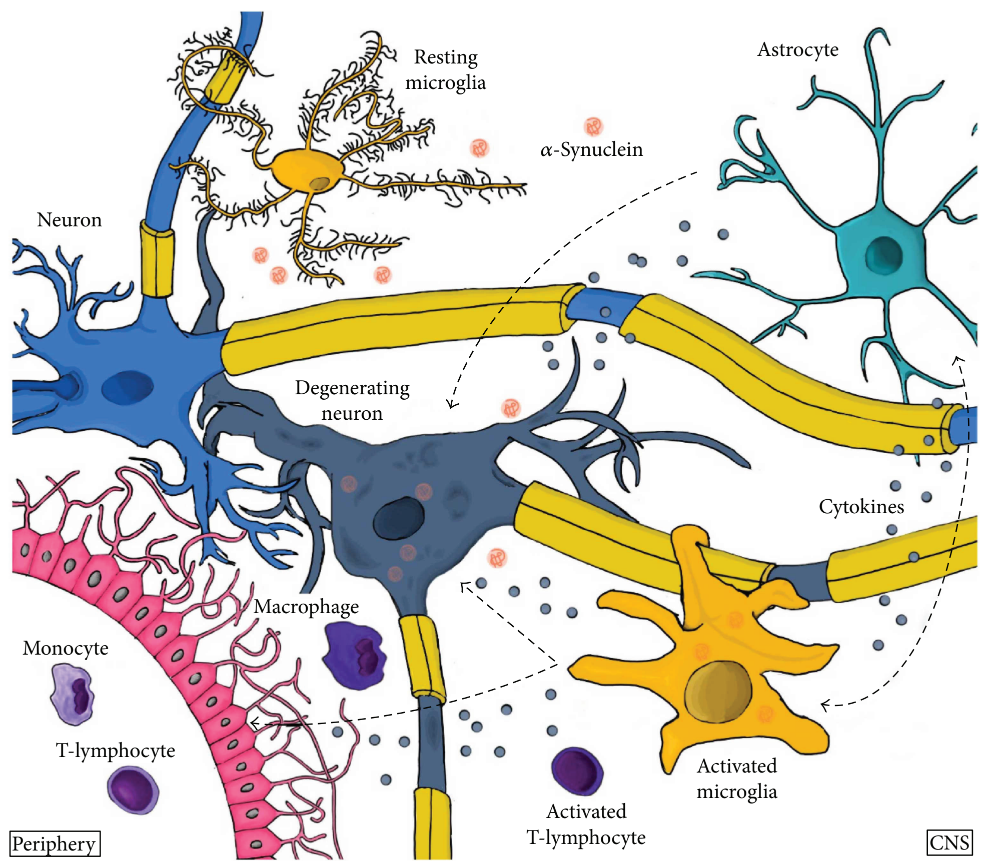

Figure 2: Possible link between α-synuclein, neuroinflammatory processes, and neurodegeneration in Parkinson's disease. In the presence of specific α-synuclein conformations, microglia get activated and induce a complex immune response by increasing the expression of toll-like receptors and several proinflammatory mediators, which consequently activates peripheral immune cells like monocytes or T cells. These peripheral immune cells might actively contribute to neurodegeneration.

motor nucleus of the vagal nerve (DMV) and extending to the midbrain and other brainstem regions in later stages of the disease. The vagal nerve receives projections from the enteric nervous system (ENS) and the spinal cord and is proposed as the connection between the peripheral nervous system (PNS) and the CNS [90].

The majority of PD patients also display nonmotor symptoms such as dysphagia, constipation, and gastroesophageal reflux [91–93]. These symptoms that may reflect a prodromal stage of the disease appear to coincide with gastrointestinal pathology involving the deposition of phosphorylated α-synuclein in enteric neurons [94]. Histological analyses have shown that the PNS and the gastrointestinal (GI) tract are affected by both pathological and inflammatory events in early stages in PD [90, 93, 95]. Intestinal hyperpermeability along with increased exposure to intestinal bacteria and bacterial endotoxins, oxidative stress (nitrotyrosine), and α-synuclein deposits have been observed in the intestinal mucosa of patients suffering from PD using immunohistochemical and serological analyses [95]. Moreover, recent studies have reported alterations in the composition of the gut microbiota in patients with PD [96]. Although the precise nature of these alterations has not been identified to date, these findings suggest that gut microbiota might play a role in PD pathology.

It has been suggested that prion-like cell-to-cell transmission through the vagal nerve and spinal cord may require the involvement of the immune system [97]. The presence of exogenous pathogens in the GI tract may lead to the activation of macrophages, which consequently secrete inflammatory mediators inducing oxidative stress and affecting the surrounding tissues and consequently initiating the synucleinopathy in the ENS. These events may influence the intestinal function as evidenced by the premotor symptoms. Cell-to-cell transmission of misfolded α-synuclein aggregates through the ENS into the brain may further contribute to the sustained activation of local macrophages as well as the progression of the pathology. Further research is necessary to unravel the involvement of the ENS and the role of the immune system in disease initiation and propagation towards the CNS.

7. Immunomodulatory Strategies for PD

Considering the potential role of neuroinflammation in the initiation and progression of PD, targeting the immune system is a promising strategy for treating PD. Several studies targeting the inflammatory pathways mediated by microglial cells have been carried out. Nigral overexpression of dominant negative TNF in the 6-OHDA rat model attenuated

microglial activation and resulted in decreased neuronal degeneration and improved motor behavior [60, 98]. Therapeutic approaches with the aim of modulating the peripheral immune system have been designed to trigger T cells *in vivo* upon exposure to distinct compounds and transfer them to preclinical models of PD. For example, Benner and coworkers reported that adoptive transfer of T cells immunized with glatiramer acetate (a synthetic random amino acid polymer used as an immunization-based antigen) to MPTP-treated mice led to the infiltration of T cells in the SN, suppressed microglial activation, and increased synthesis of astrocyte-associated glial cell line-derived neurotrophic factor, resulting in neuroprotection of dopaminergic neurons [99]. NSAIDs, such as aspirin, salicylic acid, and ibuprofen, have been shown in certain studies to have neuroprotective effects on dopaminergic neurons and have been suggested as a preventive treatment for PD [10, 48, 49, 100]. However, more research is necessary on the possible correlation between the use of anti-inflammatory drugs and developing PD. Additional anti-inflammatory compounds, like naloxone, minocycline, pioglitazone, and FK506 have been shown to reduce microglial activation and neuronal cell death in different models of PD [13, 58, 101, 102].

The recently described transmissible nature of α-synuclein underlying the progression of the disease has opened new possibilities for therapeutic strategies [89]. In an attempt to target (extracellular) α-synuclein, two immunotherapeutic strategies have been explored: active immunization using the patient's own immune system to generate antibodies against α-synuclein or passive immunization using the direct administration of antibodies against different domains of α-synuclein. Masliah et al. pioneered experimental active immunization targeting α-synuclein. They reported that immunization of WT α-synuclein transgenic mice with α-synuclein decreased protein accumulation in neuronal cell bodies and synapses and reduced neurodegeneration [103]. Later, Sanchez-Guajardo et al. used a viral vector-based α-synuclein rat model to test the active immunization against α-synuclein. Vaccination with recombinant α-synuclein 6 to 10 weeks before intracerebral injection of α-synuclein reduced protein inclusions in the SN associated with an increase in $CD4^+$ T cells and microglial activation [104]. Another vaccination-based approach consisted of administrating short fragments of α-synuclein conjugated to a carrier, also known as the AFFITOPE® AFF1 vaccine, which resulted in the stimulation of antibodies against the C-terminal part of α-synuclein in two mouse models of PD [105]. This strategy has been reported to reduce neuropathology and increase microglial activation and anti-inflammatory cytokine expression. Based on these preclinical results, AFFITOPE vaccines are in a phase I clinical trial for PD and a phase II trial for multiple system atrophy (MSA). Masliah and coworkers also conducted the first study implementing passive immunization. Passive immunization gives the possibility to reduce the dose or to stop the treatment in case of adverse effects. They showed that passive immunization with a novel monoclonal antibody against the C-terminus of α-synuclein (9E4), able to cross the BBB, improved α-synuclein pathology and motor symptoms [106]. These observations support the idea that passive immunization with monoclonal antibodies might be therapeutically relevant for PD and other neurodegenerative disorders. It has been postulated that the antibody-mediated clearance of extracellular α-synuclein mainly occurs in microglia through the Fcγ receptor as shown by the increased localization of α-synuclein as well as the antibody in microglia [107]. A phase Ib trial is currently ongoing to evaluate the humanized form of 9E4, called PRX002, and assess the safety and pharmacokinetics in patients with idiopathic PD. Fagerqvist et al. generated antibodies against different α-synuclein conformations (oligomeric or protofibrillar α-synuclein). This resulted in a decrease of these possible toxic species in the mouse brain as well as in human postmortem brain samples [108]. Although preclinical studies assessing both immunization strategies have been successful, further research is warranted to design and investigate α-synuclein conformation-specific antibodies.

8. Conclusions

Neurodegenerative disorders threaten our society with a substantial economic burden. There is an urgent need to develop novel therapeutic strategies acting on the underlying disease pathogenesis in order to slow down or halt disease progression. In this regard, it is of uttermost importance to better understand how neuroinflammation plays a role in the initiation and progression of PD. Animal models and human studies have generated multiple evidence for the involvement of microglia and T lymphocytes in PD; however, their specific role in disease initiation and progression remains elusive. Immune alterations in response to different α-synuclein conformations may play a critical role in modulating disease progression and outcome. Identifying the immuno-pathogenic conformational state of α-synuclein might open novel therapeutic options. The recruitment of peripheral monocytes has been reported to contribute to PD-associated neurodegeneration, but the exact role of these peripheral monocytes in the disease process remains to be determined. Further insights into the α-synuclein pathology occurring in the CNS or in the ENS as well as the role of the immune cells in this process will be particularly important considering early therapeutic interventions. Recent findings concerning the involvement of LRRK2 in microglial and monocytic activation may provide valuable information about its interactions with α-synuclein and the link to neuroinflammation in PD. In conclusion, targeted interventions aiming at modifying the pathological immune response in PD may prove efficient in slowing disease progression. Future research should focus on identifying new drug targets by broadening our understanding of neuroinflammatory processes in PD-associated disease initiation and progression.

Conflicts of Interest

The authors declare that they have no competing financial interests.

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References

[1] E. R. Dorsey, R. Constantinescu, J. P. Thompson et al., "Projected number of people with Parkinson disease in the most populous nations, 2005 through 2030," *Neurology*, vol. 68, no. 5, pp. 384–386, 2007.

[2] S. Lesage and A. Brice, "Parkinson's disease: from monogenic forms to genetic susceptibility factors," *Human Molecular Genetics*, vol. 18, no. R1, pp. R48–R59, 2009.

[3] A. Ascherio and M. A. Schwarzschild, "The epidemiology of Parkinson's disease: risk factors and prevention," *The Lancet Neurology*, vol. 15, no. 12, pp. 1257–1272, 2016.

[4] D. W. Dickson, H. Braak, J. E. Duda et al., "Neuropathological assessment of Parkinson's disease: refining the diagnostic criteria," *The Lancet Neurology*, vol. 8, no. 12, pp. 1150–1157, 2009.

[5] M. G. Spillantini, M. L. Schmidt, V. M. Y. Lee, J. Q. Trojanowski, R. Jakes, and M. Goedert, "α-Synuclein in Lewy bodies," *Nature*, vol. 388, no. 6645, pp. 839-840, 1997.

[6] H. Braak, K. D. Tredici, U. Rüb, R. A. I. de Vos, E. N. H. Jansen Steur, and E. Braak, "Staging of brain pathology related to sporadic Parkinson's disease," *Neurobiology of Aging*, vol. 24, no. 2, pp. 197–211, 2003.

[7] M. G. Tansey and M. S. Goldberg, "Neuroinflammation in Parkinson's disease: its role in neuronal death and implications for therapeutic intervention," *Neurobiology of Disease*, vol. 37, no. 3, pp. 510–518, 2010.

[8] P. L. McGeer, S. Itagaki, B. E. Boyes, and E. G. McGeer, "Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains," *Neurology*, vol. 38, no. 8, pp. 1285–1291, 1988.

[9] A. Gerhard, N. Pavese, G. Hotton et al., "In vivo imaging of microglial activation with $[^{11}C](R)$-PK11195 PET in idiopathic Parkinson's disease," *Neurobiology of Disease*, vol. 21, no. 2, pp. 404–412, 2006.

[10] H. Chen, E. Jacobs, M. A. Schwarzschild et al., "Nonsteroidal antiinflammatory drug use and the risk for Parkinson's disease," *Annals of Neurology*, vol. 58, no. 6, pp. 963–967, 2005.

[11] S. Theodore, S. Cao, P. J. McLean, and D. G. Standaert, "Targeted overexpression of human α-synuclein triggers microglial activation and an adaptive immune response in a mouse model of Parkinson disease," *Journal of Neuropathology & Experimental Neurology*, vol. 67, no. 12, pp. 1149–1158, 2008.

[12] V. Sanchez-Guajardo, F. Febbraro, D. Kirik, and M. Romero-Ramos, "Microglia acquire distinct activation profiles depending on the degree of α-synuclein neuropathology in a rAAV based model of Parkinson's disease," *PLoS One*, vol. 5, no. 1, article e8784, 2010.

[13] A. Van der Perren, F. Macchi, J. Toelen et al., "FK506 reduces neuroinflammation and dopaminergic neurodegeneration in an α-synuclein-based rat model for Parkinson's disease," *Neurobiology of Aging*, vol. 36, no. 3, pp. 1559–1568, 2015.

[14] A. S. Harms, A. D. Thome, Z. Yan et al., "Peripheral monocyte entry is required for alpha-synuclein induced inflammation and neurodegeneration in a model of Parkinson disease," *Experimental Neurology*, vol. 300, pp. 179–187, 2018.

[15] M. L. Block, L. Zecca, and J. S. Hong, "Microglia-mediated neurotoxicity: uncovering the molecular mechanisms," *Nature Reviews Neuroscience*, vol. 8, no. 1, pp. 57–69, 2007.

[16] V. Sanchez-Guajardo, N. Tentillier, and M. Romero-Ramos, "The relation between α-synuclein and microglia in Parkinson's disease: recent developments," *Neuroscience*, vol. 302, pp. 47–58, 2015.

[17] M. Shimoji, F. Pagan, E. B. Healton, and I. Mocchetti, "CXCR4 and CXCL12 expression is increased in the nigro-striatal system of Parkinson's disease," *Neurotoxicity Research*, vol. 16, no. 3, pp. 318–328, 2009.

[18] M. Mogi, M. Harada, H. Narabayashi, H. Inagaki, M. Minami, and T. Nagatsu, "Interleukin (IL)-1β, IL-2, IL-4, IL-6 and transforming growth factor-α levels are elevated in ventricular cerebrospinal fluid in juvenile parkinsonism and Parkinson's disease," *Neuroscience Letters*, vol. 211, no. 1, pp. 13–16, 1996.

[19] M. Mogi, M. Harada, T. Kondo, H. Narabayashi, P. Riederer, and T. Nagatsu, "Transforming growth factor-β1 levels are elevated in the striatum and in ventricular cerebrospinal fluid in Parkinson's disease," *Neuroscience Letters*, vol. 193, no. 2, pp. 129–132, 1995.

[20] M. Mogi, M. Harada, P. Riederer, H. Narabayashi, K. Fujita, and T. Nagatsu, "Tumor necrosis factor-α (TNF-α) increases both in the brain and in the cerebrospinal fluid from parkinsonian patients," *Neuroscience Letters*, vol. 165, no. 1-2, pp. 208–210, 1994.

[21] S. Hunot, N. Dugas, B. Faucheux et al., "FcεRII/CD23 is expressed in Parkinson's disease and induces, *in vitro*, production of nitric oxide and tumor necrosis factor-α in glial cells," *The Journal of Neuroscience*, vol. 19, no. 9, pp. 3440–3447, 1999.

[22] B. Brodacki, J. Staszewski, B. Toczyłowska et al., "Serum interleukin (IL-2, IL-10, IL-6, IL-4), TNFα, and INFγ concentrations are elevated in patients with atypical and idiopathic parkinsonism," *Neuroscience Letters*, vol. 441, no. 2, pp. 158–162, 2008.

[23] Z. Katsarou, S. Bostantjopoulou, O. Hatzizisi, E. Giza, A. Soler-Cardona, and G. Kyriazis, "Immune factors or depression? Fatigue correlates in Parkinson's disease," *Revista de Neurologia*, vol. 45, no. 12, pp. 725–728, 2007.

[24] J. R. Pereira, L. V. . Santos, R. M. S. Santos et al., "IL-6 serum levels are elevated in Parkinson's disease patients with fatigue compared to patients without fatigue," *Journal of the Neurological Sciences*, vol. 370, pp. 153–156, 2016.

[25] A. Nicoletti, P. Fagone, G. Donzuso et al., "Parkinson's disease is associated with increased serum levels of macrophage migration inhibitory factor," *Cytokine*, vol. 55, no. 2, pp. 165–167, 2011.

[26] D. Blum-Degena, T. Müller, W. Kuhn, M. Gerlach, H. Przuntek, and P. Riederer, "Interleukin-1β and interleukin-6 are elevated in the cerebrospinal fluid of Alzheimer's and de novo Parkinson's disease patients," *Neuroscience Letters*, vol. 202, no. 1-2, pp. 17–20, 1995.

[27] L. Hamelin, J. Lagarde, G. Dorothée et al., "Early and protective microglial activation in Alzheimer's disease: a prospective study using ^{18}F-DPA-714 PET imaging," *Brain*, vol. 139, no. 4, pp. 1252–1264, 2016.

[28] P. R. Territo, J. A. Meyer, J. S. Peters et al., "Characterization of ^{11}C-GSK1482160 for targeting the P2X7 receptor as a biomarker for neuroinflammation," *The Journal of Nuclear Medicine*, vol. 58, no. 3, pp. 458–465, 2017.

[29] C. Venkateshappa, G. Harish, R. B. Mythri, A. Mahadevan, M. M. Srinivas Bharath, and S. K. Shankar, "Increased oxidative damage and decreased antioxidant function in aging human substantia nigra compared to striatum: implications for Parkinson's disease," *Neurochemical Research*, vol. 37, no. 2, pp. 358–369, 2012.

[30] P. Damier, E. C. Hirsch, P. Zhang, Y. Agid, and F. Javoy-Agid, "Glutathione peroxidase, glial cells and Parkinson's disease," *Neuroscience*, vol. 52, no. 1, pp. 1–6, 1993.

[31] H. Braak, M. Sastre, and K. Del Tredici, "Development of α-synuclein immunoreactive astrocytes in the forebrain parallels stages of intraneuronal pathology in sporadic Parkinson's disease," *Acta Neuropathologica*, vol. 114, no. 3, pp. 231–241, 2007.

[32] M. Z. Gomes, R. Raisman-Vozari, and E. A. Del Bel, "A nitric oxide synthase inhibitor decreases 6-hydroxydopamine effects on tyrosine hydroxylase and neuronal nitric oxide synthase in the rat nigrostriatal pathway," *Brain Research*, vol. 1203, pp. 160–169, 2008.

[33] R. Bianchi, C. Adami, I. Giambanco, and R. Donato, "S100B binding to RAGE in microglia stimulates COX-2 expression," *Journal of Leukocyte Biology*, vol. 81, no. 1, pp. 108–118, 2007.

[34] P. L. McGeer, S. Itagaki, and E. G. McGeer, "Expression of the histocompatibility glycoprotein HLA-DR in neurological disease," *Acta Neuropathologica*, vol. 76, no. 6, pp. 550–557, 1988.

[35] V. Brochard, B. Combadière, A. Prigent et al., "Infiltration of CD4^{+} lymphocytes into the brain contributes to neurodegeneration in a mouse model of Parkinson disease," *The Journal of Clinical Investigation*, vol. 119, no. 1, pp. 182–192, 2009.

[36] J. Bas, M. Calopa, M. Mestre et al., "Lymphocyte populations in Parkinson's disease and in rat models of parkinsonism," *Journal of Neuroimmunology*, vol. 113, no. 1, pp. 146–152, 2001.

[37] D. Rosenkranz, S. Weyer, E. Tolosa et al., "Higher frequency of regulatory T cells in the elderly and increased suppressive activity in neurodegeneration," *Journal of Neuroimmunology*, vol. 188, no. 1-2, pp. 117–127, 2007.

[38] J. A. H. Saunders, K. A. Estes, L. M. Kosloski et al., "CD4+ regulatory and effector/memory T cell subsets profile motor dysfunction in Parkinson's disease," *Journal of Neuroimmune Pharmacology*, vol. 7, no. 4, pp. 927–938, 2012.

[39] D. Sulzer, R. N. Alcalay, F. Garretti et al., "T cells from patients with Parkinson's disease recognize α-synuclein peptides," *Nature*, vol. 546, no. 7660, pp. 656–661, 2017.

[40] D. G. Hernandez, X. Reed, and A. B. Singleton, "Genetics in Parkinson disease: Mendelian versus non-Mendelian inheritance," *Journal of Neurochemistry*, vol. 139, Supplement 1, pp. 59–74, 2016.

[41] C. F. Orr, D. B. Rowe, Y. Mizuno, H. Mori, and G. M. Halliday, "A possible role for humoral immunity in the pathogenesis of Parkinson's disease," *Brain*, vol. 128, no. 11, pp. 2665–2674, 2005.

[42] M. A. Gruden, R. D. E. Sewell, K. Yanamandra et al., "Immunoprotection against toxic biomarkers is retained during Parkinson's disease progression," *Journal of Neuroimmunology*, vol. 233, no. 1-2, pp. 221–227, 2011.

[43] K. K. Papachroni, N. Ninkina, A. Papapanagiotou et al., "Autoantibodies to alpha-synuclein in inherited Parkinson's disease," *Journal of Neurochemistry*, vol. 101, no. 3, pp. 749–756, 2007.

[44] K. Yanamandra, M. A. Gruden, V. Casaite, R. Meskys, L. Forsgren, and L. A. Morozova-Roche, "α-Synuclein reactive antibodies as diagnostic biomarkers in blood sera of Parkinson's disease patients," *PLoS One*, vol. 6, no. 4, article e18513, 2011.

[45] N. K. Majbour, N. N. Vaikath, K. D. van Dijk et al., "Oligomeric and phosphorylated alpha-synuclein as potential CSF biomarkers for Parkinson's disease," *Molecular Neurodegeneration*, vol. 11, no. 1, p. 7, 2016.

[46] I. Horvath, I. A. Iashchishyn, L. Forsgren, and L. A. Morozova-Roche, "Immunochemical detection of α-synuclein autoantibodies in Parkinson's disease: correlation between plasma and cerebrospinal fluid levels," *ACS Chemical Neuroscience*, vol. 8, no. 6, pp. 1170–1176, 2017.

[47] S. Abd-Elhadi, M. Basora, D. Vilas, E. Tolosa, and R. Sharon, "Total α-synuclein levels in human blood cells, CSF, and saliva determined by a lipid-ELISA," *Analytical and Bioanalytical Chemistry*, vol. 408, no. 27, pp. 7669–7677, 2016.

[48] M. A. Hernán, G. Logroscino, and L. A. G. Rodríguez, "Nonsteroidal anti-inflammatory drugs and the incidence of Parkinson disease," *Neurology*, vol. 66, no. 7, pp. 1097–1099, 2006.

[49] T. G. Ton, S. R. Heckbert, W. T. Longstreth Jr et al., "Nonsteroidal anti-inflammatory drugs and risk of Parkinson's disease," *Movement Disorders*, vol. 21, no. 7, pp. 964–969, 2006.

[50] P. Holmans, V. Moskvina, L. Jones et al., "A pathway-based analysis provides additional support for an immune-related genetic susceptibility to Parkinson's disease," *Human Molecular Genetics*, vol. 22, no. 5, pp. 1039–1049, 2013.

[51] J. Greenbaum, J. Sidney, J. Chung, C. Brander, B. Peters, and A. Sette, "Functional classification of class II human leukocyte antigen (HLA) molecules reveals seven different supertypes and a surprising degree of repertoire sharing across supertypes," *Immunogenetics*, vol. 63, no. 6, pp. 325–335, 2011.

[52] T. H. Hamza, C. P. Zabetian, A. Tenesa et al., "Common genetic variation in the *HLA* region is associated with late-onset sporadic Parkinson's disease," *Nature Genetics*, vol. 42, no. 9, pp. 781–785, 2010.

[53] M. Saiki, A. Baker, C. H. Williams-Gray et al., "Association of the human leucocyte antigen region with susceptibility to Parkinson's disease," *Journal of Neurology, Neurosurgery, & Psychiatry*, vol. 81, no. 8, pp. 890-891, 2010.

[54] W. T. Wissemann, E. M. Hill-Burns, C. P. Zabetian et al., "Association of Parkinson disease with structural and regulatory variants in the *HLA* region," *American Journal of Human Genetics*, vol. 93, no. 5, pp. 984–993, 2013.

[55] M. Zhang, H. Mu, Z. Shang et al., "Genome-wide pathway-based association analysis identifies risk pathways associated with Parkinson's disease," *Neuroscience*, vol. 340, pp. 398–410, 2017.

[56] C. Villegas-Llerena, A. Phillips, P. Garcia-Reitboeck, J. Hardy, and J. M. Pocock, "Microglial genes regulating

neuroinflammation in the progression of Alzheimer's disease," *Current Opinion in Neurobiology*, vol. 36, pp. 74–81, 2016.

[57] H. Akiyama and P. L. McGeer, "Microglial response to 6-hydroxydopamine-induced substantia nigra lesions," *Brain Research*, vol. 489, no. 2, pp. 247–253, 1989.

[58] Y. He, S. Appel, and W. Le, "Minocycline inhibits microglial activation and protects nigral cells after 6-hydroxydopamine injection into mouse striatum," *Brain Research*, vol. 909, no. 1-2, pp. 187–193, 2001.

[59] R. W. P. Rodrigues, V. C. Gomide, and G. Chadi, "Astroglial and microglial reaction after a partial nigrostriatal degeneration induced by the striatal injection of different doses of 6-hydroxydopamine," *International Journal of Neuroscience*, vol. 109, no. 1-2, pp. 91–126, 2009.

[60] M. K. McCoy, K. A. Ruhn, T. N. Martinez, F. E. McAlpine, A. Blesch, and M. G. Tansey, "Intranigral lentiviral delivery of dominant-negative TNF attenuates neurodegeneration and behavioral deficits in hemiparkinsonian rats," *Molecular Therapy*, vol. 16, no. 9, pp. 1572–9, 2008.

[61] G. T. Liberatore, V. Jackson-Lewis, S. Vukosavic et al., "Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson disease," *Nature Medicine*, vol. 5, no. 12, pp. 1403–1409, 1999.

[62] P. L. McGeer, C. Schwab, A. Parent, and D. Doudet, "Presence of reactive microglia in monkey substantia nigra years after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine administration," *Annals of Neurology*, vol. 54, no. 5, pp. 599–604, 2003.

[63] D. C. Wu, V. Jackson-Lewis, M. Vila et al., "Blockade of microglial activation is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson disease," *The Journal of Neuroscience*, vol. 22, no. 5, pp. 1763–1771, 2002.

[64] E. J. Benner, R. Banerjee, A. D. Reynolds et al., "Nitrated α-synuclein immunity accelerates degeneration of nigral dopaminergic neurons," *PLoS One*, vol. 3, no. 1, article e1376, 2008.

[65] A. D. Reynolds, R. Banerjee, J. Liu, H. E. Gendelman, and R. Lee Mosley, "Neuroprotective activities of CD4+CD25+ regulatory T cells in an animal model of Parkinson's disease," *Journal of Leukocyte Biology*, vol. 82, no. 5, pp. 1083–1094, 2007.

[66] M. B. Watson, F. Richter, S. K. Lee et al., "Regionally-specific microglial activation in young mice over-expressing human wildtype alpha-synuclein," *Experimental Neurology*, vol. 237, no. 2, pp. 318–334, 2012.

[67] R. M. Miller, G. L. Kiser, T. Kaysser-Kranich et al., "Wild-type and mutant α-synuclein induce a multi-component gene expression profile consistent with shared pathophysiology in different transgenic mouse models of PD," *Experimental Neurology*, vol. 204, no. 1, pp. 421–432, 2007.

[68] X. Su, K. A. Maguire-Zeiss, R. Giuliano, L. Prifti, K. Venkatesh, and H. J. Federoff, "Synuclein activates microglia in a model of Parkinson's disease," *Neurobiology of Aging*, vol. 29, no. 11, pp. 1690–1701, 2008.

[69] G. K. Tofaris, P. Garcia Reitböck, T. Humby et al., "Pathological changes in dopaminergic nerve cells of the substantia nigra and olfactory bulb in mice transgenic for truncated human α-synuclein(1-120): implications for Lewy body disorders," *The Journal of Neuroscience*, vol. 26, no. 15, pp. 3942–3950, 2006.

[70] L. Bousset, L. Pieri, G. Ruiz-Arlandis et al., "Structural and functional characterization of two alpha-synuclein strains," *Nature Communications*, vol. 4, article 2575, 2013.

[71] W. Peelaerts, L. Bousset, A. van der Perren et al., "α-Synuclein strains cause distinct synucleinopathies after local and systemic administration," *Nature*, vol. 522, no. 7556, pp. 340–344, 2015.

[72] Y. Couch, L. Alvarez-Erviti, N. R. Sibson, M. J. A. Wood, and D. C. Anthony, "The acute inflammatory response to intranigral α-synuclein differs significantly from intranigral lipopolysaccharide and is exacerbated by peripheral inflammation," *Journal of Neuroinflammation*, vol. 8, no. 1, p. 166, 2011.

[73] H. Wilms, P. Rosenstiel, M. Romero-Ramos et al., "Suppression of MAP kinases inhibits microglial activation and attenuates neuronal cell death induced by α-synuclein protofibrils," *International Journal of Immunopathology and Pharmacology*, vol. 22, no. 4, pp. 897–909, 2009.

[74] A. Sznejder-Pachołek, I. Joniec-Maciejak, A. Wawer, A. Ciesielska, and D. Mirowska-Guzel, "The effect of α-synuclein on gliosis and IL-1α, TNFα, IFNγ, TGFβ expression in murine brain," *Pharmacological Reports*, vol. 69, no. 2, pp. 242–251, 2017.

[75] A. S. Harms, V. Delic, A. D. Thome et al., "α-Synuclein fibrils recruit peripheral immune cells in the rat brain prior to neurodegeneration," *Acta Neuropathologica Communications*, vol. 5, no. 1, p. 85, 2017.

[76] H. J. Lee, J. E. Suk, E. J. Bae, and S. J. Lee, "Clearance and deposition of extracellular α-synuclein aggregates in microglia," *Biochemical and Biophysical Research Communications*, vol. 372, no. 3, pp. 423–428, 2008.

[77] S. Higashi, D. J. Moore, R. Yamamoto et al., "Abnormal localization of leucine-rich repeat kinase 2 to the endosomal-lysosomal compartment in lewy body disease," *Journal of Neuropathology & Experimental Neurology*, vol. 68, no. 9, pp. 994–1005, 2009.

[78] J. Umeno, K. Asano, T. Matsushita et al., "Meta-analysis of published studies identified eight additional common susceptibility loci for Crohn's disease and ulcerative colitis," *Inflammatory Bowel Diseases*, vol. 17, no. 12, pp. 2407–2415, 2011.

[79] A. Gardet, Y. Benita, C. Li et al., "LRRK2 is involved in the IFN-γ response and host response to pathogens," *The Journal of Immunology*, vol. 185, no. 9, pp. 5577–5585, 2010.

[80] J. Schapansky, J. D. Nardozzi, F. Felizia, and M. J. LaVoie, "Membrane recruitment of endogenous LRRK2 precedes its potent regulation of autophagy," *Human Molecular Genetics*, vol. 23, no. 16, pp. 4201–4214, 2014.

[81] M. S. Moehle, P. J. Webber, T. Tse et al., "LRRK2 inhibition attenuates microglial inflammatory responses," *The Journal of Neuroscience*, vol. 32, no. 5, pp. 1602–1611, 2012.

[82] I. Russo, G. Berti, N. Plotegher et al., "Leucine-rich repeat kinase 2 positively regulates inflammation and down-regulates NF-κB p50 signaling in cultured microglia cells," *Journal of Neuroinflammation*, vol. 12, no. 1, p. 230, 2015.

[83] X. Lin, L. Parisiadou, X. L. Gu et al., "Leucine-rich repeat kinase 2 regulates the progression of neuropathology induced by Parkinson's-disease-related mutant α-synuclein," *Neuron*, vol. 64, no. 6, pp. 807–827, 2009.

[84] M. C. Herzig, M. Bidinosti, T. Schweizer et al., "High LRRK2 levels fail to induce or exacerbate neuronal alpha-

synucleinopathy in mouse brain," *PLoS One*, vol. 7, no. 5, article e36581, 2012.

[85] J. P. L. Daher, O. Pletnikova, S. Biskup et al., "Neurodegenerative phenotypes in an A53T α-synuclein transgenic mouse model are independent of LRRK2," *Human Molecular Genetics*, vol. 21, no. 11, pp. 2420–2431, 2012.

[86] J. P. L. Daher, L. A. Volpicelli-Daley, J. P. Blackburn, M. S. Moehle, and A. B. West, "Abrogation of α-synuclein-mediated dopaminergic neurodegeneration in LRRK2-deficient rats," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 25, pp. 9289–9294, 2014.

[87] J. P. L. Daher, H. A. Abdelmotilib, X. Hu et al., "Leucine-rich repeat kinase 2 (LRRK2) pharmacological inhibition abates α-synuclein gene-induced neurodegeneration," *The Journal of Biological Chemistry*, vol. 290, no. 32, pp. 19433–19444, 2015.

[88] H. Braak, E. Ghebremedhin, U. Rüb, H. Bratzke, and K. del Tredici, "Stages in the development of Parkinson's disease-related pathology," *Cell and Tissue Research*, vol. 318, no. 1, pp. 121–134, 2004.

[89] C. Hansen, E. Angot, A. L. Bergström et al., "α-Synuclein propagates from mouse brain to grafted dopaminergic neurons and seeds aggregation in cultured human cells," *The Journal of Clinical Investigation*, vol. 121, no. 2, pp. 715–725, 2011.

[90] C. H. Hawkes, K. Del Tredici, and H. Braak, "A timeline for Parkinson's disease," *Parkinsonism & Related Disorders*, vol. 16, no. 2, pp. 79–84, 2010.

[91] T. Lebouvier, M. Neunlist, S. Bruley des Varannes et al., "Colonic biopsies to assess the neuropathology of Parkinson's disease and its relationship with symptoms," *PLoS One*, vol. 5, no. 9, article e12728, 2010.

[92] L. L. Edwards, E. M. M. Quigley, and R. F. Pfeiffer, "Gastrointestinal dysfunction in Parkinson's disease: frequency and pathophysiology," *Neurology*, vol. 42, no. 4, pp. 726–732, 1992.

[93] H. Braak, R. A. I. de Vos, J. Bohl, and K. del Tredici, "Gastric α-synuclein immunoreactive inclusions in Meissner's and Auerbach's plexuses in cases staged for Parkinson's disease-related brain pathology," *Neuroscience Letters*, vol. 396, no. 1, pp. 67–72, 2006.

[94] T. G. Beach, C. H. Adler, L. I. Sue et al., "Multi-organ distribution of phosphorylated α-synuclein histopathology in subjects with Lewy body disorders," *Acta Neuropathologica*, vol. 119, no. 6, pp. 689–702, 2010.

[95] C. B. Forsyth, K. M. Shannon, J. H. Kordower et al., "Increased intestinal permeability correlates with sigmoid mucosa alpha-synuclein staining and endotoxin exposure markers in early Parkinson's disease," *PLoS One*, vol. 6, no. 12, article e28032, 2011.

[96] F. Scheperjans, V. Aho, P. A. B. Pereira et al., "Gut microbiota are related to Parkinson's disease and clinical phenotype," *Movement Disorders*, vol. 30, no. 3, pp. 350–358, 2015.

[97] A. Aguzzi, F. L. Heppner, M. Heikenwalder et al., "Immune system and peripheral nerves in propagation of prions to CNS," *British Medical Bulletin*, vol. 66, no. 1, pp. 141–159, 2003.

[98] A. S. Harms, C. J. Barnum, K. A. Ruhn et al., "Delayed dominant-negative TNF gene therapy halts progressive loss of nigral dopaminergic neurons in a rat model of Parkinson's disease," *Molecular Therapy*, vol. 19, no. 1, pp. 46–52, 2011.

[99] E. J. Benner, R. L. Mosley, C. J. Destache et al., "Therapeutic immunization protects dopaminergic neurons in a mouse model of Parkinson's disease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 25, pp. 9435–9440, 2004.

[100] J. Couzin, "Halt of Celebrex study threatens drug's future, other trials," *Science*, vol. 306, no. 5705, p. 2170, 2004.

[101] X. Lu, G. Bing, and T. Hagg, "Naloxone prevents microglia-induced degeneration of dopaminergic substantia nigra neurons in adult rats," *Neuroscience*, vol. 97, no. 2, pp. 285–291, 2000.

[102] T. Dehmer, M. T. Heneka, M. Sastre, J. Dichgans, and J. B. Schulz, "Protection by pioglitazone in the MPTP model of Parkinson's disease correlates with IκBα induction and block of NFκB and iNOS activation," *Journal of Neurochemistry*, vol. 88, no. 2, pp. 494–501, 2004.

[103] E. Masliah, E. Rockenstein, A. Adame et al., "Effects of α-synuclein immunization in a mouse model of Parkinson's disease," *Neuron*, vol. 46, no. 6, pp. 857–868, 2005.

[104] V. Sanchez-Guajardo, A. Annibali, P. H. Jensen, and M. Romero-Ramos, "α-Synuclein vaccination prevents the accumulation of parkinson disease-like pathologic inclusions in striatum in association with regulatory T cell recruitment in a rat model," *Journal of Neuropathology & Experimental Neurology*, vol. 72, no. 7, pp. 624–645, 2013.

[105] M. Mandler, E. Valera, E. Rockenstein et al., "Next-generation active immunization approach for synucleinopathies: implications for Parkinson's disease clinical trials," *Acta Neuropathologica*, vol. 127, no. 6, pp. 861–879, 2014.

[106] E. Masliah, E. Rockenstein, M. Mante et al., "Passive immunization reduces behavioral and neuropathological deficits in an alpha-synuclein transgenic model of Lewy body disease," *PLoS One*, vol. 6, no. 4, article e19338, 2011.

[107] E. J. Bae, H. J. Lee, E. Rockenstein et al., "Antibody-aided clearance of extracellular α-synuclein prevents cell-to-cell aggregate transmission," *The Journal of Neuroscience*, vol. 32, no. 39, pp. 13454–13469, 2012.

[108] T. Fagerqvist, V. Lindström, E. Nordström et al., "Monoclonal antibodies selective for α-synuclein oligomers/protofibrils recognize brain pathology in Lewy body disorders and α-synuclein transgenic mice with the disease-causing A30P mutation," *Journal of Neurochemistry*, vol. 126, no. 1, pp. 131–144, 2013.

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Review Article

NLRP3: A Novel Mediator in Cardiovascular Disease

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Cardiovascular disease is a major cause of death worldwide. Inflammasome infiltration has been identified to play a central role in the pathological progression of certain cardiovascular diseases, such as vascular damage spanning atherosclerosis, aneurysm, or arteritis; ischemic heart disease; and other nonischemic heart diseases including diabetic cardiomyopathy, chronic heart failure, and hypertension- or virus-induced cardiac dysfunction. The NLRP3 inflammasome, a key participant in the innate immune response, requires both priming and activation signals for the initiation of inflammation. Piling evidence has revealed that the NLRP3 inflammasome could exert an inflammatory effect by inducing the secretion of proinflammatory cytokines (i.e., IL-1β, IL-18) or could cause pyroptosis, a novel programmed cell death process, in a caspase-1-dependent manner. The importance of the NLRP3 inflammasome in cardiac disease has been broadly investigated. In this review, we present the current knowledge regarding the function of NLRP in vascular disease, ischemic heart disease, and nonischemic heart disease and discuss the potential therapeutic options targeting the NLRP3 inflammasome.

1. Introduction

Cardiovascular disease remains prevalent in both developing and developed countries, presenting high morbidity and mortality rates. Vascular damage such as atherosclerosis, aneurysm, and following ischemic heart diseases accounts for the majority of cardiovascular diseases. Atherosclerosis is a chronically progressive disease characterized by abnormal lipid deposition in large arteries and obstructed blood flow, followed by possible plaque rupture, which could cause embolism in vital organs such as the brain, heart, and kidney [1]. In the last three decades, mounting evidence has supported the notion that inflammation contributes to the pathological process of atherosclerosis in pivotal ways [2]. The initial step of atherosclerosis involves the expression of adhesive factors by vascular endothelial cells and the subsequent accumulation of monocytes and lymphocytes. The next step comprises foam cell formation, a critical feature in atherosclerotic plaque. In this process, monocytes differentiate into macrophages, swallow abundant lipoproteins, and finally eat themselves to death. Under the action of proinflammatory cytokines, atherosclerotic plaque is susceptible to rupture and embolization [2]. Taken together, inflammation participates in every step of atherosclerosis. When embolism occurs in the coronary arteries, the patient is likely to have a heart attack owing to a disrupted blood supply. However, the role of inflammation in myocardial ischemia and reperfusion is rather complicated [3]. Neutrophils have been found to be recruited to the ischemic and reperfused myocardium. Neutrophils can exert harmful effects by secreting proteolytic enzymes, whereas macrophages can exhibit beneficial effects such as promoting angiogenesis and cell proliferation [3]. Moreover, cardiac dysfunction often arises at later stages of metabolic disease. For example, diabetic cardiomyopathy

(DCM), characterized by loss of cardiomyocytes and dysfunction of the left ventricle, is a major contributor to the fatality of patients with diabetes [4]. Interestingly, Luo et al.'s work could help us better understand DCM from an inflammatory perspective [4].

The innate immune system, the first line of defense in the human body, responds to stimuli such as infection or danger signals released from cells. Pattern recognition receptors (PRRs) expressed by macrophages, neutrophils, and other inflammatory cells of the innate immune system can recognize these danger signals, which can be divided into two categories: pathogen-associated molecular patterns (PAMPs) and damage/danger-associated molecular patterns (DAMPs) [5]. PRRs are classified into five families: toll-like receptors (TLRs), nucleotide-binding and oligomerization domain- (NOD-) like receptors (NLRs), retinoic acid inducible gene-I- (RIG-I-) like receptors (RLRs), C-type lectins (CTLs), and absent-in-melanoma- (AIM-) like receptors (ALRs) [6]. TLR is the first identified PRR and functions as a transmembrane receptor. Although NLRs resemble TLRs, they function as cytoplasmic receptors instead [6]. Proteins from the NLR family primarily consist of three parts: the NOD (NACHT), the C-terminal leucine-rich repeat (LRR) domain, and the N-terminal effector domain [6, 7]. Based on different types of N-terminal domains, a major site responsible for binding to other proteins, the NLR family is further divided into four groups: NLRs containing the acidic transactivation domain (named NLRA), the baculoviral inhibitory repeat-like domain (named NLRB), the caspase activation and recruitment domain (CARD; named NLRC), and the pyrin domain (named NLRP) [6–8]. The biochemical properties of NLRs have been discussed in detail by MacDonald et al. [8]. The NLRP family comprises 14 members, which mainly participate in inflammasome formation [6]. In this review, we present the link between the NLRP subfamily and cardiovascular disease, with a focus on NLRP3, the most widely investigated member.

2. NLRP and NLRP Inflammasomes

The NLRP subfamily, characterized by a pyrin-containing domain in the NLRs, is primarily involved in inflammasome formation [6, 7]. According to Amin et al. [7], an inflammasome complex is generally composed of three parts: sensor receptors that could be triggered by PAMPs or DAMPs, adaptors that could facilitate an inflammatory reaction, and effectors that could initiate the inflammatory cascade. Most members of the NLRP family are equipped with a pyrin domain, which serves to interact with another pyrin domain in apoptosis-associated speck-like protein containing a CARD (ASC), a NACHT domain, and an LRR domain, suggested to function as a PAMP-sensing detector [8]. When a PAMP or DAMP is detected by NLRPs, the pyrin domain in the NLRPs binds to the pyrin domain in ASC, which later combines with procaspase-1 via a CARD-CARD interaction. Subsequently, procaspase-1 is converted into caspase-1, and the inflammasome induces either cytokine secretion of IL-1β and IL-18 or pyroptosis (a newly defined programmed cell death) [7]. The release of IL-1 requires two steps: a priming step known to modulate the transcription of pro-IL-1β and NLRP3 (signal 1) and an activation step known to assemble the NLRP3 inflammasome complex, which help process pro-IL-1β into mature IL-1β (signal 2) [5]. Moreover, researchers had discovered that caspase-8 and caspase-1 show similar effects in IL-1β conversion [7]. However, two members in the NLRP subfamily are structurally different from the others. NLRP10 does not possess an LRR domain and hence probably plays a part in signaling rather than in sensing; NLRP1 possesses an extra CARD, which enables it to directly bind to procaspase-1 without any involvement of ASC [6]. Apart from the well-known inflammasome-dependent proinflammatory function, Willingham et al. proved that NLRP3 facilitated macrophage necrosis and release of high-mobility group box 1 protein (HMGB1), another proinflammatory factor, in response to pulmonary infection [9]. Moreover, attenuated inflammation was observed in NLRP3-deficient mice, along with a declined survival rate, suggesting a protective role of NLRP3 [9].

With regard to the regulation of NLRP3 activation in atherosclerosis, this issue can be discussed in two respects: the priming step and the activation step. Duewell et al. revealed cholesterol crystals to be an activator of the NLRP3 inflammasome in macrophages, probably via induction of lysosomal damage. Oxidized LDL (oxLDL) was also demonstrated to promote atherosclerosis, since it facilitated cholesterol crystallization, induced NLRP3 and pro-IL-1β transcription, and thus acted as both signals 1 and 2 [10]. Similar to oxLDL, IL-1β, TNF-α, and other stimuli recognized by TLRs could serve as priming factors and further induce NLRP3 and pro-IL-1 transcription via the NF-κB pathway [1]. Except for cholesterol crystals, there are several common substances and receptors sufficient to induce activation. It has been reported that ATP and various toxins including nigericin and maitotoxin could trigger the activation of the NLRP3 inflammasome via the purinergic 2X7 receptor (P2X7R), subsequently leading to ROS formation, potassium efflux, and mitochondrial DNA release [11–13]. However, ATP also exerts a negative regulatory effect in a rather indirect manner. When ATP binds to P2X7R, it also induces acetylcholine influx, restraining mitochondrial DNA release and NLRP3 activation via the α7 nicotinic acetylcholine receptor signaling pathway [13]. Interestingly, researchers also found that NLRP inflammasome formation occurs voluntarily when the cellular potassium level is below 90 mM and is suppressed at high potassium levels, indicating an indispensable role of K^+ [14]. Under stimulation of oxLDL, CD36 helps to facilitate the intracellular conversion from soluble factors to crystals, thus causing lysosomal damage and rupture. Concomitantly, statistics also revealed that CD36-deficient macrophages generated much lesser IL-1β [15]. Other common crystals include silica and aluminum salts [16]. The overall scheme of NLRP3 inflammasome activation is presented in Figure 1.

3. Role of NLRP in Vascular Disease

3.1. Atherosclerosis. Characterized by endothelium dysfunction, foam cell formation, and lymphocyte infiltration,

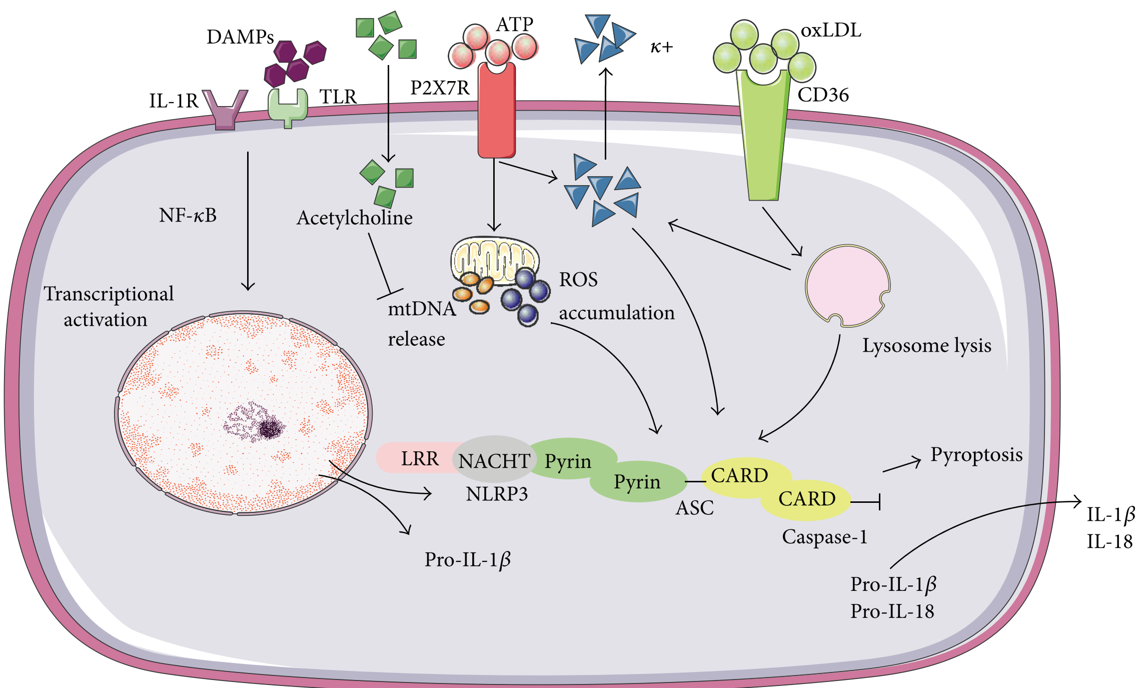

FIGURE 1: Overall scheme of NLRP3 inflammasome activation.

atherosclerosis is widely accepted to be a pathological process of inflammation [2]. However, the crucial role of the NLRP3 inflammasome in atherosclerosis was first proved by Duewell et al. [10]. They employed low-density lipoprotein receptor- (LDLR-) deficient mice with wild-type bone marrow or NLRP3$^{-/-}$, ASC$^{-/-}$, and IL-1$\alpha/\beta^{-/-}$ bone marrow. After 8 weeks of high fat diet, lower level of IL-18, an important biomarker of inflammasome, and attenuated atherosclerotic lesion were observed in the NLRP3$^{-/-}$, ASC$^{-/-}$, and IL-1$\alpha/\beta^{-/-}$ bone marrow group [10]. Consistently, caspase-1/11 depletion in bone marrow of the LDLR-deficient mice fed with high-fat diet also showed significant reduced atherosclerosis plaque [17].

In another study involving atherosclerosis-prone apolipoprotein E-null (Apoe$^{-/-}$) mice, contradictory outcomes were observed. In 2001, researchers found that IL-18-binding protein, an IL-18 inhibitor, could not only attenuate the progress of plaque formation in the aorta but also decrease lymphocyte infiltration and lipid content in the lesion, thus exerting protective effects against atherosclerosis on Apoe$^{-/-}$ mice [18]. Similarly, silencing IL-1α, IL-1β, and caspase-1 respectively in Apoe$^{-/-}$ mice could attenuate atherosclerosis development [19–21]. However, Menu et al. reported contradictory results [22]. After analyzing NLRP3$^{-/-}$, ASC$^{-/-}$, or caspase-1$^{-/-}$ on Apoe-null mice fed with a high-fat diet for 11 weeks, the team observed no significant change in cell infiltration, plaque stability, and atherosclerosis progression [22]. The mechanisms underlying such discrepancies remain undefined; however, Baldrighi et al. [1] suggested that this might be related to the duration of high-fat diet administration to the Apoe-null mice. Compared to mice with Apoe deficiency alone, Apoe$^{-/-}$ mice fed with a high-fat diet and high-methionine diet (a hyperhomocysteinemia- (HHcy-) induced atherosclerosis model) showed an increased atherosclerotic plaque size. Silencing *NLRP3* in such models also reduced macrophage infiltration and HHcy-induced atherosclerosis lesions [23]. Noteworthily, in a transcriptomic analysis of human samples, we found that the mRNA level of NLRP3, ASC, caspase-1, IL-1β, and and IL-18 were all significantly increased in atherosclerotic plaque [24].

In addition to the fact that lacking components of NLRP inflammasomes or proinflammatory cytokines would probably exert protective effects against atherosclerosis, it is suggested that modulating correlative regulators could also exhibit similar effects. Increased P2X7 expression was observed in human atherosclerotic plaque and atherosclerosis-prone mouse models. Moreover, knocking down *P2X7* in Apoe$^{-/-}$ mice delayed the progression of atherosclerosis [25]. Lectin-like oxLDL receptor-1 (LOX-1), a receptor for oxLDL, contributes to the lipid accumulation process of atherosclerosis. Several studies confirmed that in vivo deletion of *LOX-1* in LDLR$^{-/-}$ mice fed with a high-fat diet for 18 weeks resulted in enhanced collagen deposition and attenuated atherosclerosis, while in vitro silencing of *LOX-1* in macrophages reduced mtDNA damage, ROS accumulation, and NLRP3 activation [26–28]. Given that mtDNA enrichment in the cytoplasm is detrimental, Tumurkhuu et al. [29] found a link between OGG1, an important DNA glycosylase that eliminates oxidized DNA, and atherosclerosis, possibly involving the NLRP3 inflammasome. Compared to LDLR$^{-/-}$ mice fed with a western diet, OGG1$^{-/-}$ LDLR$^{-/-}$ mice displayed increased mtDNA accumulation, more severe inflammatory response, and larger atherosclerotic plaques. However, such phenomena could be reversed by silencing *NLRP3* [29], indicating that OGG1 is indeed a negative regulator of atherosclerosis. Furthermore, miR-9 has also been identified as a negative

modulator that deactivates the NLRP3 inflammasome and reduces the atherosclerotic inflammatory response [30]. Although macrophages form a core component of atherosclerotic plaque and NLRP3 inflammasomes mostly reside in macrophages, recent evidence suggests that NLRP3 inflammasomes are also present in endothelial cells (ECs) [31]. EC dysfunction might be triggered by irregular blood flow or cytokines. Microparticles from macrophages induce the expression of adhesion molecules on ECs through the NLRP3 inflammasome, which later attract more inflammatory cells such as macrophages, thus forming an activation loop [31]. Sterol regulatory element-binding protein (SREBP) is a key regulator of cholesterol synthesis and an inducer of inflammation in ECs, which could provide both signals 1 and 2 for NLRP3 inflammasome formation [31]. When the activated form of SREBP was overexpressed in mice with Apoe deficiency, augmented atherosclerosis lesions were observed [31].

3.2. Others. Cholesterol crystals were found in both atherosclerosis plaques and the arterial wall of patients with abdominal aortic aneurysm (AAA). After conducting a genetic test of the NLRP3 inflammasome complex in more than 1000 AAA patients and control group, Roberts et al. reported a possible link between genetic variations of the NLRP3 inflammasome and the pathophysiology of AAA [32]. In a mouse model of coronary arteritis, Chen et al. found that inflammasome complex formation was hindered by silencing *NLRP3*, stabilizing the lysosome membrane or inhibiting cathepsin B, a critical factor released into the cytoplasm after lysosomal decomposition [33]. When mice were subjected to chronic exposure of aldosterone, vascular damage and elevated IL-1β level were observed. However, such effect could be abolished by silencing NLRP3, IL-1R, or caspase-1 [34]. Together, we can conclude that the NLRP3 inflammasome is critically involved in the pathological process of coronary arteritis as well as aldosterone-induced vascular damage [33, 34].

4. Role of NLRP in Ischemic Heart Disease

Considering that the heart tissue is susceptible to ischemia and has limited regenerative ability, reperfusion therapy has been employed in the treatment of ischemic heart disease. Reperfusion is typically accompanied by inflammation. On the one hand, inflammation is indispensable in wound healing and scar formation. On the other hand, excessive inflammatory response may result in adverse remodeling [35]. In 2011, Kawaguchi et al. examined cardiac tissues from patients who died of myocardial infarction (MI). Most of the infiltrated cells were found to be macrophages and neutrophils, showing a high expression of ASC [36]. Similarly, when wild-type (WT) mice and $ASC^{-/-}$ mice were subjected to ischemia/reperfusion (I/R) injury, $ASC^{-/-}$ mice showed a smaller infarcted area, decreased inflammatory cell infiltration, and improved cardiac remodeling. Given that inflammatory cell infiltration was not determined until 6 hours after I/R, an in vitro experiment revealed that the inflammatory response was activated in cardiac fibroblasts, but not cardiomyocytes, under the stimulation of LPS and that the underlying mechanism may involve cellular ROS generation and potassium efflux [36]. Moreover, expression of the NLRP3 inflammasome and its downstream inflammatory cytokines such as IL-1 and IL-18 was elevated in patients with coronary artery diseases or MI [37]. Consistently, Sandanger et al. [38] reported that the expression of the NLRP3 inflammasome and proinflammatory cytokines IL-1 and IL-18 was upregulated in mice that underwent MI surgery. Their data confirmed that inflammasome activation primarily existed in fibroblasts. When $NLRP3^{-/-}$, $ASC^{-/-}$, and WT hearts were exposed to ex vivo I/R injury, heart function was preserved and cell apoptosis was ameliorated in $NLRP3^{-/-}$ hearts, but not in the $ASC^{-/-}$ group [38]. However, contradictory results were presented later in 2016. Sandanger et al. observed an increased infarct size in $NLRP^{-/-}$ mice 24 hours after I/R, while no significant difference was observed in lymphocyte infiltration [39]. When mice were pretreated with a cardioprotective compound before I/R, the due beneficial outcome was not observed in $NLRP^{-/-}$ mice and $ASC^{-/-}$ mice, suggesting a surprisingly protective role of the NLRP inflammasome in an I/R model [39]. The different timepoints chosen for assessment in these studies might account for this discrepancy, since the NLRP inflammasome was not abundantly activated in the early stage [40, 41]. Accordingly, when NLRP3 inhibitors were administered to the MI model, a decreased infarct size was seen at 24 hours after reperfusion, but not at 3 hours. Moreover, the beneficial effects were only observed when the NLRP3 inhibitor was injected immediately or 1 hour after reperfusion, and these effects were subverted when the NLRP3 inhibitor was injected 3 hours after reperfusion, indicating that pharmacological inhibition of NLRP3 has a limited therapeutic time window [41].

Apart from the well-known inflammatory damage induced by the NLRP3 inflammasome complex, caspase-1 has been reported to exert negative effects in an MI model by initiating pyroptosis, a type of programmed cell death in the form of cellular lysis. This damage was observed to be exacerbated in mice with a diabetic background [42]. Regarding the specific cell types in which inflammation occurs after I/R, Liu et al. recently identified cardiac microvascular endothelial cells to be involved, apart from fibroblasts [43]. NLRP3 inflammasome activation in endothelial cells is suggested to be mediated by thioredoxin-interacting/inhibiting protein (TXINP), indicated by the fact that employment of TXINP siRNA would disturb the formation of the NLRP3 inflammasome. Moreover, the NLRP3 inflammasome activation process is suggested to be ROS dependent [43].

5. Role of NLRP in Nonischemic Heart Disease

5.1. Diabetic Cardiomyopathy. DCM is a complication commonly observed at the terminal stage of diabetes; the inflammasome has been found to be pivotal in this process. Given that glucose is an essential inducer of the NLRP3 inflammasome, we speculate that the NLRP3 inflammasome may contribute to the pathological process of DCM [4]. In Luo et al.'s work [44], they employed a gene silencing approach to investigate the role of the NLRP3 inflammasome in type 2 diabetic rats fed with a high-fat diet. As expected, silencing *NLRP*

Table 1: Possible therapeutic approaches targeting the NLRP3 inflammasome.

Experiment types	Model	Treatment	Effects	Ref.
Animal experiments or in vitro experiments	$Apoe^{-/-}$ mouse model, high-fat diet	Arglabin	Reduced the secretion of IL-1β and IL-18, convert proinflammatory M1 macrophage into anti-inflammatory M2 macrophage, induce autophagy, decrease cholesterol level in plasma, reduce atherosclerosis size	[51]
	Type 2 diabetic rat model	Rosuvastatin	Inhibited the NLRP3 inflammasome and suppressed the MAPK pathway	[53]
	AMI mouse model	16673-34-0	Reduced the NLRP3 inflammasome activation in cardiomyocytes, decreased the infarct size	[57]
	AMI mouse model	Colchicine	Inhibited the mRNA expression level of NLRP3 inflammasome components, improved the survival rate	[58]
	In vitro hypoxia model	Pigment epithelium-derived factor (PEDF)	Inhibited the NLRP3 inflammasome by eliminating mitochondrial damage and thus mtROS accumulation	[56]
Clinical trials	Patients with coronary artery disease	Atorvastatin or rosuvastatin for 8 months	Reduced the expression level of the NLRP3 inflammasome and slowed the progression of atherosclerosis in the atorvastatin, but not the rosuvastatin, group	[52]
	Patients with ST-elevation MI	Colchicine	Reduced the infarct size	[59]
	Patients with coronary disease	Colchicine	Decreased the incidence of cardiovascular events	[60]

attenuated cardiac inflammation, fibrosis, cell pyroptosis, and cardiac dysfunction in diabetic rats. Similar effects were noticed in H9C2 cells cultured in a high-glucose medium [44]. Moreover, ROS was found to be a critical mediator in inflammasome activation, as inhibiting ROS accumulation could abolish the activation of the NF-κB and TXINP pathway and further diminish proinflammatory cytokine secretion [44].

5.2. Others. To further assess the role of the NLRP inflammasome in heart failure, Bracey et al. [45] constructed a mouse model in which the calcineurin transgene (CNTg) was heterozygously overexpressed specifically in the heart, mimicking chronic heart failure. Elevated *NLRP3* mRNA levels were observed along with cardiac hypertrophy, inflammation, and ventricular dilatation in CNTg mice. Concurrent with this finding, the team also discovered that genetic ablation of NLRP3 or administration of the IL-1 receptor antagonist could attenuate cardiac inflammation and rescue systolic dysfunction [45]. Moreover, a vital role of the NLRP3 inflammasome in a mouse model of hypertension has also been confirmed [46]. Li et al. performed transverse aortic constriction (TAC) in C57/BL6 mice to induce hypertension. Expression of the NLRP3 inflammasome and its downstream effectors was significantly increased in the TAC group, along with impaired cardiac function; triptolide could attenuate myocardial remodeling and improve cardiac function of the TAC mice by suppressing the NLRP3 inflammasome [46]. In addition, activation of the NLRP3 inflammasome and upregulation of IL-1 were observed both in cardiac fibroblasts pretreated with lipopolysaccharide (LPS) and in a sepsis mouse model [47]. Inhibition of the NLRP3 inflammasome by using glyburide could ameliorate myocardiac dysfunction induced by sepsis [47]. In the background of sepsis, Xie et al. found that *PKM2* knockout hindered the formation of the NLRP3 inflammasome and sepsis cell death [48]. In a coxsackievirus B3- (CVB3-) induced viral myocarditis model, the NLRP3 inflammasome, probably triggered by ROS and K^+ efflux, was found to be critical in the pathogenesis process [49]. Surprisingly, in the aged heart, sustained increase in VEGF-A was found to exert negative effects on cardiac function. Such adverse impacts could be partially abolished by inactivating NLRP3, but not IL-1R or IL-18, indicating that NLRP3 may participate in regulating cardiac function independent of the inflammasome [50].

6. Possible Therapeutic Targets

Since it has been proven that the NLRP3 inflammasome is crucial in the pathological progression of various cardiac diseases, numerous experiments have been conducted to verify different approaches targeting either the NLRP3 inflammasome or its upstream regulators and downstream effectors. For example, in an $Apoe^{-/-}$ mouse model fed with a high-fat diet, arglabin was demonstrated to effectively reduce the secretion of IL-1β and IL-18, convert proinflammatory M1 macrophage into anti-inflammatory M2 macrophage, induce autophagy, decrease the cholesterol level in plasma, and thus reduce the atherosclerosis size [51]. Moreover, in a randomized clinical trial, patients with coronary artery disease were administered atorvastatin or rosuvastatin for 8 months. Results showed that the expression level of the NLRP3 inflammasome was lower in the atorvastatin than in the rosuvastatin group. Such effects might be implicated in the slower progression of atherosclerosis [52]. Interestingly, rosuvastatin, on the other hand, was proven to be potent in the treatment of DCM by inhibiting the NLRP3

inflammasome and suppressing the MAPK pathway [53]. With regard to the beneficial effects of statin, Yu et al. proposed that statin might be utilized in the treatment of atherosclerosis and myocardial I/R injury, since it could regulate both the NLRP1 and NLRP3 inflammasomes [54, 55]. Furthermore, in vitro hypoxia-induced NLRP3 inflammasome activation in cardiomyocytes was abolished by the administration of pigment epithelium-derived factor (PEDF), a glycoprotein known to possess anti-inflammatory effects. PEDF is known to inhibit the NLRP3 inflammasome by eliminating mitochondrial damage and thus mtROS accumulation, the upstream regulator of inflammatory response [56].

In regard to the treatment of I/R injury, a novel small molecule named 16673-34-0 was identified as an effective pharmacological agent to reduce NLRP3 inflammasome activation in cardiomyocytes and decrease the infarct size in a mouse model of acute myocardial infarction (AMI) [57]. Similarly, colchicine was demonstrated to exert cardioprotective effects and improve the survival rate in an AMI mouse model, by inhibiting the mRNA expression level of NLRP3 inflammasome components [58]. Two clinical trials confirmed that colchicine is effective in reducing the infarct size in patients with ST-elevation MI and in decreasing cardiovascular events in patients with coronary disease [59, 60]. Apart from interfering with NLRP3 inflammasome assembly, blocking IL-1 function also serves as an alternative therapeutic approach [35].

The possible therapeutic targets are summarized in Table 1.

7. Conclusions

NLRPs are critical participants in the formation of inflammasomes and initiation of the immune response, which could be triggered by various DAMPs and PAMPs. While the indispensable role of inflammation was established in cardiovascular diseases such as atherosclerosis, aneurysm, cardiac ischemia/reperfusion injury, diabetic cardiomyopathy, chronic heart failure, and hypertension- or virus-induced cardiac dysfunction, the mechanism by which NLRP functions in these diseases has been widely investigated. Until date, we have deduced that the NLRP3 inflammasome contributes significantly to the pathological process of atherosclerosis, cardiac I/R injury, and other nonischemic cardiac diseases. In atherosclerosis and DCM models, silencing NLRP3 or other inflammasome components by using different approaches showed overall beneficial effects. However, outcomes from experiments manipulating the NLRP inflammasome in I/R models remain indefinite. In conclusion, targeting the NLRP inflammasome in cardiovascular disease treatment holds promise, and the optimization of therapeutic approaches requires further clarification regarding the precise role of NLRP in cardiovascular disease.

Conflicts of Interest

The authors declare no competing financial interests.

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References

[1] M. Baldrighi, Z. Mallat, and X. Li, “NLRP3 inflammasome pathways in atherosclerosis,” *Atherosclerosis*, vol. 267, pp. 127–138, 2017.

[2] P. Libby, “Inflammation in atherosclerosis,” *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 32, no. 9, pp. 2045–2051, 2012.

[3] N. G. Frangogiannis, C. W. Smith, and M. L. Entman, “The inflammatory response in myocardial infarction,” *Cardiovascular Research*, vol. 53, no. 1, pp. 31–47, 2002.

[4] B. Luo, F. Huang, Y. Liu et al., “NLRP3 Inflammasome as a molecular marker in diabetic cardiomyopathy,” *Frontiers in Physiology*, vol. 8, p. 519, 2017.

[5] T. Karasawa and M. Takahashi, “Role of NLRP3 inflammasomes in atherosclerosis,” *Journal of Atherosclerosis and Thrombosis*, vol. 24, no. 5, pp. 443–451, 2017.

[6] Y. K. Kim, J.-S. Shin, and M. H. Nahm, “NOD-like receptors in infection, immunity, and diseases,” *Yonsei Medical Journal*, vol. 57, no. 1, pp. 5–14, 2016.

[7] J. Amin, D. Boche, and S. Rakic, “What do we know about the inflammasome in humans?,” *Brain Pathology*, vol. 27, no. 2, pp. 192–204, 2017.

[8] J. A. MacDonald, C. P. Wijekoon, K.-C. Liao, and D. A. Muruve, “Biochemical and structural aspects of the ATP-binding domain in inflammasome-forming human NLRP proteins,” *IUBMB Life*, vol. 65, no. 10, pp. 851–862, 2013.

[9] S. B. Willingham, I. C. Allen, D. T. Bergstralh et al., “NLRP3 (NALP3, Cryopyrin) facilitates in vivo caspase-1 activation, necrosis, and HMGB1 release via inflammasome-dependent and -independent pathways,” *The Journal of Immunology*, vol. 183, no. 3, pp. 2008–2015, 2009.

[10] P. Duewell, H. Kono, K. J. Rayner et al., “NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals,” *Nature*, vol. 464, no. 7293, pp. 1357–1361, 2010.

[11] S. Mariathasan, D. S. Weiss, K. Newton et al., “Cryopyrin activates the inflammasome in response to toxins and ATP,” *Nature*, vol. 440, no. 7081, pp. 228–232, 2006.

[12] P. Kankkunen, E. Välimäki, J. Rintahaka et al., “Trichothecene mycotoxins activate NLRP3 inflammasome through a $P2X_7$ receptor and Src tyrosine kinase dependent pathway,” *Human Immunology*, vol. 75, no. 2, pp. 134–140, 2014.

[13] B. Lu, K. Kwan, Y. A. Levine et al., “α7 nicotinic acetylcholine receptor signaling inhibits inflammasome activation by preventing mitochondrial DNA release,” *Molecular Medicine*, vol. 20, pp. 350–358, 2014.

[14] V. Pétrilli, S. Papin, C. Dostert, A. Mayor, F. Martinon, and J. Tschopp, “Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration,” *Cell Death & Differentiation*, vol. 14, no. 9, pp. 1583–1589, 2007.

[15] F. J. Sheedy, A. Grebe, K. J. Rayner et al., “CD36 coordinates NLRP3 inflammasome activation by facilitating intracellular

nucleation of soluble ligands into particulate ligands in sterile inflammation," *Nature Immunology*, vol. 14, no. 8, pp. 812–820, 2013.

[16] V. Hornung, F. Bauernfeind, A. Halle et al., "Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization," *Nature Immunology*, vol. 9, no. 8, pp. 847–856, 2008.

[17] T. Hendrikx, M. L. J. Jeurissen, P. J. van Gorp et al., "Bone marrow-specific caspase-1/11 deficiency inhibits atherosclerosis development in $Ldlr^{-/-}$ mice," *The FEBS journal*, vol. 282, no. 12, pp. 2327–2338, 2015.

[18] Z. Mallat, A. Corbaz, A. Scoazec et al., "Interleukin-18/Interleukin-18 binding protein signaling modulates atherosclerotic lesion development and stability," *Circulation Research*, vol. 89, no. 7, pp. e41–e45, 2001.

[19] H. Kirii, T. Niwa, Y. Yamada et al., "Lack of interleukin-1β decreases the severity of atherosclerosis in ApoE-deficient mice," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 23, no. 4, pp. 656–660, 2003.

[20] Y. Kamari, A. Shaish, S. Shemesh et al., "Reduced atherosclerosis and inflammatory cytokines in apolipoprotein-E-deficient mice lacking bone marrow-derived interleukin-1α," *Biochemical and Biophysical Research Communications*, vol. 405, no. 2, pp. 197–203, 2011.

[21] J. Gage, M. Hasu, M. Thabet, and S. C. Whitman, "Caspase-1 deficiency decreases atherosclerosis in apolipoprotein E-null mice," *Canadian Journal of Cardiology*, vol. 28, no. 2, pp. 222–229, 2012.

[22] P. Menu, M. Pellegrin, J.-F. Aubert et al., "Atherosclerosis in ApoE-deficient mice progresses independently of the NLRP3 inflammasome," *Cell Death & Disease*, vol. 2, no. 3, article e137, 2011.

[23] R. Wang, Y. Wang, N. Mu et al., "Activation of NLRP3 inflammasomes contributes to hyperhomocysteinemia-aggravated inflammation and atherosclerosis in apoE-deficient mice," *Laboratory Investigation*, vol. 97, no. 8, pp. 922–934, 2017.

[24] G. Paramel Varghese, L. Folkersen, R. J. Strawbridge et al., "NLRP3 Inflammasome expression and activation in human atherosclerosis," *Journal of the American Heart Association*, vol. 5, no. 5, article e003031, 2016.

[25] K. Peng, L. Liu, D. Wei et al., "P2X7R is involved in the progression of atherosclerosis by promoting NLRP3 inflammasome activation," *International Journal of Molecular Medicine*, vol. 35, no. 5, pp. 1179–1188, 2015.

[26] Z. Ding, S. Liu, X. Wang et al., "LOX-1, mtDNA damage, and NLRP3 inflammasome activation in macrophages: implications in atherogenesis," *Cardiovascular Research*, vol. 103, no. 4, pp. 619–628, 2014.

[27] C. Hu, A. Dandapat, L. Sun et al., "LOX-1 deletion decreases collagen accumulation in atherosclerotic plaque in low-density lipoprotein receptor knockout mice fed a high-cholesterol diet," *Cardiovascular Research*, vol. 79, no. 2, pp. 287–293, 2008.

[28] J. L. Mehta, N. Sanada, C. P. Hu et al., "Deletion of LOX-1 reduces atherogenesis in LDLR knockout mice fed high cholesterol diet," *Circulation Research*, vol. 100, no. 11, pp. 1634–1642, 2007.

[29] G. Tumurkhuu, K. Shimada, J. Dagvadorj et al., "*Ogg1*-dependent DNA repair regulates NLRP3 inflammasome and prevents atherosclerosis," *Circulation Research*, vol. 119, no. 6, pp. e76–e90, 2016.

[30] Y. Wang, Z. Han, Y. Fan et al., "MicroRNA-9 inhibits NLRP3 inflammasome activation in human atherosclerosis inflammation cell models through the JAK1/STAT signaling pathway," *Cellular Physiology and Biochemistry*, vol. 41, no. 4, pp. 1555–1571, 2017.

[31] Z. Chen, M. Martin, Z. Li, and J. Y. J. Shyy, "Endothelial dysfunction," *Current Opinion in Lipidology*, vol. 25, no. 5, pp. 339–349, 2014.

[32] R. L. Roberts, A. M. Van Rij, L. V. Phillips et al., "Interaction of the inflammasome genes *CARD8* and *NLRP3* in abdominal aortic aneurysms," *Atherosclerosis*, vol. 218, no. 1, pp. 123–126, 2011.

[33] Y. Chen, X. Li, K. M. Boini et al., "Endothelial Nlrp3 inflammasome activation associated with lysosomal destabilization during coronary arteritis," *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, vol. 1853, no. 2, pp. 396–408, 2015.

[34] T. Bruder-Nascimento, N. S. Ferreira, C. Z. Zanotto et al., "NLRP3 inflammasome mediates aldosterone-induced vascular damage," *Circulation*, vol. 134, no. 23, pp. 1866–1880, 2016.

[35] S. Toldo and A. Abbate, "The NLRP3 inflammasome in acute myocardial infarction," *Nature Reviews Cardiology*, vol. 15, no. 4, pp. 203–214, 2017.

[36] M. Kawaguchi, M. Takahashi, T. Hata et al., "Inflammasome activation of cardiac fibroblasts is essential for myocardial ischemia/reperfusion injury," *Circulation*, vol. 123, no. 6, pp. 594–604, 2011.

[37] L. Wang, P. Qu, J. Zhao, and Y. Chang, "NLRP3 and downstream cytokine expression elevated in the monocytes of patients with coronary artery disease," *Archives of Medical Science*, vol. 10, no. 4, pp. 791–800, 2014.

[38] Ø. Sandanger, T. Ranheim, L. E. Vinge et al., "The NLRP3 inflammasome is up-regulated in cardiac fibroblasts and mediates myocardial ischaemia–reperfusion injury," *Cardiovascular Research*, vol. 99, no. 1, pp. 164–174, 2013.

[39] Ø. Sandanger, E. Gao, T. Ranheim et al., "NLRP3 inflammasome activation during myocardial ischemia reperfusion is cardioprotective," *Biochemical and Biophysical Research Communications*, vol. 469, no. 4, pp. 1012–1020, 2016.

[40] W. M. C. Jong, J. C. Leemans, N. C. Weber et al., "Nlrp3 plays no role in acute cardiac infarction due to low cardiac expression," *International Journal of Cardiology*, vol. 177, no. 1, pp. 41–43, 2014.

[41] S. Toldo, C. Marchetti, A. G. Mauro et al., "Inhibition of the NLRP3 inflammasome limits the inflammatory injury following myocardial ischemia–reperfusion in the mouse," *International Journal of Cardiology*, vol. 209, pp. 215–220, 2016.

[42] Z. Qiu, S. Lei, B. Zhao et al., "NLRP3 inflammasome activation-mediated pyroptosis aggravates myocardial ischemia/reperfusion injury in diabetic rats," *Oxidative Medicine and Cellular Longevity*, vol. 2017, Article ID 9743280, 17 pages, 2017.

[43] Y. Liu, K. Lian, L. Zhang et al., "TXNIP mediates NLRP3 inflammasome activation in cardiac microvascular endothelial cells as a novel mechanism in myocardial ischemia/reperfusion injury," *Basic Research in Cardiology*, vol. 109, no. 5, p. 415, 2014.

[44] B. Luo, B. Li, W. Wang et al., "NLRP3 gene silencing ameliorates diabetic cardiomyopathy in a type 2 diabetes rat model," *PLoS One*, vol. 9, no. 8, article e104771, 2014.

[45] N. A. Bracey, P. L. Beck, D. A. Muruve et al., "The Nlrp3 inflammasome promotes myocardial dysfunction in structural cardiomyopathy through interleukin-1β," *Experimental Physiology*, vol. 98, no. 2, pp. 462–472, 2013.

[46] R. Li, K. Lu, Y. Wang et al., "Triptolide attenuates pressure overload-induced myocardial remodeling in mice via the inhibition of NLRP3 inflammasome expression," *Biochemical and Biophysical Research Communications*, vol. 485, no. 1, pp. 69–75, 2017.

[47] W. Zhang, X. Xu, R. Kao et al., "Cardiac fibroblasts contribute to myocardial dysfunction in mice with sepsis: the role of NLRP3 inflammasome activation," *PLoS One*, vol. 9, no. 9, article e107639, 2014.

[48] M. Xie, Y. Yu, R. Kang et al., "PKM2-dependent glycolysis promotes NLRP3 and AIM2 inflammasome activation," *Nature Communications*, vol. 7, 2016.

[49] Y. Wang, B. Gao, and S. Xiong, "Involvement of NLRP3 inflammasome in CVB3-induced viral myocarditis," *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 307, no. 10, pp. H1438–H1447, 2014.

[50] A. G. Marneros, "Effects of chronically increased VEGF-A on the aging heart," *The FASEB Journal*, 2017.

[51] A. Abderrazak, D. Couchie, D. F. D. Mahmood et al., "Anti-inflammatory and antiatherogenic effects of the NLRP3 inflammasome inhibitor arglabin in $ApoE_2$.Ki mice fed a high-fat diet," *Circulation*, vol. 131, no. 12, pp. 1061–1070, 2015.

[52] M. Satoh, T. Tabuchi, T. Itoh, and M. Nakamura, "NLRP3 inflammasome activation in coronary artery disease: results from prospective and randomized study of treatment with atorvastatin or rosuvastatin," *Clinical Science*, vol. 126, no. 3, pp. 233–241, 2014.

[53] B. Luo, B. Li, W. Wang et al., "Rosuvastatin alleviates diabetic cardiomyopathy by inhibiting NLRP3 inflammasome and MAPK pathways in a type 2 diabetes rat model," *Cardiovascular Drugs and Therapy*, vol. 28, no. 1, pp. 33–43, 2014.

[54] S.-y. Yu, B. Dong, L. Tang, and S.-h. Zhou, "Statin regulates NLRP1 inflammasome expression through SREBP1: a novel anti-atherosclerotic mechanism," *International Journal of Cardiology*, vol. 247, p. 11, 2017.

[55] S.-y. Yu, L. Tang, G.-j. Zhao, and S.-h. Zhou, "Statin protects the heart against ischemia-reperfusion injury via inhibition of the NLRP3 inflammasome," *International Journal of Cardiology*, vol. 229, pp. 23-24, 2017.

[56] Z. Zhou, Z. Wang, Q. Guan et al., "PEDF inhibits the activation of NLRP3 inflammasome in hypoxia cardiomyocytes through PEDF receptor/phospholipase A2," *International Journal of Molecular Sciences*, vol. 17, no. 12, 2016.

[57] C. Marchetti, J. Chojnacki, S. Toldo et al., "A novel pharmacologic inhibitor of the NLRP3 inflammasome limits myocardial injury after ischemia–reperfusion in the mouse," *Journal of Cardiovascular Pharmacology*, vol. 63, no. 4, pp. 316–322, 2014.

[58] K. Fujisue, K. Sugamura, H. Kurokawa et al., "Colchicine improves survival, left ventricular remodeling, and chronic cardiac function after acute myocardial infarction," *Circulation Journal*, vol. 81, no. 8, pp. 1174–1182, 2017.

[59] S. Deftereos, G. Giannopoulos, C. Angelidis et al., "Anti-inflammatory treatment with colchicine in acute myocardial Infarction," *Circulation*, vol. 132, no. 15, pp. 1395–1403, 2015.

[60] S. M. Nidorf, J. W. Eikelboom, C. A. Budgeon, and P. L. Thompson, "Low-dose colchicine for secondary prevention of cardiovascular disease," *Journal of the American College of Cardiology*, vol. 61, no. 4, pp. 404–410, 2013.

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Research Article

Parameters of Somatosensory Evoked Potentials in Patients with Primary Sjögren's Syndrome: Preliminary Results

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Primary Sjogren's syndrome (pSS) is a chronic autoimmune disease. The aim of the study was to establish whether in patients with pSS without central nervous system (CNS) involvement, the function of the central portion of the sensory pathway can be challenged. In 33 patients with pSS without clinical features of CNS damage and normal head computed tomography scan, somatosensory evoked potentials (SEP) were studied. The results were compared to other clinical parameters of the disease, particularly to immunological status. The control group consisted of 20 healthy volunteers. Mean latency of all components of SEP was considerably prolonged in patients compared to the control group. Mean interpeak latency N20-N13 (duration of central conduction TT) did not differ significantly between the groups. However, in the study group, mean amplitude of N20P22 and N13P16 was significantly higher compared to healthy individuals. In patients with pSS, significant differences in SEP parameters depending on the duration of the disease and presence of SSA and SSB antibodies were noted. The authors confirmed CNS involvement often observed in patients with pSS. They also showed dysfunction of the central sensory neuron as a difference in the amplitude of cortical response, which indicates subclinical damage to the CNS.

1. Introduction

Primary Sjogern's syndrome (pSS) is a chronic autoimmune exocrinopathy characterized by dysfunction of exocrine glands resulting from lymphocytic infiltration. Inflammation can occur in other organs as well. The pathogenesis of this disease is not fully understood. pSS affects 0.09–3.5% of the general population, and morbidity is estimated at 3.9–5.3/100000 population. Females are more often affected [1, 2]. Concomitance of pSS and B-cell lymphomas is also observed [3].

Central nervous system (CNS) involvement is estimated to be present in 2.5–60% of patients with pSS. The injury of the peripheral (10–60%) part is more common than that of the CNS (20%) [1–3]. Very rarely (4%), both peripheral and CNS are affected in the same patient [4]. Sensory neuropathy (50–60%), particularly sensory neuropathy of thin fibers, motor sensory neuropathy, and sensory ataxia are the major types of dysfunction. Less commonly, multifocal mononeuropathy (6–12%), polyradiculoneuropathy (4–14%), cranial neuropathy (17%), and autonomic neuropathy (50%) can develop [5, 6].

CNS involvement could have many clinical presentations and various severities. It can be presented as encephalitis, neuromyelitis optica spectrum disorder (NMOSD), or mild episodes of headache or emotional disorder [7, 8]. Radiological images, for example, computed tomography (CT) and magnetic resonance (MR), are necessary for the assessment

of severe CNS involvement. In subclinical benign cases, functional testing, for example, evoked potential of variety modality, is helpful.

Somatosensory evoked potential (SEP) study is one of the most sensitive and objective neurophysiological methods used to evaluate the function of afferent sensory pathways. SEPs are generated at different levels of the sensory pathway leading to the primary sensory cortex through the dorsal column and medial lemniscus. N9 notch is generated by the brachial plexus, and N13 by postganglionic fibers of the first sensory neuron, that is, posterior radix and/or dorsal column of the cervical spinal cord, while N20 originates in the primary sensory cortex of the contralateral parietal lobe, at the location of somatotopic representation of the hand.

The aim of the study was to establish whether in patients with pSS without symptoms of central nervous system involvement, the dysfunction of the central portion of the neurosensory pathway can be observed. The researchers also analyzed whether rheumatological prognostic factors (including skin lesions indicating vasculitis and laboratory parameters reflecting disease activity) correlate with SEP parameters.

1.1. Subjects. The study group consisted of 33 patients (including 1 male) with a mean age of 50, who fulfilled the diagnostic criteria of pSS at the time of SEP study, according to the American-European classification (2002) [9]. Retrospectively, the patients met the diagnostic criteria of pSS since 2016 [10], which were unavailable at the time of the study. The exclusion criteria included a history of neurological, metabolic, and deficiency disorders and use of drugs affecting central nervous system activity. Concomitant diseases included lipid disorder (1 patient), cholelithiasis (1 patient), controlled hypertension (3 patients), and euthyroid struma (9 patients).

The control group consisted of 20 healthy volunteers selected with respect to sex and age.

2. Methods

In all patients, neurological history was taken and physical examination was performed, as well as head CT scan. Fatigue was assessed using the FACIT questionnaire and visual analogue scale (VAS; 0–10 pts, where 0 denoted the total lack of symptom, while 10 referred to intensity affecting everyday home activity). The analysis of pSS activity was conducted using EULAR Sjogren's Syndrome Disease Activity Index (ESSDAI), EULAR Sjogren's Syndrome Patient Reported Index (ESSPRI) scales as well as focus score in minor salivary glands biopsied from the lower lip [11], and laboratory parameters including C3 and C4 component levels, antinuclear antibodies (ANA; measured by indirect immunofluorescence, IF), rheumatoid factor (RF), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), peripheral blood morphology, and total protein level in serum.

The evoked potential study was conducted using the Viking Quest equipment. The procedure was conducted according to the International Federation of Clinical Neurophysiology (IFCN) guidelines [11, 12]. The study was conducted in the supine position, in a quiet and dimmed room at 22–24 degrees Celsius. Superficial Ag/AgCl electrodes with a diameter of 10 mm by Nicolet Instrument Corporation were used and placed on the skin of the head according to the international 10–20 scheme and fixed using the adhesive-conductive Ten20 Conductive paste by D.O. Weaver and Co.

SEPs were achieved by stimulating median nerves with transdermal electric impulses. Three superficial sensory electrodes were placed at (1) Erb's point (mid-clavicular point) with contralateral reference electrode; (2) at the level of C7 vertebra with reference electrode at Fz central point; and (3) on the head skin above the cortex representation of the hand, in the right (C4/P4) and left (C3/P3) parietal regions, on the contralateral side to the stimulated median nerve with reference electrode in the frontal region at Fz point. Ground electrode was placed above the stimulating electrode on the forearm. The stimulating electrode on the wrist generated impulses with a duration of 100 ms and a frequency of 4.7 Hz and intensity resulting in thumb movement in the range of 1-2 cm. Three responses were averaged, and the analysis time was 100 ms. Each registration was performed twice in order to confirm repetitiveness of the measurement.

Responses were selected, and their characteristic components were interpreted. Latency of SEP components were analyzed: peripheral—N9 and N10, brainstem—N13 and P16, cortical—N20 and P22, and interpeak latency—N20-N13, that is, central conduction time TT. Also, the amplitudes of N9/P10, N13/P16, and N20/P22 were assessed.

The study was conducted in accordance with the principles of the Declaration of Helsinki. The locally appointed ethics committee has approved the research protocol (357/2010), and informed consent has been obtained from all the subjects.

2.1. Statistical Analysis. Statistical analysis was conducted using STATISTICA 12.0 software. All tests (for normality, homogeneity of variance, equivalence of means, and ranked tests) were conducted at the significance level of $\alpha = 0.05$. To test the normality of distribution, the Shapiro-Wilko test was used. Variables with normal distribution were tested for homogeneity of variance. After positive verification of both hypotheses (of normal distribution and homogenous variance), the hypothesis of the equivalent means between both groups was tested using Student's t-test. The comparison of variables, the distribution of which was not normal according to the Shapiro-Wilk test, was conducted using the ranked Mann–Whitney U test.

For all multiple testing, Bonferroni's correction was used [13]. Ten variables are tested, therefore the particular significance level of $\alpha = 0.005$.

3. Results

3.1. Analysis of Rheumatological Parameters. In the study group, the mean time since the diagnosis of pSS to the SEP study was 4 years (1–14 years). The first symptoms of pSS included xerophthalmia and/or xerostomia (63%), fatigue

(24%), arthralgia lasting over 30 minutes per day (21%), skin lesions typical for pSS [14] (12%), peripheral arthritis of the extremities (9%), and large swollen salivary glands (6%). At the time of the study, symptoms of arthritis were present on 28 (85%) patients, respiratory involvement in 19 (57%), swelling of major salivary glands (parotid/submandibular glands) in 14 (42%), skin lesions typical for pSS in 10 (30%), and lymphadenopathy in 5 (15%).

The extent of inflammatory infiltration expressed as the mean focus score was 2.1 (0–5) in patients while the ESSDAI score was 20 points (4–24 pts), which indicated the high activity of the disease [15]. In all patients, mild xerophthalmia according to ESSPRI scale 5.2 (2–8) persisted in all patients, while mild xerostomia rated 5.2 (0–9) on ESSPRI scale was noted in 31 patients (94%).

ANA antibodies $\geq 1:320$ were present in 26 (78%) patients with pSS, anti-SSA/Ro60 in 26 (78%), anti-Ro52 in 22 (66%), and anti-SSB/La in 21 (64%). In 3 cases (9%), the presence of RF was not confirmed.

No correlation between pSS activity expressed on the ESSDAI scale and the duration of the disease was found ($p = 0.18$).

All laboratory results are summarized in Table 1. None of the pSS patients showed anemia or thrombocytopenia.

32 patients (94%) were treated with chloroquine (250 mg per day) or hydroxychloroquine (200 mg or 400 mg per day), 28 (82%) with corticosteroids, 6 (18%) with azathioprine, 4 (13%) with cyclosporine, and 5 (15%) with methotrexate.

3.2. Neurological Examination. At the time of the study, all patients reported fatigue lasting for over 3 months, 7 patients complained about sleep disturbances, 7 had low mood, anxiety, and fear, 4 suffered headaches, and one had a history of optic neuritis (which was the primary manifestation of pSS). Mean fatigue assessed using the visual analogue scale (VAS) was 6.7 points (4–10 pts).

On neurological examination, primitive reflexes (snout, palmomental reflex grasping) were noted in 6 patients, and slightly weakened ankle jerk reflexes were observed in 12. Otherwise, the neurological status of the patients was normal, and especially, no dysfunction of position sense, vibration, stereognosis, and two-point discrimination was found. In the patients, no abnormalities on the head CT scan were found. All patients had chronic fatigue syndrome, and 20 patients had normal neurological status (Table 2).

3.3. SEP. SEPs were obtained in all patients and all healthy individuals in the control group. The mean latency of all SEP components was considerably longer in all patients compared to the control group (Table 3). Interpeak N20-N13 (central conduction time TT) did not differ significantly between both groups. In the study group, mean N13/P16 and N20/P22 amplitude was significantly higher compared to healthy individuals; however, no statistically significant difference was found with respect to N9/N10 amplitude (Table 3, Figure 1).

In pSS patients, significant differences in SEP parameters were observed depending on the duration of the disease and the presence of anti-SSA/SSB antibodies. Patients suffering from the disease for more than 10 years compared to patients with a shorter duration of the disease showed higher N9/P10 amplitude (1.2 versus 1.9; $p = 0.0026$). Patients with anti-SSA antibodies (1.4 versus 2.3; $p = 0.0016$) and anti-SSB antibodies (1.3 versus 2.0; $p = 0.0034$) showed significantly lower N13/P16 amplitude.

Table 1: Selected laboratory parameters in pSS patients.

	Number of patients (n/%)	Normal values
Decreased level of C3 component	6/18%	0.9–1.8 g/l
Decreased level of C4 component	4/12%	0.1–0.4 g/l
Hypergammaglobulinemia*	10/30%	0.7–1.4 g/dl
Elevated ESR, mm/h	10/30%	1–20 mm/h
Elevated CRP	3/9%	0–5 mg/l
Lymphopenia	19/57%	1.5–3.5 k/ul

*Hypergammaglobulinemia was measured by electrophoresis of serum.

Table 2: Neurological assessment.

	Number of patients (n/%)
(1) Subjective syndrome:	
(i) Sleep disorder	7/21%
(ii) Depressed mood	7/21%
(iii) Headache	4/12%
(iv) Optic neuritis	1/3%
(2) Changes in neurolocical examination:	
(i) Primitive reflexes	6/18%
(ii) Adynamic ankle jerk	12/33%

Table 3: SEP latency and amplitude in pSS patients and in the control group. These are significant where p value < 0.005 (Bonferroni adjustment).

SEP Value	Study group $n = 33$ Mean ± SD	Control group $n = 20$ Mean ± SD	p value
Latency (ms)			
N9	10.26 ± 0.90	9.51 ± 0.91	*0.009*
P10	12.23 ± 1.13	11.05 ± 0.96	*0.0002*
N13	13.74 ± 1.20	12.80 ± 1.10	*0.0054*
P16	16.97 ± 1.30	15.95 ± 0.89	*0.0014*
N20	20.07 ± 1.37	19.06 ± 1.17	*0.0136*
P22	23.18 ± 1.77	21.90 ± 1.60	*0.0050*
TT (N20–N13)	6.29 ± 1.22	6.26 ± 0.85	0.8507
Amplitude (μV)			
N9/P10	2.76 ± 1.26	2.79 ± 2.10	0.673
N13/P16	1.53 ± 0.62	0.99 ± 0.44	*0.0005*
N20/P22	1.29 ± 0.74	0.81 ± 0.47	*0.0030*

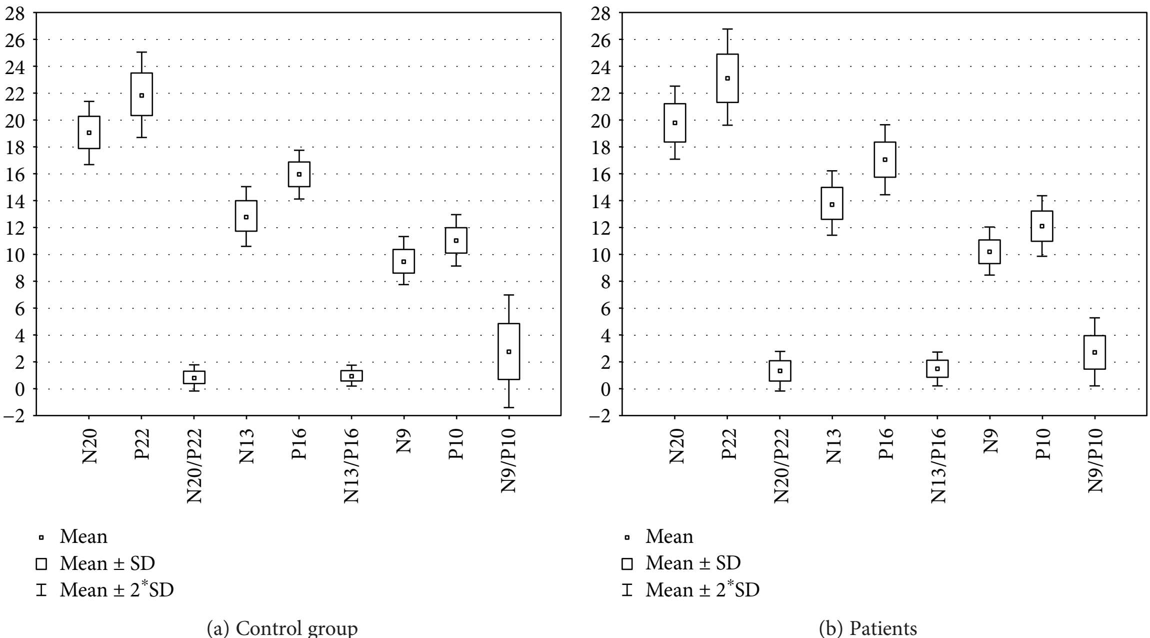

Figure 1: Box plot diagrams for SEP parameters in the (a) control group and (b) study group. Mean: mean standard deviation.

No statistically significant differences in mean SEP parameters were found depending on the presence of skin lesions, xerophthalmia, joint pain and swelling, focus score, C3 and C4 component levels, ESR, CRP, presence of Ro52 antibodies, and treatment. Patients with joint pain lasting more than 10 years show longer P10 latency (13.2 versus 11.9; $p = 0.008$).

4. Discussion

In patients with CNS involvement, in 50% of the cases, the first manifestation of pSS is neurological deficit; however, Delalande et al. [16] in a study on 82 patients showed that neurological symptoms preceded the diagnosis of pSS in 81% of cases. Karaca et al. [17] presented 11 patients, in whom the first symptoms were neurological ones: in 7 patients, CNS involvement manifested as MS-like form and optic neuritis, and in 4 cases, peripheral nerve injury as Guillain-Barre syndrome and multifocal mononeuropathy. In 2 patients, increased SEP latency was observed and in 1 reduced SEP amplitude.

In the literature from the past 20 years, there are only few reports presenting SEP in patients with pSS [18–20]. They cover single cases or very limited groups of patients. For instance, Bouraoui et al. [21] presented a case of a 67-year-old female patient with pSS, in whom reduced pinprick and vibration sensation were observed in lower extremities. In that case, SEP of tibial nerves showed prolonged latency of P40 cortical wave. However, the results of SEP study in pSS are inconsistent. Some publications describe prolonged SEP latency [19, 22], while others present normal SEP [20]. Our study covers a larger population of patients and analyzes SEP parameters in 33 patients. In all patients, the head CT scan was normal, 6 patients presented with frontal release signs, and 12 presented with weakened ankle jerk reflexes without other signs of neuropathy or CNS damage. Only using SEP, which is a sensitive neurophysiological method, allowed to detect bioelectric disturbances in both peripheral and central portions of the sensory pathway. In the study group, prolonged SEP latency at each level—brachial plexus, brainstem, and cortex—was observed. Prolonged latency of response can result from peripheral injury since there were no significant differences in central conduction time TT between the study and the control group. The lack of such differences indicates normal conduction of sensory impulses within the contralateral medial lemniscus and the hypothalamic-cortical tract. However, significantly higher amplitude of cortical response (N20/P22) demonstrates dysfunction of central neurons.

Increased amplitude of cortical response suggests increased excitability of the cortex and lowered seizure threshold. This phenomenon can be observed in autoimmune disorders, where the risk of developing epilepsy is even five times greater [23]. In pSS, seizures can be one of the neurological manifestations of the disease [24–26]. Attout et al. [24] described a 70-year-old female patient with paroxysmal loss of consciousness without clonic movements, in whom paroxysmal focal seizures were observed on electroencephalogram (EEG) from the left anterior electrodes extending to the contralateral side during the HV test.

Introduction of valproic acid resulted in regained consciousness. Seizures in pSS are often drug-resistant and do not show characteristic morphology or primary location. Their pathogenesis can be related to various etiological factors, inflammation, vascular lesions, and antibody production [23]. Although none of our patients presented with epilepsy, the mean amplitude of somatosensory cortical response was significantly higher compared to the control group. Such observations have not been described for pSS so far. However, in patients with myoclonic epilepsy, high

amplitude of N20/P25 complex, so-called gigantic SEP, was observed [27, 28]. The pathomechanism of this phenomenon is unclear. It can suggest disturbed inhibition occurring directly after excitation. There is a hypothesis of possible "wandering" impulse between the primary motor cortex and the primary sensory cortex.

Higher N13/P16 and N20/P22 amplitudes in patients with pSS compared to the control group proved in our study may be associated with glutamatergic excitatory amino acids (EAA). Many publications in recent years indicate the role of EAA receptors in the conduction of sensory and pain impulses, located in spinal nerve ganglions and sensory pathways [29]. It was established that the system contributes to the development of inflammation and rheumatic diseases [30]. The significant role of EAAs in inflammation and pain conduction at the level of the spinal cord and brain was proved in experiments conducted on animal models. For example, two days after the administration of an inflammation-triggering factor, an increase in the number of different glutamatergic receptor subtypes (AMPA, NMDA, KA) in peripheral myelinated and nonmyelinated nerve endings was proved [31]. The relationship between chronic pain and cognitive disorders has been established [32]. Although the influence of pain on neurological complications in pSS is not well known these days, it has been proven that in pSS cognitive dysfunction is the characteristic for subcortical lesions of the frontal lobe [33]. As far as we know, our research is the first one indicating an association between pain and SEP outcomes. Comparing pSS patients with varying durations of arthralgia, we found longer latency of P10 peripheral response at Erb's point. This indicates impaired conduction of the sensory stimulus along the peripheral neuron. It is possible that some cytokines or even nociceptors contributing to pain response caused by inflammation also indirectly play part in the damage of the nervous system. Hence, early treatment of the active forms of the pSS with the involvement of the musculoskeletal system is important in order to stop inflammation. This hypothesis, however, requires further observations and well statistical analysis due to the small number of the study group.

The literature describes the relationship between different immunological profiles among patients with pSS and the development of neurological complications, especially nonataxic sensory neuropathy [5, 29]. Predisposing factors include the presence of ANA antibodies, including anti-SSA/SSB antibodies and RF, as well as hypergammaglobulinemia [5]. Our study also proved, as previously reported, the relationship between the presence of anti-SSA/SSB antibodies and SEP parameters. Significantly lower N13/P16 amplitude was reported in patients with the anti-SSA/SSB antibodies compared to patients without such antibodies. It points out the significant role between inflammation and damage to sensory neurons in pSS. Interestingly, SEP changes did not correlate with lowered values of the C3 and C4 complement components and the intensity of minor salivary gland infiltration expressed by the focus score, which may be an evidence of high disease activity. ESSDAI was initially higher in patients with pSS. Although SEP examination is not part of ESSDAI evaluation, our results show that this examination should consider patients with pSS for the identification of microtraumas of the nervous system. Also, the relation between the presences of purpura, which may be a sign with vasculitis, was not proven, while the damage of small vessels is considered by some authors to be crucial to neuronal damage in pSS [4]. Our study is limited by a lack of cryoglobulinemia testing (due to technical reasons), which is an independent factor of sensorimotor neuropathy and mononeuritis multiplex.

Some authors proposed the role of muscarinic receptor activation in the pathogenesis of pSS and the modulation of certain central nervous system function, for example, cognitive functions [34, 35]. The precise immune mechanisms remain still unclear. Reina et al. postulate that early agonistic-promoting activation in two types of muscarinic acetylcholine receptors (mAChRs) M1 and M3 initiated by antibodies binds to persistently activate cerebral frontal cholinoceptors. This activity might induce desensitization, internalization, and/or intracellular degradation of the mAChR, leading to a progressive decrease of cerebral M1 and M3 mAChR expression and activity. They hypothesized that the CUN manifestations in SS might be induced by an impaired response to cholinergic stimuli by mAChR antibody-specific interactions [36].

Our results showed some SEP differences depending on the duration of pSS. Significantly higher amplitude of N13/P16 was reported in patients with pSS lasting more than 10 years compared with a shorter history of pSS, despite the fact that we did not observe any correlation between ESSDAI. This may be an evidence of insufficient inhibition of pathological processes despite therapy. The influence of the disease duration on evoked potentials has been proved earlier by analyzing P300 endogenous potentials in pSS [37]. It was found then that latency of N200 and P300 components is significantly extended in patients with pSS lasting longer than 10 years, which indicates greater cognitive deficit.

Our results showing prolonged mean latency of all SEP components in patients compared to the control group proved the involvement of the peripheral nervous system, which is often observed in pSS. Despite no differences in central conduction time, significant changes of cortical response amplitude showed dysfunctions of the central sensory neuron, which corresponds to subclinical damage of the CNS. We observed the relationship between SEP parameters and duration of pSS, duration of arthralgia, and presence of anti-SSA and SSB antibodies. Those results, however, require further observations in a larger number of pSS patients.

Conflicts of Interest

The authors declare no conflicts of interest.

References

[1] M. Morreale, P. Marchione, P. Giacomini et al., "Neurological involvement in primary Sjögren syndrome: a focus on central nervous system," *PLoS One*, vol. 9, no. 1, article e84605, 2014.

[2] I. Moreira, F. Teixeira, A. Martins Silva, C. Vasconcelos, F. Farinha, and E. Santos, "Frequent involvement of central nervous system in primary Sjögren syndrome," *Rheumatology International*, vol. 35, no. 2, pp. 289–294, 2015.

[3] G. J. Tobón, J. O. Pers, V. Devauchelle-Pensec, and P. Youinou, "Neurological disorders in primary Sjögren's syndrome," *Autoimmune Diseases*, vol. 2012, Article ID 645967, 11 pages, 2012.

[4] F. Teixeira, I. Moreira, A. M. Silva, C. Vasconcelos, F. Farinha, and E. Santos, "Neurological involvement in primary Sjögren syndrome," *Acta Reumatológica Portuguesa*, vol. 38, no. 1, pp. 29–36, 2013.

[5] D. Sène, M. Jallouli, J. P. Lefaucheur et al., "Peripheral neuropathies associated with primary Sjögren syndrome: immunologic profiles of nonataxic sensory neuropathy and sensorimotor neuropathy," *Medicine*, vol. 90, no. 2, pp. 133–138, 2011.

[6] J. Chai and E. L. Logigian, "Neurological manifestations of primary Sjogren's syndrome," *Current Opinion in Neurology*, vol. 23, no. 5, pp. 509–513, 2010.

[7] D. C. Carvalho, T. S. Tironi, D. S. Freitas, R. Kleinpaul, N. C. Talim, and M. A. Lana-Peixoto, "Sjögren syndrome and neuromyelitis optica spectrum disorder co-exist in a common autoimmune milieu," *Arquivos de Neuro-Psiquiatria*, vol. 72, no. 8, pp. 619–624, 2014.

[8] J. C. McGee and A. Minagar, "A case of neuromyelitis optica spectrum disorder presenting with undiagnosed Sjogren's syndrome and a single, atypical tumefactive lesion: a diagnostic challenge," *Journal of the Neurological Sciences*, vol. 383, pp. 219-220, 2017.

[9] C. Vitali, S. Bombardieri, R. Jonsson et al., "Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American–European consensus group," *Annals of the Rheumatic Diseases*, vol. 61, no. 6, pp. 554–558, 2002.

[10] C. H. Shiboski, S. C. Shiboski, R. Seror et al., "2016 American College of Rheumatology/European League against rheumatism classification criteria for primary Sjögren's syndrome," *Annals of the Rheumatic Diseases*, vol. 76, no. 1, pp. 9–16, 2016.

[11] G. Cruccu, M. J. Aminoff, G. Curio et al., "Recommendations for the clinical use of somatosensory-evoked potentials," *Clinical Neurophysiology*, vol. 119, no. 8, pp. 1705–1719, 2008.

[12] M. R. Nuwer, M. Aminoff, J. Desmedt et al., "IFCN recommended standards for short latency somatosensory evoked potentials. Report of an IFCN committee," *Electroencephalography and Clinical Neurophysiology*, vol. 91, no. 1, pp. 6–11, 1994.

[13] R. A. Armstrong, "When to use the Bonferroni correction," *Ophthalmic & Physiological Optics*, vol. 34, no. 5, pp. 502–508, 2014.

[14] R. Seror, S. J. Bowman, P. Brito-Zeron et al., "EULAR Sjögren's syndrome disease activity index (ESSDAI): a user guide," *RMD Open*, vol. 1, no. 1, article e000022, 2015.

[15] R. Seror, H. Bootsma, A. Saraux et al., "Defining disease activity states and clinically meaningful improvement in primary Sjögren's syndrome with EULAR primary Sjögren's syndrome disease activity (ESSDAI) and patient-reported indexes (ESSPRI)," *Annals of the Rheumatic Diseases*, vol. 75, no. 2, pp. 382–389, 2016.

[16] S. Delalande, J. de Seze, A.-L. Fauchais et al., "Neurologic manifestations in primary Sjögren syndrome," *Medicine*, vol. 83, no. 5, pp. 280–291, 2004.

[17] S. Karaca, E. D. Ersözlü Bozkirli, B. Karakurum Göksel, M. Tan, and A. E. Yücel, "If neurologists establish the diagnosis of primary Sjogren's syndrome?," *Nöro Psikiyatri Arşivi*, vol. 51, no. 2, pp. 148–156, 2014.

[18] J. Birnbaum, "Peripheral nervous system manifestations of Sjögren syndrome: clinical patterns, diagnostic paradigms, etiopathogenesis, and therapeutic strategies," *The Neurologist*, vol. 16, no. 5, pp. 287–297, 2010.

[19] S. F. Storti, A. Del Felice, L. Canafoglia et al., "Neurophysiological and BOLD signal uncoupling of giant somatosensory evoked potentials in progressive myoclonic epilepsy: a case-series study," *Scientific Reports*, vol. 7, article 44664, 2017.

[20] S. Fushimi, I. Nagano, K. Deguchi et al., "A case of subacute myelitis associated with primary Sjögren syndrome showing no MRI abnormality and diagnosed by somatosensory evoked potentials," *Nō to Shinkei*, vol. 56, no. 12, pp. 1029–1034, 2004.

[21] A. Bouraoui, S. F. Bari, J. Nash, and K. Dawson, "Unusual presentation of Sjogren's syndrome," *Case Reports*, vol. 2014, no. 1, article bcr2014206489, 2014.

[22] F. Gökçay, N. Çelebisoy, A. Gökçay et al., "Multimodal evoked potentials in primary Sjögren's syndrome without neurological manifestations," *Journal of Neurological Sciences (Turkish)*, vol. 26, pp. 390–394, 2009.

[23] I. Valencia, "Epilepsy in systemic autoimmune disorders," *Seminars in Pediatric Neurology*, vol. 21, no. 3, pp. 226–231, 2014.

[24] H. Attout, A. Martre, S. Guez, and C. Series, "Syndrome de Sjögren avec épilepsie et dysautonomie," *La Revue de Médecine Interne*, vol. 26, no. 2, pp. 141–144, 2005.

[25] Y. Dong, J. L. Yang, and N. Z. Zhang, "The manifestations of the nervous system in primary Sjogren syndrome," *Zhonghua Nei Ke Za Zhi*, vol. 30, no. 10, pp. 619–21.658, 1992.

[26] S. K. Bansal, I. M. S. Sawhney, and J. S. Chopra, "Epilepsia partialis continua in Sjögren's syndrome," *Epilepsia*, vol. 28, no. 4, pp. 362-363, 1987.

[27] B. Schmitt, L. Thun-Hohenstein, L. Molinari, A. Superti-Furga, and E. Boltshauser, "Somatosensory evoked potentials with high cortical amplitudes: clinical data in 31 children," *Neuropediatrics*, vol. 25, no. 2, pp. 78–84, 1994.

[28] D. Atakli, A. Soysal, T. Atay, H. Altintas, B. Arpaci, and S. Baybas, "Somatosensory evoked potentials and EEG findings in siblings of juvenile myoclonic epilepsy patients," *Epileptic Disorders*, vol. 1, no. 3, pp. 173–177, 1999.

[29] Y. Jamilloux, L. Magy, J. F. Hurtevent et al., "Immunological profiles determine neurological involvement in Sjögren's syndrome," *European Journal of Internal Medicine*, vol. 25, no. 2, pp. 177–181, 2014.

[30] J. Parada-Turska, "Contemporary diagnostics of the primary Sjögren's syndrome," *Wiadomości Lekarskie*, vol. 69, no. 4, pp. 680–685, 2016.

[31] S. M. Carlton and R. E. Coggeshall, "Inflammation-induced changes in peripheral glutamate receptor populations," *Brain Research*, vol. 820, no. 1-2, pp. 63–70, 1999.

[32] O. Moriarty, B. E. McGuire, and D. P. Finn, "The effect of pain on cognitive function: a review of clinical and preclinical research," *Progress in Neurobiology*, vol. 93, no. 3, pp. 385–404, 2011.

[33] V. Le Guern, C. Belin, C. Henegar et al., "Cognitive function and 99mTc-ECD brain SPECT are significantly correlated in patients with primary Sjogren syndrome: a case-control study," *Annals of the Rheumatic Diseases*, vol. 69, no. 1, pp. 132–137, 2009.

[34] R. Tandon, J. E. Shipley, J. F. Greden, N. A. Mann, W. H. Eisner, and J. A. Goodson, "Muscarinic cholinergic hyperactivity in schizophrenia," *Schizophrenia Research*, vol. 4, no. 1, pp. 23–30, 1991.

[35] S. H. Zorn, S. B. Jones, K. M. Ward, and D. R. Liston, "Clozapine is a potent and selective muscarinic M4 receptor agonist," *European Journal of Pharmacology*, vol. 269, no. 3, pp. R1–R2, 1994.

[36] S. Reina, L. Sterin-Borda, B. Orman, and E. Borda, "Autoantibodies against cerebral muscarinic cholinoceptors in Sjögren syndrome: functional and pathological implications," *Journal of Neuroimmunology*, vol. 150, no. 1-2, pp. 107–115, 2004.

[37] E. Dziadkowiak, A. Sebastian, P. Wiland et al., "Endogenous event-related potentials in patients with primary Sjögren's syndrome without central nervous system involvement," *Scandinavian Journal of Rheumatology*, vol. 44, no. 6, pp. 487–494, 2015.

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Research Article

Serological Immunoglobulin-Free Light Chain Profile in Myasthenia Gravis Patients

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Background. Serological levels of free immunoglobulin light chains (FLCs), produced in excess of heavy chains during synthesis of immunoglobulins by plasma cells, can be considered a direct marker of B cell activity in different systemic inflammatory-autoimmune conditions and may represent a useful predictor of rituximab (RTX) therapeutic efficacy, as reported for rheumatoid arthritis and systemic lupus erythematosus. Myasthenia gravis (MG) is an autoimmune disease of the neuromuscular junction with antibodies (abs) targeting the acetylcholine receptor (AChR) or the muscle-specific tyrosine kinase (MuSK), inducing muscle weakness and excessive fatigability. As MG course may be remarkably variable, we evaluated the possible use of FLCs as biomarkers of disease activity. *Subjects and Methods*. We assessed FLC levels in 34 sera from 17 AChR-MG and from 13 MuSK-MG patients, in comparison with 20 sera from patients with systemic autoimmune rheumatic diseases and 18 from healthy blood donors, along with titers of specific auto-abs and IgG subclass distribution. *Results*. We found a statistically significant increase in free κ chains in both AChR- and MuSK-MG patients, while free λ chain levels were increased only in AChR-MG. We also observed a significant reduction of both free κ and λ chains in 1/4 MuSK-MG patients along with specific abs titer, two months after RTX treatment. *Conclusions*. From our data, FLCs appear to be a sensitive marker of B cell activation in MG. Further investigations are necessary to exploit their potential as reliable biomarkers of disease activity.

1. Introduction

Activation of autoreactive B lymphocytes, leading to their differentiation into autoantibodies (auto-abs) producing plasma cells, is the most important pathogenetic mechanism in several autoimmune diseases. Immunoglobulin- (Ig-) free light chains (FLCs) are produced in excess of heavy chains during the synthesis of intact Ig by plasma cells [1] and contribute to inflammation in experimental disease models [2]. In serum, these excess polyclonal FLCs have a short half-life (2–6 hours), and they are excreted by the kidney [3]. Therefore, an increase in their circulating levels reflects either a decreased clearance because of kidney failure [4] or an increased production. Thanks to their short half-life, and in subjects with normal kidney function, their serum levels can be considered as a direct marker of B cell activity, which

is otherwise difficult to measure in routine clinical practice. As a matter of fact, the quantitative assay of κ and λ FLCs and the κ/λ ratio is a useful diagnostic tool in plasma cell dyscrasias, such as mixed cryoglobulinemia, multiple myeloma, monoclonal gammopathy of undetermined significance, and amyloidosis [5–7]. In the last few years, elevated concentrations of polyclonal FLCs in the serum and urine have been reported in patients with rheumatoid arthritis (RA), systemic sclerosis (SS), primary Sjogren syndrome (pSS), and systemic lupus erythematosus (SLE) [8–11]. Serum FLCs have no significant antigen-binding activity and, therefore, are not consumed in immune-inflammatory reactions unlike other molecules (complement, immune complexes, Ig, and auto-abs) that are used as biomarkers of disease activity. Because of these characteristics, they may outperform more widely used biomarkers in evaluating disease activity and predict flares in RA and SLE patients [2, 9].

Myasthenia gravis (MG) is a rare autoimmune disorder with an incidence estimated to be 1-2 per 100,000 and a prevalence of 7–20 per 100,000 [12]. Auto-abs bind to well-defined antigens in the postsynaptic membrane at the neuromuscular junction and impair nerve-muscle transmission, which in turn induces muscle weakness and excessive fatigability. In approximately 85% of the patients, abs are directed against the nicotinic acetylcholine receptors (AChR) [13] while a smaller portion of MG patients produce abs against the muscle specific tyrosine kinase (MuSK) [14, 15] or the low-density lipoprotein receptor-related protein 4 [16]. Along with age at onset and thymus pathology, the auto- abs status is used in the definition of disease subgroups [17].

Due to the fluctuating nature and heterogeneity of the disease, the diagnosis of MG can be puzzling. It is confirmed by the combination of typical symptoms and signs, electromyographic and pharmacological tests, as well as by the detection of specific auto-abs. Disease management can be difficult: therapy must be tailored on the single patient, but the clinical course may be unpredictable and the therapeutic response is highly variable. In order to find a measure of disease activity, many groups focused on the analysis of pro-inflammatory and anti-inflammatory cytokines and molecules: many of them showed significant differences between patients and controls [18–20] but there is no validated commercial kit to routinely measure them. Also, changes of T and B cell subsets were evaluated as possible biomarkers of disease activity and eventually as therapeutic targets [21].

FLCs have never been investigated in MG but could be useful to predict possible variations of B cell activity, which can influence the clinical picture both in the short and in the long range. In this study, we explored the levels of FLCs in MG patients, along with titers of specific auto-abs and IgG subclasses. These results were compared with those from patients with systemic autoimmune rheumatic diseases (SARD) and from healthy blood donors (HBD).

2. Materials and Methods

2.1. Patients and Controls. We collected 34 sera from 30 MG patients referred to the Department of Neurosciences of the Fondazione Policlinico Agostino Gemelli in Rome from June 2007 to April 2016. All patients had generalized MG, as defined by the Myasthenia Gravis Foundation of America (MGFA) classification [22]. MG patients were treated according to the accepted guidelines [23] and are detailed in Supplementary Tables 1 and 2. Seventeen patients had AChR-abs and 13 had anti-MuSK abs; in 4 MuSK-MG patients, a second sample was collected after rituximab (RTX) treatment.

The SARD control group included 12 SLE patients (positive for antinuclear abs with homogeneous pattern and with high levels of anti-double-stranded DNA abs) and 8 RA patients (positive for antinuclear abs with coarse speckled pattern and with high levels of anticyclic citrullinated peptide abs) naïve to drug treatment. Sera from 18 HBD were used as negative controls.

Five MuSK-MG patients (#1, #10, #11, #12, and #13) received RTX at a dose of 375 mg/m2 once a week for 4 consecutive weeks [24]. Efficacy of RTX treatment was verified by cytofluorimetric CD19+ cell count before and after treatment.

All patients had an estimated glomerular filtration rate (eGFR) ≥ 60 mL/min/1.73 m^2. None of them underwent plasmapheresis, nor received high dose intravenous immunoglobulins, during this study. The whole study was conducted according to the Declaration of Helsinki and approved by the Ethical Committee of the Università Cattolica; all the participants provided written informed consent before enrollment. All samples were processed anonymously.

2.2. Laboratory Testing. Sera were obtained by standard centrifugation, divided into aliquots, and stored frozen until analysis. Samples were thawed only once and immediately assayed in a blinded fashion and in a single batch.

FLCs were assessed using the Freelite™ Human Kappa and Lambda Free Kits (The Binding Site, Birmingham, UK) on a SPAPLUS instrument (The Binding Site, UK; free κ normal range: 3.3–19.4 mg/L; free λ normal range: 5.7–26.3 mg/L). A ratio of $\kappa/\lambda < 0.26$ or >1.65 is abnormal, according to the manufacturer's recommendations.

The four IgG subclasses concentration were measured by turbidimetry (Human IgG and IgG subclass liquid reagent kits, The Binding Site) on the SPAPLUS instrument according to the manufacturer's recommendations. Normal range for subclasses: 3.82–9.29 g/L for IgG1; 2.42–7.0 g/L for IgG2; 0.22–1.76 g/L for IgG3; and 0.04–0.86 g/L for IgG4.

Anti-AChR and Anti-MuSK antibodies were detected by radioimmunoprecipitation assay using, respectively, AChR-Ab RIA Kit (cut-off ≥ 0.5 nmol/L) and MuSK-Ab RIA Kit (RSR Cardiff, UK) (cut-off ≥ 0.05 nmol/L) according to the manufacturer's instructions.

2.3. Statistical Analysis. Comparison of mean values was performed by Student's *t*-test; p values < 0.05 were considered significant. A correlation analysis was carried out using the Pearson correlation coefficient.

3. Results

3.1. Serum FLC Assessment in Patients and Controls. Determinations of antibody titer, free κ, free λ, κ/λ ratio, and IgG subclasses in all samples from AChR- and MuSK-MG

Table 1: κ, λ, and k/λ ratio mean values in patients and controls.

	κ-free mg/L	λ-free mg/L	κ/λ
AChR-MG	33.14 ± 26.29 ($p < 0.05$)	18.76 ± 11.79 ($p < 0.05$)	1.69 ± 0.47 ($p < 0.05$)
MuSK-MG	27.02 ± 16.98 ($p < 0.05$)	14.54 ± 8.24 (p n.s.)	1.75 ± 0.52 ($p < 0.05$)
SARD	71.65 ± 115.55 ($p < 0.05$)	52.14 ± 101.54 (p n.s.)	1.76 ± 0.73 ($p < 0.05$)
HBD	16.09 ± 4.28	11.82 ± 3.07	1.39 ± 0.28

Normal range for FLCs: 3.3–19.4 mg/L for κ and 5.7–26.3 mg/L for λ. A ratio of $\kappa/\lambda < 0.26$ or >1.65 is abnormal; p was calculated between each patient group and HBD. n.s.: not significant.

patients are reported in Supplementary Tables 1 and 2, respectively. In AChR-MG patients, 11 and 4 out of 17 samples had a free κ or λ value, respectively, above the range of normality, while 1/17 had a λ value below the range of normality; in MuSK-MG patients, 8 out of the 13 samples displayed a free κ value above the range of normality while 2/13 had a value of free λ above and 2/13 below the range of normality.

The mean values (±standard deviation) of FCLs and κ/λ ratio are reported in Table 1. The statistical analysis revealed significant differences between patients and HBD: the mean values of free κ were above the cut-off and significantly higher in both MG subgroups and in SARD patients as compared to HBD. The mean value of λ-free chain levels was significantly different only in AChR-MG patients when compared to HBD, even if it was still within the normal value (as determined by the manufacturer); SARD patients had higher λ levels both when compared to MG patients and to HBD, but differences were not statistically significant because of the high standard deviation. The κ/λ ratio was significantly higher in both MG subgroups and in the SARD group when compared to HBD.

3.2. Correlation between Serum FLC Levels and Antibody Titer in MG Patients. We evaluated if levels of serum FLCs in MG patients correlated with the specific antibody titer. We found that both free κ and λ chains had a moderate, but significant, correlation with anti-AChR abs titer ($R = 0.388462$, $p = 0.004661$ for κ; $R = 0.345413$, $p = 0.046207$ for λ), while only free κ chains had a weak correlation with anti-MuSK abs titer ($R = 0.203086$, $p = 0.000127$) (Figure 1).

3.3. Serum IgG Subclass Levels among Patients and Controls. The mean values (± standard deviation) of IgG subclasses are reported in Table 2 and visualized in Figures 2 and 3. Only serum IgG1 levels in SARD patients displayed a mean value (9.77 ± 5.68 g/L) which was above the normal range (3.82–9.29 g/L) and statistically different from our HBD mean value (6.46 ± 1.64 g/L, $p = 0.020658$).

3.4. Serological Parameters in Rituximab-Treated MuSK-MG Patients. We were able to measure retrospectively specific auto-abs and FLCs in two blood samples of 4 MuSK-MG patients treated with rituximab (#10–13, Supplementary Table 2), collected at different time points before first infusion (8–60 months) and 3–8 weeks after first RTX treatment (Table 3).

Cytofluorimetric CD19+ cell count displayed a reduction greater than 90% after RTX in all 4 patients. As shown in Table 3, only patient #13 displayed a strong decrease in all serological parameters, along with MGFA score, 8 weeks after the first infusion of RTX while the other 3/4 patients, who were examined at a shorter interval, had variable, but moderate, variations.

4. Discussion

Quantitative analysis of immunoglobulin chain synthesis by B cells demonstrated that there is an excess of light chain production [1] which are then released in the general circulation. Being a by-product of intact immunoglobulin synthesis, they may represent a marker of overall B cell activity, particularly in those diseases where there is an increased formation of immune complexes like systemic autoimmune disorders. In these conditions, characterized by chronic inflammatory reactions, FLCs may play a pathogenetic role thanks to their enzymatic activities and binding to intra- and extracellular proteins, which, in turn, can initiate and maintain the inflammatory cascade [25]. Previous reports confirmed that patients with systemic autoimmune diseases have FLC levels significantly higher than the normal population, with a normal κ/λ ratio. These studies focused on relatively common SARD, in particular, SLE, RA, and SS: concentrations of FLCs were found to be significantly increased and have been investigated as possible biomarkers of the progression and severity of these chronic inflammatory diseases [8–11] as well as a potential therapeutic target [25]. In these conditions, the normality of κ/λ ratio, while free κ and λ chains were elevated, has been explained with their polyclonal production.

Little, if nothing, is known about FLC levels in organ-specific autoimmune diseases. Here, we report our results on FLC analysis in MG patients with anti-AChR and anti-MuSK auto-abs, and compare them with HBD and SARD patients. Our data demonstrate a statistically significant increase in free κ chains in both AChR- and MuSK-MG, as well as in SARD, when compared to HBD, with mean values more elevated in SARD than in MG (Table 1). When we analyzed free λ chain levels, we found that only in AChR-MG there was a little but statistically significant increase if compared to HBD, while they were normal in MuSK-MG: this result could be ascribed to the different immunosuppressive therapies that have been used in these two groups of patients. These differences in the trend of κ FLCs (definite increased in both MG subgroups) and λ FLCs (increased to less extents only in AChR-MG) can ultimately explain why we observe a statistically significant increase of the κ/λ ratio in both MG subgroups. SARD patients had greatly increased

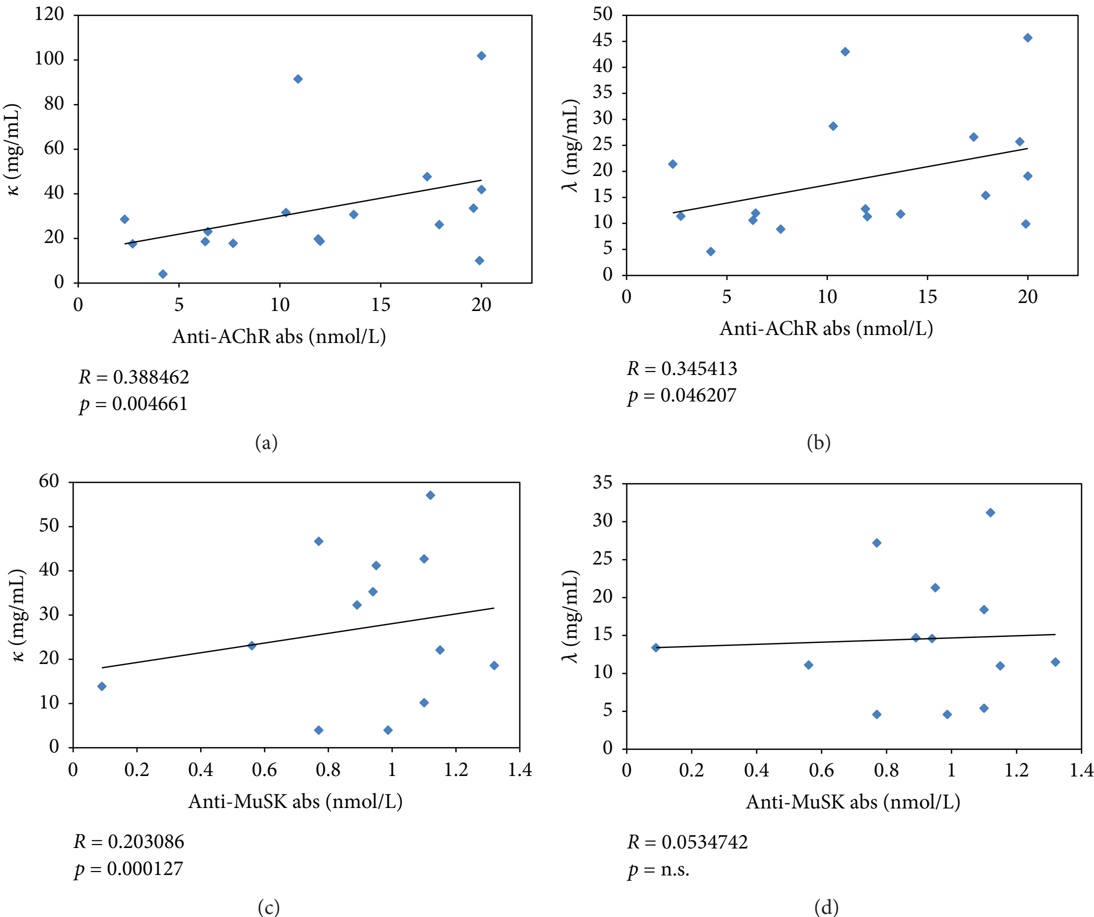

Figure 1: Correlation between κ and λ levels and anti-AChR (a and b) and anti-MuSK (c and d) antibodies.

Table 2: IgG subclasses distribution in patients and controls.

	AChR-MG	MuSK-MG	SARD	HBD
IgG1, g/L	7.93 ± 5.73	6.43 ± 4.75	9.77 ± 5.68*	6.46 ± 1.64
IgG2, g/L	4.57 ± 2.68	4.92 ± 2.90	4.28 ± 1.53	4.39 ± 1.06
IgG3, g/L	0.64 ± 0.45	0.57 ± 0.49	0.87 ± 0.60	0.81 ± 0.31
IgG4, g/L	0.35 ± 0.37	0.36 ± 0.34	0.43 ± 0.42	0.32 ± 0.19

Normal range for subclasses: 3.82–9.29 g/L for IgG1; 2.42–7.0 g/L for IgG2; 0.22–1.76 g/L for IgG3; and 0.04–0.86 g/L for IgG4. *$p = 0.02$.

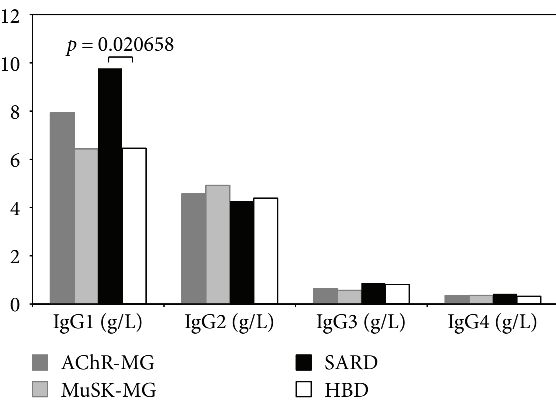

Figure 2: Serum IgG subclass levels in AChR-MG, MuSK-MG, SARD, and HBD.

free λ levels, compared to both HBD and MG patients, but differences were not significant because of higher standard deviations (Table 1).

The finding of a correlation of anti-AChR abs with both κ and λ FLCs, as opposed to a weaker correlation of only κ FLCs with anti-MuSK abs, can be explained by the demonstrated different antibody repertoire in MuSK-MG as compared to AChR-MG [26].

Our finding of a small, but significant, increase of FLCs in MG may reflect the different pathogenetic mechanism between organ specific and systemic autoimmune diseases like SLE and RA: in the latter conditions, FLCs can increase the inflammatory reaction, which is a relevant pathogenic aspect of these diseases [25] that is not present in an organ-specific autoimmune disease like MG.

Rituximab emerged as an effective option in those MG patients refractory to conventional immunosuppression, with particular benefit for patients with MuSK-MG [27]. It is a chimeric mouse/human monoclonal antibody against the surface antigen CD20, which is expressed during early pre-B cell development: it is present on naïve and memory

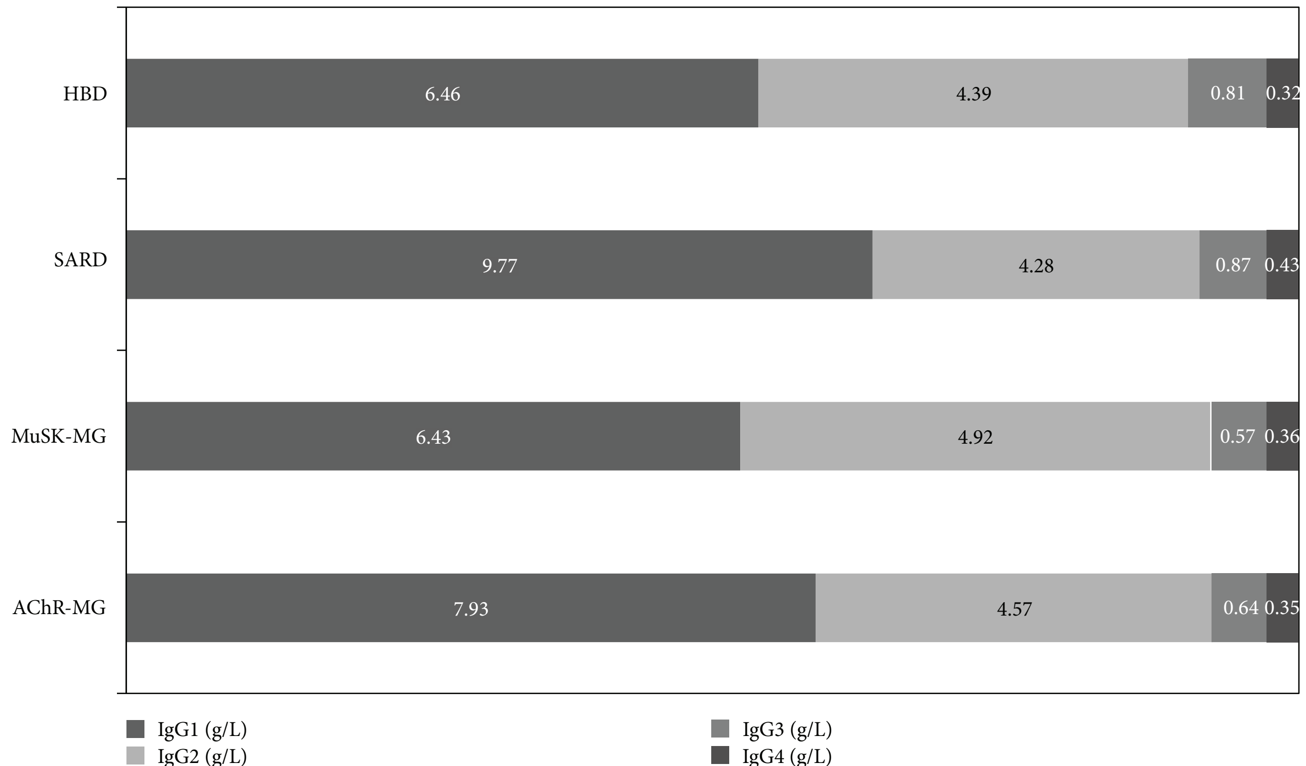

Figure 3: Serum IgG1–4/IgG distribution in HBD, SARD, MuSK-MG, and AChR-MG.

Table 3: Serological parameters in four MuSK-MG patients pre and post rituximab therapy.

Patients	RTX therapy	Anti-MuSK abs nmol/L	κ-free mg/mL	λ-free mg/mL	κ/λ	CD19+ cell count %	MGFA score
#10	Pre	0.77	4.00	4.60	0.87	9.40	III b
	Post 3 weeks	0.83	4.00	4.60	0.87	0.40	III b
Δ%		+7.79	0.00	0.00	0.00	−95.74	
#11	Pre	1.32	18.60	11.50	1.62	11.00	III b
	Post 4 weeks	1.41	27.60	14.90	1.85	0.10	II b
Δ%		+6.82	+48.39	+29.57	+14.2	−99.09	
#12	Pre	1.12	57.10	31.20	1.83	15.00	III b
	Post 6 weeks	1.09	49.80	41.10	1.21	1.00	III b
Δ%		−2.68	−12.78	+31.73	−33.88	−93.34	
#13	Pre	0.89	32.30	14.70	2.20	4.00	IV b
	Post 8 weeks	0.49	13.50	7.50	1.80	0.20	III b
Δ%		−44.94	−58.20	−48.98	−18.18	−95.00	

RTX therapy: the pretherapy sample was collected 8–60 months before first infusion. CD19+ cell count: percent over total peripheral blood lymphocytes. Δ% represents the percent variation over the pretherapy sample.

B cells, but not on stem cells or fully differentiated plasma cells. The human protein is part of a multimeric complex regulating Calcium transport across the cell membrane, thus controlling B lymphocytes activation and proliferation; accordingly, RTX binding to CD20 interferes with these processes [28]. Circulating Ig-producing long-lived plasma cells are not depleted by RTX [29]. A proposed model for MuSK-MG responsiveness to RTX suggests that the consistent reduction in MuSK-MG auto-abs titer, seen as early as 3 months after first infusion, depends on short-lived antibody-secreting plasmablasts. As only a small fraction of these cells is CD20+, MuSK-MG outcome may depend on depletion of a pool of plasmablast-progenitor CD20+ memory B cells; alternatively, the direct depletion of the CD20+ fraction of plasmablasts by rituximab could contribute to clinical response [30, 31]. B cells and plasmablasts seem to be key players in several autoimmune, RTX-responsive disorders [32]. Recent studies evaluated the role

of FLCs as a marker of the therapeutic efficacy of RTX in patients with SLE and RA and their results seemed promising [33, 34]. To investigate if serum FLCs can be considered a biomarker of both disease activity and effectiveness of RTX treatment, we retrospectively analyzed the serological profile in 4 (#10–13) out of our 13 MuSK-MG patients, before and 3–8 weeks after RTX infusion, when CD19+ cell count was decreased by more than 90%. We observed a significant reduction of both free κ and λ chains (−58.20% and −48.98%, resp.) only in patient #13, 8 weeks after the first RTX infusion. Even if it remained above the cut-off of positivity, also specific anti-MuSK auto-abs titer decreased from 0.89 to 0.49 nmol/L (−44.94%) along with a reduction of all IgG subclasses (Supplementary Table S2). In the other three RTX-treated patients, the post-RTX samples were drawn after a shorter interval (3–6 weeks), so that it was perhaps too early to observe the effect of CD20+ cells depletion, either on FLCs or on anti-MuSK abs titers. This finding is consistent with previous reports [30, 33]. On the whole, our results suggest that serum FLCs may represent a new marker of B cell activation in MG, which parallels auto-abs titer variations in response to B cell depleting therapy.

Most of the auto-abs are class G Ig (IgG), which includes 4 subclasses (IgG1–4). These IgG subclasses could contribute to the immunopathogenesis by modulating interaction of Ig, Fc-γ receptor and complement. Differences in serum IgG subclasses distribution between patients with autoimmune diseases and healthy controls have been described only recently, with distinct patterns in different conditions [35–37]. Here, we reported the IgG subclass mean levels and distribution among AChR-MG, MuSK-MG, SARD, and HBD (Table 2, Figures 2 and 3): only IgG1 in SARD had a statistically significant difference when compared to HBD and was above the range of normality. Our results do not suggest that IgG subclasses can have a diagnostic role in MG, and they further strengthen the hypothesis that serum IgG subclass distribution has peculiar characteristics in different autoimmune disease as already reported in a cross-sectional study in other autoimmune diseases [37]. Further elucidating these characteristics could lead to a better understanding of their pathogenetic roles in autoimmune disease development.

During the last twenty years, a novel systemic, chronic, and inflammatory disease entity with specific features has been described: IgG4-related disease (IgG4-RD) [38]. It is an uncommon immune-mediated inflammatory condition that affects a wide variety of organs, including the nervous system, characterized by tissue infiltration with IgG4+ plasma cells, storiform fibrosis, and frequent increase of serum IgG4 levels. Most anti-MuSK auto-abs belong to the IgG4 subclass [39], which cause MG in mice and humans by directly interfering with MuSK function [40, 41]. However, MuSK-MG does not fulfill IgG4-RD criteria, even if a recent case report opens the question of a possible link between the two diseases [42]. Our results did not show any significant increase in IgG4 mean values in all patients (AChR-, MuSK-MG, and SARD) when compared to HBD.

There is a great need for biomarkers in MG to identify patients at risk for disease flares, monitor response to treatment, and be a guide to a better management of immunosuppression. Different groups had tried to identify such markers: they analyzed large panels of proinflammatory and anti-inflammatory cytokines and molecules, finding many analytes which can be promising [18–20]. Several reports, analysing T and B cell subsets in MG patients, showed a disequilibrium between follicular and regulatory T cells together with a lower frequency of regulatory B cells. Longitudinal studies, evaluating changes in these cell subsets in different MG phases and in response to treatment, will clarify their role as markers of disease activity [21].

A truly useful biomarker should meet strict criteria: ideally, it should be central to the pathophysiology of the disease, it should be related to the disease activity and severity, it should fluctuate only with clinical flares or progression, and it should be sensitive to treatments known to be effective. We are conscious that there are limitations to our study. First, due to the small sample size, only a few potential confounders could be controlled, mainly those variables known to influence FLC levels, like kidney failure and plasmapheresis. Second, we could not include an active control group of subjects with acute viral and/or bacterial infections. Finally, our analytical method was consistent with previously reported studies [2, 9], but we acknowledge that it may not be the optimal one [43]. For these reasons, at the present time, we cannot suggest that the determination of FLCs has clinical relevance, with a cost/benefit ratio justifying its use in MG clinical management. Further investigations using longitudinal analysis will be necessary to validate it as a valuable tool to predict MG fluctuations and to monitor clinical management. Our goal, however, was to conduct a pilot study on serum levels of FLCs and IgG subclasses in an organ-specific autoimmune disease like MG. We report for the first time an increase of serum free κ chains in both AChR- and MuSK-MG patients, not correlated with an increase of circulating IgG: this can be explained with a continuous activation of the immune response which, as it is aimed at a specific autoantigen, does not reach levels as high as to increase total IgG. In this scenario, FLC determination seems to be a sensitive index of B cell activation even in organ-specific disorders where the autoimmune response is limited at a well-defined, single autoantigen.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Umberto Basile, Mariapaola Marino, Carlo Provenzano, and Emanuela Bartoccioni designed the study and analyzed the data. Umberto Basile, Cecilia Napodano, Krizia Pocino, and Francesca Gulli performed the analysis. Paolo Emilio Alboini and Amelia Evoli collected patients' serum samples and clinical data. Mariapaola Marino, Carlo Provenzano, and Emanuela Bartoccioni wrote the manuscript. Umberto Basile and Mariapaola Marino equally contributed to this work.

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Supplementary Materials

Supplementary 1. Table S1: the anonymous data set of AChR-MG patients.

Supplementary 2. Table S2: the anonymous data set of MuSK-MG patients.

References

[1] J. E. Hopper and E. Papagiannes, "Evidence by radioimmunoassay that mitogen-activated human blood mononuclear cells secrete significant amounts of light chain Ig unassociated with heavy chain," *Cellular Immunology*, vol. 101, no. 1, pp. 122–131, 1986.

[2] A. Burmeister, L. K. Assi, C. J. Ferro et al., "The relationship between high-sensitivity CRP and polyclonal free light chains as markers of inflammation in chronic disease," *International Journal of Laboratory Hematology*, vol. 36, no. 4, pp. 415–424, 2014.

[3] T. A. Waldmann, W. S. Strober, and R. P. Mogielnicki, "The renal handling of low molecular weight proteins. *II. Disorders of serum protein catabolism in patients with tubular proteinuria, the nephrotic syndrome, or uremia*," *Journal of Clinical Investigation*, vol. 51, no. 8, pp. 2162–2174, 1972.

[4] C. A. Hutchison, S. Harding, P. Hewins et al., "Quantitative assessment of serum and urinary polyclonal free light chains in patients with chronic kidney disease," *Clinical Journal of the American Society of Nephrology*, vol. 3, no. 6, pp. 1684–1690, 2008.

[5] U. Basile, L. Gragnani, A. Piluso et al., "Assessment of free light chains in HCV-positive patients with mixed cryoglobulinaemia vasculitis undergoing rituximab treatment," *Liver International*, vol. 35, no. 9, pp. 2100–2107, 2015.

[6] P. Milani, G. Palladini, and G. Merlini, "Serum-free light-chain analysis in diagnosis and management of multiple myeloma and related conditions," *Scandinavian Journal of Clinical and Laboratory Investigation*, vol. 76, Supplement 245, pp. S113–S118, 2016.

[7] J. L. García de Veas Silva, C. Bermudo Guitarte, P. Menéndez Valladares, J. C. Rojas Noboa, K. Kestler, and R. Duro Millán, "Prognostic value of serum free light chains measurements in multiple myeloma patients," *PLoS One*, vol. 11, no. 11, article e0166841, 2016.

[8] K. Sølling, J. Sølling, and F. K. Rømer, "Free light chains of immunoglobulins in serum from patients with rheumatoid arthritis, sarcoidosis, chronic infections and pulmonary cancer," *Acta Medica Scandinavica*, vol. 209, no. 1-6, pp. 473–477, 1981.

[9] R. Aggarwal, W. Sequeira, R. Kokebie et al., "Serum free light chains as biomarkers for systemic lupus erythematosus disease activity," *Arthritis Care & Research*, vol. 63, no. 6, pp. 891–898, 2011.

[10] X. Deng, C. S. Crowson, S. V. Rajkumar et al., "Elevation of serum immunoglobulin free light chains during the preclinical period of rheumatoid arthritis," *The Journal of Rheumatology*, vol. 42, no. 2, pp. 181–187, 2015.

[11] J. E. Gottenberg, F. Aucouturier, J. Goetz et al., "Serum immunoglobulin free light chain assessment in rheumatoid arthritis and primary Sjögren's syndrome," *Annals of the Rheumatic Diseases*, vol. 66, no. 1, pp. 23–27, 2007.

[12] J. S. Yi, J. T. Guptill, P. Stathopoulos, R. J. Nowak, and K. C. O'Connor, "B cells in the pathophysiology of myasthenia gravis," *Muscle & Nerve*, vol. 57, no. 2, pp. 172–184, 2018.

[13] A. Vincent and J. Newsom-Davis, "Acetylcholine receptor antibody characteristics in myasthenia gravis. III. Patients with low anti-AChR antibody levels," *Clinical and Experimental Immunology*, vol. 60, no. 3, pp. 631–636, 1985.

[14] W. Hoch, J. McConville, S. Helms, J. Newsom-Davis, A. Melms, and A. Vincent, "Auto-antibodies to the receptor tyrosine kinase MuSK in patients with myasthenia gravis without acetylcholine receptor antibodies," *Nature Medicine*, vol. 7, no. 3, pp. 365–368, 2001.

[15] F. Scuderi, M. Marino, L. Colonna et al., "Anti-p110 autoantibodies identify a subtype of "seronegative" myasthenia gravis with prominent oculobulbar involvement," *Laboratory Investigation*, vol. 82, no. 9, pp. 1139–1146, 2002.

[16] M. Marino, F. Scuderi, D. Samengo et al., "Flow cytofluorimetric analysis of anti-LRP4 (LDL receptor-related protein 4) autoantibodies in Italian patients with myasthenia gravis," *PLoS One*, vol. 10, no. 8, article e0135378, 2015.

[17] N. E. Gilhus, "Myasthenia gravis," *The New England Journal of Medicine*, vol. 375, no. 26, pp. 2570–2581, 2016.

[18] A. Uzawa, T. Kanai, N. Kawaguchi, F. Oda, K. Himuro, and S. Kuwabara, "Changes in inflammatory cytokine networks in myasthenia gravis," *Scientific Reports*, vol. 6, no. 1, article 25886, 2016.

[19] C. J. Molin, E. Westerberg, and A. R. Punga, "Profile of upregulated inflammatory proteins in sera of myasthenia gravis patients," *Scientific Reports*, vol. 7, article 39716, 2017.

[20] A. Uzawa, N. Kawaguchi, K. Himuro, T. Kanai, and S. Kuwabara, "Serum cytokine and chemokine profiles in patients with myasthenia gravis," *Clinical & Experimental Immunology*, vol. 176, no. 2, pp. 232–237, 2014.

[21] A. Evoli, "Myasthenia gravis: new developments in research and treatment," *Current Opinion in Neurology*, vol. 30, no. 5, pp. 464–470, 2017.

[22] Task Force of the Medical Scientific Advisory Board of the Myasthenia Gravis Foundation of America, A. Jaretzki, R. J. Barohn et al., "Myasthenia gravis: recommendations for clinical research standards," *Neurology*, vol. 55, no. 1, pp. 16–23, 2000.

[23] G. O. Skeie, S. Apostolski, A. Evoli et al., "Guidelines for treatment of autoimmune neuromuscular transmission disorders," *European Journal of Neurology*, vol. 17, no. 7, pp. 893–902, 2010.

[24] R. Tandan, M. K. Hehir 2nd, W. Waheed, and D. B. Howard, "Rituximab treatment of myasthenia gravis: a systematic review," *Muscle & Nerve*, vol. 56, no. 2, pp. 185–196, 2017.

[25] M. Thio, B. Blokhuis, F. Nijkamp, and F. Redegeld, "Free immunoglobulin light chains: a novel target in the therapy of inflammatory diseases," *Trends in Pharmacological Sciences*, vol. 29, no. 4, pp. 170–174, 2008.

[26] J. A. Vander Heiden, P. Stathopoulos, J. Q. Zhou et al., "Dysregulation of B cell repertoire formation in myasthenia

gravis patients revealed through deep sequencing," *The Journal of Immunology*, vol. 198, no. 4, pp. 1460–1473, 2017.

[27] J. Diaz-Manera, E. Martinez-Hernandez, L. Querol et al., "Long-lasting treatment effect of rituximab in MuSK myasthenia," *Neurology*, vol. 78, no. 3, pp. 189–193, 2012.

[28] D. G. Maloney, "Anti-CD20 antibody therapy for B-cell lymphomas," *The New England Journal of Medicine*, vol. 366, no. 21, pp. 2008–2016, 2012.

[29] R. A. Manz, A. E. Hauser, F. Hiepe, and A. Radbruch, "Maintenance of serum antibody levels," *Annual Review of Immunology*, vol. 23, no. 1, pp. 367–386, 2005.

[30] P. Stathopoulos, A. Kumar, R. J. Nowak, and K. C. O'Connor, "Autoantibody-producing plasmablasts after B cell depletion identified in muscle-specific kinase myasthenia gravis," *JCI Insight*, vol. 2, no. 17, 2017.

[31] M. Lanzillotta, E. Della-Torre, and J. H. Stone, "Roles of plasmablasts and B cells in IgG4-related disease: implications for therapy and early treatment outcomes," *Current Topics in Microbiology and Immunology*, vol. 401, pp. 85–92, 2017.

[32] S. H. Kim, S. Y. Huh, S. J. Lee, A. Joung, and H. J. Kim, "A 5-year follow-up of rituximab treatment in patients with neuromyelitis optica spectrum disorder," *JAMA Neurology*, vol. 70, no. 9, pp. 1110–1117, 2013.

[33] L. Chiche, J. M. Cournac, J. Mancini et al., "Normalization of serum-free light chains in patients with systemic lupus erythematosus upon rituximab treatment and correlation with biological disease activity," *Clinical Rheumatology*, vol. 30, no. 5, pp. 685–689, 2011.

[34] T. G. Kormelink, J. Tekstra, R. M. Thurlings et al., "Decrease in immunoglobulin free light chains in patients with rheumatoid arthritis upon rituximab (anti-CD20) treatment correlates with decrease in disease activity," *Annals of the Rheumatic Diseases*, vol. 69, no. 12, pp. 2137–2144, 2010.

[35] G. G. Lin and J. M. Li, "IgG subclass serum levels in systemic lupus erythematosus patients," *Clinical Rheumatology*, vol. 28, no. 11, pp. 1315–1318, 2009.

[36] Y. Liu and J. Li, "Preferentially immunoglobulin (IgG) subclasses production in primary Sjögren's syndrome patients," *Clinical Chemistry and Laboratory Medicine (CCLM)*, vol. 50, no. 2, pp. 345–349, 2011.

[37] H. Zhang, P. Li, D. Wu et al., "Serum IgG subclasses in autoimmune diseases," *Medicine*, vol. 94, no. 2, article e387, 2015.

[38] J. H. Stone, A. Khosroshahi, V. Deshpande et al., "Recommendations for the nomenclature of IgG4-related disease and its individual organ system manifestations," *Arthritis & Rheumatism*, vol. 64, no. 10, pp. 3061–3067, 2012.

[39] P. Tsiamalos, G. Kordas, A. Kokla, K. Poulas, and S. J. Tzartos, "Epidemiological and immunological profile of muscle-specific kinase myasthenia gravis in Greece," *European Journal of Neurology*, vol. 16, no. 8, pp. 925–930, 2009.

[40] R. Klooster, J. J. Plomp, M. G. Huijbers et al., "Muscle-specific kinase myasthenia gravis IgG4 autoantibodies cause severe neuromuscular junction dysfunction in mice," *Brain*, vol. 135, no. 4, pp. 1081–1101, 2012.

[41] M. G. Huijbers, W. Zhang, R. Klooster et al., "MuSK IgG4 autoantibodies cause myasthenia gravis by inhibiting binding between MuSK and Lrp4," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 51, pp. 20783–20788, 2013.

[42] P. Raibagkar, J. A. Ferry, and J. H. Stone, "Is MuSK myasthenia gravis linked to IgG4-related disease?," *Journal of Neuroimmunology*, vol. 305, pp. 82-83, 2017.

[43] R. Sabatino, A. Perrone, M. Cuomo et al., "Analytical criticalities associated to different immunological methods for serum free light chain detection in plasma cell dyscrasias: a description of particular clinical cases," *International Journal of Molecular Sciences*, vol. 18, no. 4, 2017.