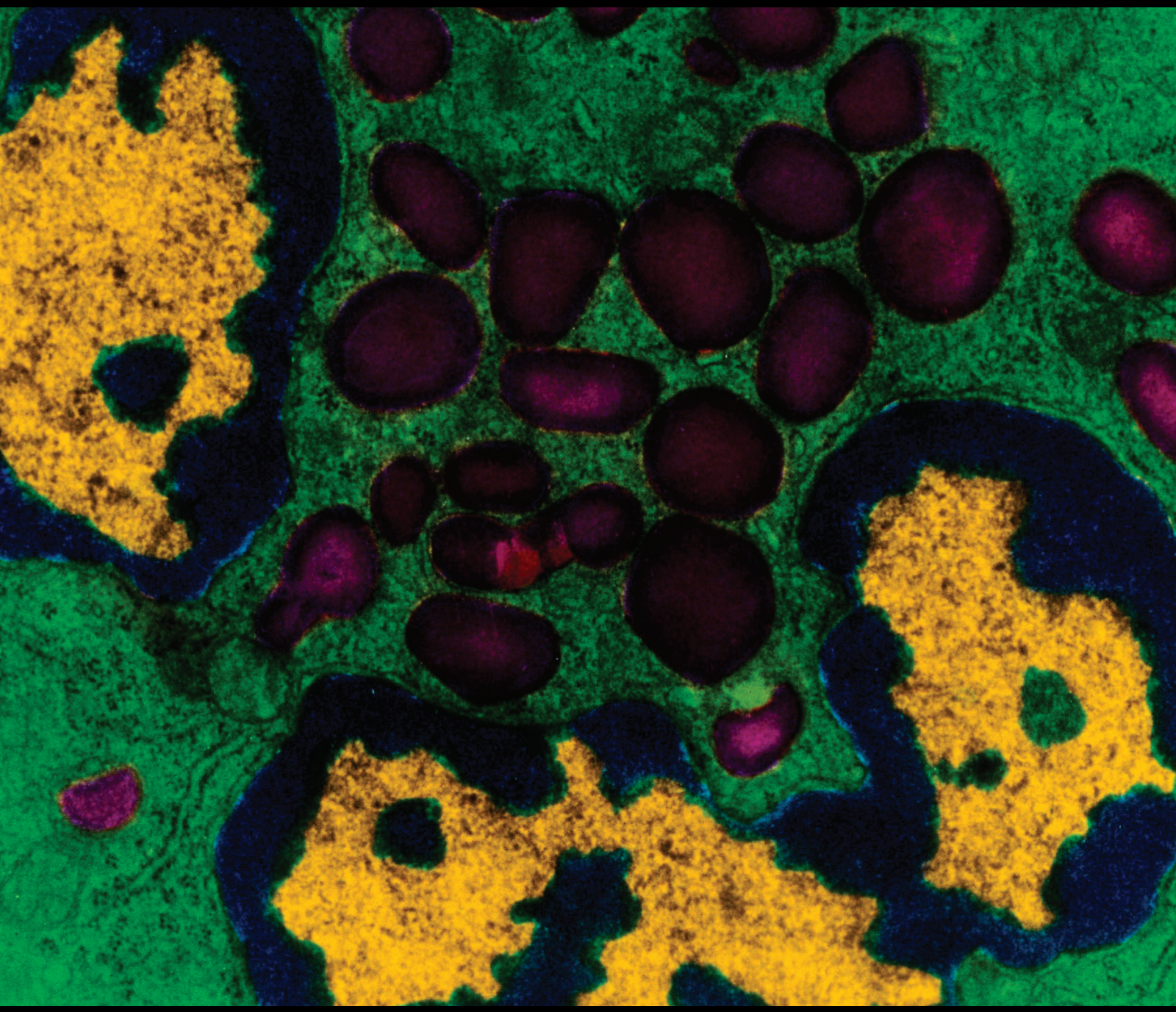
Mediators of Inflammation

Inflammation in the Disease: Mechanism and Therapies 2014

Guest Editors: M. Seelaender, J. C. Rosa Neto, G. D. Pimentel, R. S. Goldszmid, and F. S. de Lira



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Editorial Board

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Editorial

Inflammation in the Disease: Mechanism and Therapies 2014

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Inflammation in the face of harming stimuli protects the organism; as a result, it is an essential route for survival, in which both innate and adaptive immunity are involved. This process must be tightly controlled and terminated in order to warrant the reestablishment of body homeostasis. Therefore, activation of resident inflammatory cells and the recruitment and modulation of migrating inflammatory cells must be ceased. When failure in neutralizing acute inflammation occurs, there is augmented risk of development of chronic inflammation, leading to several metabolic consequences. In the present special issue, original research studies as well as review articles address the inflammatory process as a key contributor to disease onset and progression. In addition, pharmacological and nonpharmacological therapies are examined and the molecular and physiological mechanisms of the treatments are discussed.

Among the 24 accepted papers, 7 approach nutritional therapy and inflammation (J.-Y. Jhun et al.; E. A. Lima et al.; H. B. F. Silva et al.; G. I. G. Souza et al.; B. M. Mohammed et al.; C. A. Morais et al.; S. Arora et al.). The selected papers discuss the effects of Macadamia oil supplementation attenuation of adipocyte hypertrophy and of the inflammatory response of adipose tissue macrophages. The protection induced by supplementation of chitosan coacervate whey protein against metabolic changes and obesity-related inflammation is investigated. Other papers examine Jussara supplementation-associated reversal of the adverse effects of perinatal intake of transfatty acids, the beneficial effect of red genistein extract on autoimmune arthritis, and the role of vitamin C in the resolution of inflammation. Finally, the promotion, by nutritional intervention, of the recovery of glucose homeostasis is discussed. In addition, results on the effect of microencapsulated probiotics on alcoholic liver disease are presented. Taken together, these papers show new encouraging results of nutritional therapy in counteracting the symptoms of obesity and insulin resistance.

The ability of exercise to modulate chronic inflammation was the center of two papers (C. P. Papini et al. and B. Koc et al.,). These studies report that a community-based exercise program results in decrease or maintenance of inflammatory biomarkers after 1 year and thus presents strong potential in public health approaches for chronic disease prevention.

Inflammation and its mechanisms are discussed in the development and progression of many diseases, such as in ectopic fat deposition (L. Liu et al.), acute and chronic kidney disease (P. Ranganathan et al.), and polymicrobial sepsis (G. Pizzino et al.). The role of interleukin- (IL-) 18 in the regulation of toll-like receptors and mannose receptor expression (L. A. Dias-Melicio et al.) is examined. Inflammation in the metabolic syndrome (E. Hopps et al.) and the effect of metformin on autoimmune arthritis (H.-J. Son et al.) are debated. Gut microbiota participation in inflammation is addressed (Mingming Sun et al.; Yu Lijuan et al.), and its regulation by short-chain fatty acids in diabetes (A. Puddu et al.)

contemplated. The contribution of circulating LL-37 and inflammatory cytokines in the setting of plaque and guttate psoriasis (Y. J. Hwang et al.) is commented on. Other papers consider ionotropic and metabotropic proton-sensing receptors in allergic asthma (H. Aoki et al.) and autologous bone marrow stem cell transplantation in myocardial infarction (E. B. Furenes et al.). Flavocoxid and infliximab are examined in the context of classical and nutraceutical therapies (A. Bitto et al.; L. Yu et al.). The role of IL-38 and related cytokines in inflammation (X. Yuan et al.) is discussed, in addition to gingival inflammation and pregnancy (M. Wu et al.) and the contribution of the immune system in triplet repeat expansion diseases (M. Olejniczak et al.).

Collectively, this issue provides insight on the role of acute and chronic inflammation in different diseases and discusses mechanisms and new treatment strategies.

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Review Article

The Role of the Immune System in Triplet Repeat Expansion Diseases

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Trinucleotide repeat expansion disorders (TREDs) are a group of dominantly inherited neurological diseases caused by the expansion of unstable repeats in specific regions of the associated genes. Expansion of CAG repeat tracts in translated regions of the respective genes results in polyglutamine- (polyQ-) rich proteins that form intracellular aggregates that affect numerous cellular activities. Recent evidence suggests the involvement of an RNA toxicity component in polyQ expansion disorders, thus increasing the complexity of the pathogenic processes. Neurodegeneration, accompanied by reactive gliosis and astrocytosis is the common feature of most TREDs, which may suggest involvement of inflammation in pathogenesis. Indeed, a number of immune response markers have been observed in the blood and CNS of patients and mouse models, and the activation of these markers was even observed in the premanifest stage of the disease. Although inflammation is not an initiating factor of TREDs, growing evidence indicates that inflammatory responses involving astrocytes, microglia, and the peripheral immune system may contribute to disease progression. Herein, we review the involvement of the immune system in the pathogenesis of triplet repeat expansion diseases, with particular emphasis on polyglutamine disorders. We also present various therapeutic approaches targeting the dysregulated inflammation pathways in these diseases.

1. Introduction

A number of human inherited neurological disorders are caused by the expansion of trinucleotide repeats in specific, functionally unrelated genes [1, 2]. Based on the localization of repeats in transcripts, triplet repeat expansion diseases (TREDs) are classified into coding and noncoding repeat expansion disorders. Mutant proteins and transcripts are toxic factors in a group of polyglutamine (polyQ) diseases, for example, Huntington's disease (HD), that are caused by the expansion of CAG repeats in open reading frames (ORFs) of implicated genes. An RNA *gain-of-function* mechanism is thought to be responsible for noncoding repeat expansion disorders, including myotonic dystrophy type 1 (DM1) and fragile X-associated tremor ataxia syndrome (FXTAS). The age of onset and the severity of symptoms correlate with the size of the expansion, with a threshold of approximately 40 CAG repeats in most polyQ diseases and more than 50 CTG/CGG repeats in nontranslated repeat disorders. The functions of the proteins, the main sites of pathogenesis, and the characteristics of the clinical features are presented in Supplementary Table 1 (see Supplementary Material available online at http://dx.doi.org/10.1155/2015/873860). Although these late-onset diseases are characterized predominantly by neurological manifestations, several peripheral tissues show abnormalities in morphology and function, for example, skeletal muscles, hepatocytes, kidney, and blood cells [3, 4].

The common feature of most TREDs is the loss of neurons in specific brain regions accompanied by reactive gliosis and astrocytosis, which may suggest involvement of inflammation in pathogenesis. The inflammatory response on the CNS level is reflected by the interplay between neurons, microglial cells, and astrocytes, and the effects observed in the CNS may be further modulated by blood cells that fulfill immunological functions in the periphery.

The innate immune response plays a key role in recognition of pathogen-associated molecular patterns (PAMPs), exogenous signals derived from microorganisms (e.g., unmethylated CpG DNA, viral RNA, and 5′-triphosphate RNA, as well as lipoproteins, surface glycoproteins, and membrane components, e.g., lipopolysaccharide (LPS)). Cells release also endogenous danger signals, known as damage-associated molecular pattern molecules (DAMPs) that alert the innate immune system in response to stress. PAMPs as well as DAMPs, such as nucleic acids, ATP, and aggregated or misfolded proteins, are recognized in cells by conserved sensors, known as pattern-recognition receptors (PRRs). These include, for example, Toll-like receptors, an IFN-inducible dsRNA-activated protein kinase (PKR), retinoid acid-inducible gene I- (RIG-I-) like receptors (RLRs), AIM2 like receptors (ALRs), and NOD-like receptors (NLRs) that trigger inflammasome assembly and caspase activation. Stimulation of PRRs leads to activation of intracellular signaling pathways, including transcription factors (e.g., nuclear factor-κB (NF-κB), AP-1 and IFN regulatory factors (IRFs)), and the synthesis of signaling molecules, such as cytokines, chemokines, and immunoreceptors [5]. Persistent stimulation of immune system and synthesis of cytokines such as IL-6, IL-8, or TNF-α can contribute to degeneration of cells and disease progression.

In neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS), the role of inflammation in neurodegeneration is well documented [6–8]. Similarly, in TREDs, chronic stimulation of the immune system by a mutant protein/transcript may play an important role in disease progression. A number of immune response markers, including elevated cytokine, reactive oxygen species (ROS), and nitric oxide (NO) levels, activation of caspases, and changes in gene expression have been observed in the blood and CNS of patients and mouse models. Some of these effects are even observed in the premanifest stage of the disease and may have diagnostic significance [9, 10]. It seems that ageing and environmental factors, such as infections, may influence and modulate this response, thus affecting the development of the disease [11, 12].

In this review, we present the current state of knowledge regarding the role of the immune system in the pathogenesis of TREDs. We discuss the topic from the perspective of not only the toxic proteins but also mutant transcripts, which may be an important and overlooked factor participating in this response. We also present therapeutic approaches targeting toxic RNAs and proteins that trigger pathological effects and induce various immune responses.

2. Inflammation in Polyglutamine Diseases

Currently, there are nine known inherited neurodegenerative disorders caused by the expansion of CAG repeats within the coding region of associated genes. These include HD, spinocerebellar ataxias types 1, 2, 3, 6, 7, and 17 (SCA), spinobulbar muscular atrophy (SBMA), and dentatorubral-pallidoluysian atrophy (DRPLA). The accumulation of polyglutamine-rich proteins that affect various cellular functions and cause selective neurodegeneration in specific brain regions is a common feature of polyQ diseases [13–15]. Most studies have focused on protein toxicity in the disease pathomechanism, but recent findings demonstrate that mutant transcripts also contribute to the disease *via* an RNA *gain-of-function* mechanism [16, 17]. This mechanism was initially described for noncoding repeat expansion disorders, such as myotonic dystrophy type 1, FXTAS, or myotonic dystrophy type 2 (DM2), reviewed in [18]. The RNA-mediated mechanism assumes that nuclear foci formed by mutant CAG-containing transcripts sequester specific RNA-binding proteins, leading to loss of their normal function.

Immune activation in polyQ diseases is found both in the central nervous system and in the blood (peripheral level) of patients and mouse models of the diseases. The crosstalk between these levels as well as between the innate and adaptive immune responses needs to be better recognized. Currently, most of the information concerning the immune response in polyQ diseases refers to Huntington's disease [19], but it is unclear whether HD is unique in this respect. There are still many unresolved questions concerning the role of the immune response in the pathogenesis of polyQ diseases. What is the contribution of the toxic entities, that is, proteins and transcripts, in the stimulation of the immune system? Are the changes in the immune system the cause or the consequence of neurodegeneration? What is the role of aging and exposure to environmental factors, such as infections (priming), in the pathogenesis? Is the immune system a good target for therapeutic interventions for polyQ diseases? We hope that the answers to at least some of these questions can be found in this review.

2.1. Inflammation in HD-CNS Level. Huntington's disease, the most common of the polyQ disorders, is caused by expansion of CAG repeats in exon 1 of the *HTT* gene, which encodes the huntingtin protein (The Huntington's Disease Collaborative Research Group, 1993). In the healthy population, the number of CAG repeats lies between 6 and 35 CAG units. Alleles with repeat lengths from 36 to 39 exhibit reduced penetrance, whereas forty or more repeats are fully penetrant and associated with the development of HD [20–22]. Selective loss of neurons in the striatum and cortex leads to progressive movement abnormalities, dementia, and eventually death, typically in the fourth or fifth decade of life. Mutant huntingtin is widely expressed, and its posttranslationally modified versions, frameshifting products [23, 24] or N-terminal cleavage fragments, may form toxic aggregates in cells [25]. The presence of a mutant protein leads to many abnormalities, such as mitochondrial dysfunction and oxidative stress, ubiquitin-proteasome system dysregulation, chaperone protein and autophagy inhibition, synaptic dysfunction, glutamate-induced excitotoxicity, and transcriptional dysregulation [12, 26, 27].

Mutant HTT is expressed in immune cells at high levels, and persistent stimulation of the immune system at the CNS level is manifested by microglia activation and reactive gliosis [26, 28]. Microglia (the macrophages of the CNS)

surrounded by astrocytes and neurons are the major resident immune cells in the brain and serve as the frontline defense of the innate immune system. Under physiological conditions, microglia play roles in the programmed elimination of neural cells during development and in maintaining their survival by removing toxic cellular debris [29]. In response to a stimulus, microglia proliferate, migrate toward an immune stimulus, and induce a cascade of proinflammatory cytokines (e.g., IL-6, IL-12, TNF-α, and IL-1β). These, in turn, lead to caspase activation, changes in intracellular calcium levels, and free radical production. Excessive stimulation of these pathways may lead to neurodegeneration [30].

The microglia activation observed in postmortem HD brain tissue [31] is also detectable in the presymptomatic stage in HD gene carriers [9] and mouse models [27, 32]. This activation increases over the duration of the disease and correlates with the severity of disease progression [33, 34], suggesting a close relationship between microglial activation and neuronal death [26]. Microglia that express mutant HTT produce increased levels of proinflammatory cytokines, including IL-6, IL-8, and TNF-α [10], neurotoxic kynurenine metabolites [35], and have an impaired response to brain injury and migration to chemotactic stimuli [36]. Massive transcriptional induction of several chemokines, including monocyte chemoattractant protein-1 (MCP-1) and murine chemokine (KC), was detected in mouse neuroblastoma cells expressing mutant HTT [37]. This upregulation is explained by HTT-induced, mild proteasomal dysfunction and activity of the NFκB transcription factor in neuronal cells.

Mutant huntingtin also disturbs the normal functions of other glial cells, mainly astrocytes [4, 38] that play many important roles in amino acid, nutrient, and ion metabolism in the brain, maintaining homeostasis at the synapse, regulating neuronal signaling, and protecting neurons from oxidative damage. Reactive astrocytes observed in brains of patients with HD are characterized by hypertrophy and upregulation of several molecules including GFAP, S100B, iNOS, and NFκB. The role of astrocytes in inflammation is of great importance because reactive gliosis even occurs in HD models that do not express mutant HTT in neurons [38]. In addition, LPS-induced activation of proinflammatory cytokines in the brain was not observed in a mouse model that expressed mutant HTT in neurons (N171-82Q), but not in glial cells [39]. Hsiao et al. reported that mutant huntingtin enhanced the activity of IκB kinase (IKK), leading to enhanced activation of transcription factor NFκB in astrocytes of patients and mouse models of HD, but not in microglia and neurons. Such an IKK-NFκB-mediated immune response leads to upregulation of inflammatory genes, caspase 3 activation, and neuron apoptosis. In another study using a mouse model of HD (R6/2 brains), it has been shown that effector molecules, such as caspases [40], iNOS [41], and proinflammatory nitric oxide, contribute to the observed astrogliosis and apoptosis in neighboring cells [40, 42]. Caspases are proteases that play essential roles in apoptosis. In the late presymptomatic stage of HD, mutant huntingtin-induced toxicity results in caspase-1 activation and IL-1β production. As disease progresses, caspase-3 is upregulated. This picture is further complicated by the fact that huntingtin is itself a substrate of caspases 1 and 3 [43–45], and its cleavage precedes neurodegeneration in HD [46]. Some evidence indicates that the complement system, which connects the innate and adaptive immune responses (e.g., C3 and C9 factors), is upregulated in brains of HD patients [47]. However, research on the involvement of the adaptive immune system in HD is still in its infancy.

Taken together, the results of numerous studies show that immune system activation contributes to the neurodegeneration observed in HD and that the interplay between neurons, astrocytes, and microglia is responsible for these effects at the CNS level (Figure 1).

2.2. Inflammation in HD-Peripheral Level. Growing evidence supports the role of the peripheral immune system in HD pathogenesis. The immunological effects observed in the blood of patients and mouse models of HD are similar to those in the CNS and appear long before neurological symptoms (Figure 1). It has been shown that the level of mutant HTT in leukocytes increases with disease progression [48] and may act as a chronic stimulator of these cells. Elevated cytokines, for example, IL-6, IL-8, IL-4, IL-10, TNF-α, and IL-1β [10, 34], and chemokines, for example, eotaxin-3, MIP-1β, eotaxin, MCP-1, and MCP-4 [49], were observed in the plasma of HD patients and mouse models, such as YAC128 [10, 50], R6/2, and *Hdh* [10, 39] (Supplementary Table 2). Surprisingly, in the BACHD mouse model, the authors did not observe elevated cytokine levels. Monocytes and macrophages from patients with HD are hyperreactive in response to IFNγ/LPS stimulation, producing increased levels of IL-6, IL-8, and TNF-α [10, 51]. The TNF-α and IL-1β levels were also elevated in the serum and liver of mouse models after stimulation with LPS [39]. Interestingly, this hyperactivity of myeloid cells was abrogated by silencing HTT expression with siRNA [51], which suggested that immune cell activation was caused by a cell-autonomous effect of mutant HTT expression, rather than a secondary response to other extracellular factors. Furthermore, a significant association between CAG repeat length and the level of TNF-α produced by HD monocytes was observed [51], suggesting direct involvement of mutant HTT in triggering these effects. Träger et al. demonstrated that mutant huntingtin influenced the activity of the NFκB transcription factor. Altered transcription of NFκB target genes results in increased cytokine (IL-6 and TNF-α) production by immune cells. These results are consistent with those obtained by Hsiao et al., showing astrocyte-mediated IKK-NFκB-dependent inflammation in brains [39]. The other dysfunctions observed in the blood of HD patients and mouse models include increased apoptosis [52], autophagy and caspase activation [53], transcriptional dysregulation [54, 55], and elevated levels of mitochondrial dysfunction markers [56]. Additionally, the kynurenine/tryptophan ratio, which is an indicator of ongoing inflammation, is elevated in the serum of HD patients and correlates with disease progression [35, 57].

Thus, peripheral immune system activation reflects the processes observed in the CNS; however, the direct role of these systems in the pathogenesis of HD must be better recognized.

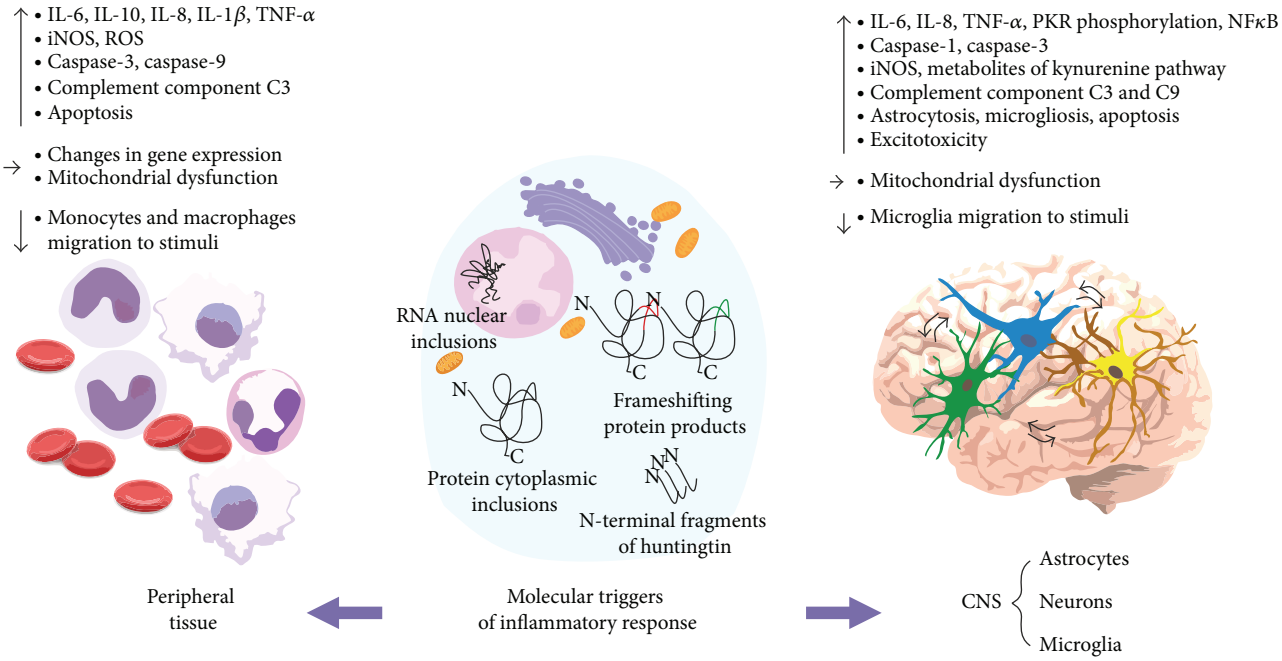

Figure 1: Inflammation in Huntington's disease. The mutant HTT transcript and protein are expressed in many cell types, including neurons, astrocytes, and blood cells of HD patients. The pathogenic effect may be triggered by expanded CAG repeat hairpins, cytoplasmic protein aggregates, N-terminal fragments of huntingtin, or toxic frameshifting products, and so forth. It is currently not clear what pathways are primarily involved in inducing the inflammatory response observed in the CNS and peripheral immune system. This effect is observed in the brain and peripheral tissues, indicating crosstalk in the signaling between distant tissues. The observed immune effects include elevated cytokine levels, caspase pathways activation, induction of apoptosis, dysregulation of gene expression, or decreased immune cell migration.

2.3. Inflammation in Other PolyQ Diseases. Toxic transcripts and proteins containing polyglutamine tracts are common factors that may trigger pathogenic pathways in polyQ disorders. Several polyQ proteins, such as huntingtin, ataxin-3, ataxin-7, AR, and atrophin-1, are substrates for caspases. Truncated fragments of these proteins are more toxic than their full-length forms; therefore, they play crucial roles in the pathogenesis of each disease [58–60]. However, whether these toxic protein fragments induce immune responses similar to those for HD must be determined. In addition, during the translation of mutant HD and SCA3 transcripts, chimeric polyQ/polyAla proteins may be formed due to ribosomal frameshifting, thus increasing the variety of toxic entities [23, 24, 61]. The role of the immune response in the pathogenesis of other polyQ diseases is less explored than in the case of HD. The existing data are incomplete and limited mainly to spinocerebellar ataxia type 3 (SCA3), known also as Machado-Joseph disease (MJD), which is caused by expansion of CAG repeats in exon 10 of the *ATXN3* gene [62]. The CAG repeat length normally varies from 10 to 51, with 55–87 CAG repeats being reported to associate with the disease [2, 63]. Ataxin-3 is an ubiquitously expressed enzyme that functions in the proteasomal protein degradation pathway and in transcription regulation (Supplementary Table 1). Toxic intracellular aggregates of mutant ataxin-3 are observed in neurons from different regions of the brains of SCA3 patients and in cell and animal models of SCA3 [14, 64].

Studies on cell lines and brain tissues confirmed the involvement of inflammatory processes in the pathogenesis of SCA3 [65–67]. The authors identified genes directly or indirectly involved in the immune response that were upregulated in a mutant ataxin-3-expressing rat cell line (SCA3-Q70). These genes included endopeptidase matrix metalloproteinase 2 (*MMP-2*), transmembrane protein amyloid precursor protein (*APP*), interleukin-1 receptor-related Fos-inducible transcript (*Fit-1S*), and cytokine stromal cell-derived factor 1α (*SDF1α*). Increased expression of the corresponding (MMP-2 and SDF1) or associated proteins, including anti-inflammatory interleukin-1 receptor antagonist (IL-1ra), the proinflammatory cytokine IL-1β, and the proinflammatory chemokine SDF1, was also demonstrated in human SCA3 pontine neurons. Evert et al. confirmed these results in their later study and demonstrated that in addition to the Fit-1S and IL-1ra cytokines, IL-6 and two cytokine-inducible transcription factors (C/EBPβ and C/EBPδ) were also upregulated in mutant ataxin-3-expressing cell lines and pontine neurons of SCA3 patients [66]. The presence of nuclear inclusions is not a prerequisite for these transcriptional changes because increased expression of cytokines was observed in neurons with and without inclusions [67]. The identified activation of inflammatory pathways corresponds well with the observed neurodegeneration [65, 66]. Immunostaining performed on human brain tissues with the use of microglial and astrocytic markers (CD68 and GFAP, resp.) has shown increased

numbers of activated microglial cells and reactive astrocytes in the pons of SCA3 patients. Microglial dysfunction and reactive astrocytosis were also observed in other SCAs [68–70].

Expanded ataxin-3 and ataxin-7 induce the apoptotic death of cultured cerebellar neurons by upregulation of the proapoptotic proteins Bax and PUMA and downregulation of Bcl-x_L (an antiapoptotic protein) [71–73]. Mutant ataxin 7 decreases the nuclear translocation of NF-κB p65 and impairs NF-κB activity by inhibiting proteasome activity in cerebellar neurons, leading to reduced Bcl-x_L expression, caspase activation, and cerebellar neuronal death. Moreover, the misfolded androgen receptor protein in the cytosol induces the Bax-dependent apoptotic cascade that is initiated by the JNK signaling pathway in cultured primary neurons from mice [74].

The role of the polyQ tract in triggering the immune response is still unclear. A SCA3 gene trap mouse model that expresses a truncated N-terminal region of the endogenous mouse ataxin-3 protein was generated to study the pathomechanism of SCA3. Despite the fact that the C-terminal region, which contains the polyQ tract, is missing, homozygous mutant mice still develop neurological symptoms and prematurely die. In contrast to observations of an HD mouse model [10], the levels of circulating cytokines were unchanged but showed high interindividual variability. The only observation that indicated some changes in the immune system, although unexplained by the authors, was the increased number of granulocytes and decreased number of B cells at 12 months [75].

The results described above confirm the involvement of the immune response in the pathomechanism of polyQ diseases, especially HD. However, the molecular mechanism and molecular triggers of these signaling cascades are still unknown.

3. RNA as a Trigger of the Immune Response

Most studies on the pathogenesis of polyQ diseases have traditionally focused on protein-based mechanisms of toxicity. However, growing evidence suggests that mutant transcripts may also play an important role in neurodegeneration [76–82], as was shown for untranslated trinucleotide diseases [79, 83, 84]. In contrary to normal transcripts, where CAG repeats either form unstable hairpins or do not form such structures at all, expanded repeats of mutant transcripts fold into more stable hairpin structures, which may interfere with normal cellular processes [85]. Such abnormal endogenous RNAs may serve as PAMPs recognized by pattern recognition receptors in cells. It cannot be excluded that stem-loop structures formed by mutant transcripts, products of bidirectional transcription, alternatively spliced transcripts, and RNAs released from necrotic cells are recognized as "non-self" molecules by cellular sensors of foreign RNA. In the next step, the activation of signal transduction pathways (e.g., MyD88, TRIF, NF-κB, IRFs) may lead to the production of effector molecules such as cytokines and reactive oxygen species (ROS) that amplify the immune response and recruit additional immune cells.

There are at least 7 cytoplasmic and endosomal sensors of foreign RNA, and these include IFN-inducible dsRNA-activated protein kinase (PKR) [86], $2'$-$5'$-oligoadenylate synthetase (OAS) [87], retinoic acid-inducible gene I (RIG-I), melanoma differentiation associated gene-5 (MDA5), and Toll-like receptors (TLR3, TLR7, and TLR8) [88, 89]. These sensors are highly expressed in cells that play important roles in innate immune responses, such as macrophages and microglia.

Activation of the PKR and OAS signaling pathways by long dsRNA results in the general inhibition of protein synthesis and the degradation of cellular RNA, respectively. It has been shown that PKR binds CUG repeat-containing DM1 transcripts *in vitro* in a length-dependent manner (with a minimal length of 15 CUG repeats) and is activated by pathologically expanded repeats [84]. CUG repeats cause stress in DM1 cells through the PKR-phospho-eIF2α pathway and inhibits translation of mRNAs associated with cytoplasmic stress granules (SGs) [90].

Similarly, mutant huntingtin mRNA also binds PKR in human brain tissue extracts, and the strength of binding increases with the length of the CAG tract [91]. In postautopsy human brains and mouse tissues, the activated form of PKR (phospho-PKR) was detected, and the increased immunoreactivity was more pronounced in areas affected by the disease. Strong induction of phospho-PKR in hippocampal neurons was also observed in another study of brain tissues from HD patients [92]. Interestingly, in the FXTAS caused by the expansion of CGG repeats in the $5'$ UTR of the *FMR1* gene, RNA hairpins were not shown to activate PKR *in vitro* or *in vivo* [93].

There is no direct evidence that other cellular sensors of dsRNA are activated in cells expressing mutant transcripts; however, Toll-like receptors are identified as potential sensors involved in self-RNA recognition [94, 95].

Indirect evidence of immune system activation by toxic RNA originates mainly from studies of noncoding repeat expansion diseases, such as DM1, DM2, FXS, and FXTAS. Rhodes et al. demonstrated the upregulation of interferon-regulated genes and genes associated with the response to dsRNA as well as the innate immune response in lens epithelium samples obtained from DM1 and DM2 cataracts patients [96]. Many of these genes were dysregulated in both types of DM, suggesting a common causative mechanism. The authors hypothesized that toxic dsRNAs containing expanded CUG and CCUG repeats in DM1 and DM2, respectively, serve as triggers of the interferon response. However, the direct activation of cellular RNA sensors as well as the main elements of the related signaling pathways has not yet been shown. Transcriptional dysregulation of genes involved in the immune response was also detected in the blood of FXTAS carriers [97].

In addition to transcriptional dysregulation, elevated plasma levels of TNF-α, IL-6 [98, 99], and IL-1β cytokines [99] were demonstrated in DM1 patients. The TNF-α levels directly correlated with the length of the CTG expansion and were significantly associated with the disease stage [99]. Interestingly, a later study suggested that elevated levels of TNF-α might result from CUGBP1-mediated increased stability

of the TNF-α mRNA in skeletal muscles and not from a response of immune cells to the disease [100]. CUGBP1 function is affected in DM1, and depletion of CUGBP1, which regulates the stability of the TNF-α mRNA, may explain the elevated levels of serum TNF-α that is observed in DM1 patients.

Plasma cytokine and chemokine profiles were also studied in FXS patients [101] and *FMR1* knockout mice [102]. The IL-1α cytokine level was elevated, the IL-6 level was unchanged, and the RANTES and IP-10 levels were decreased [101]. Different results were obtained when premutation carriers (FXTAS) were studied [103, 104]. The level of the anti-inflammatory cytokine IL-10 in the supernatant of PBMCs derived from premutation carriers was elevated, was correlated with the number of CGG repeats, and was observed before the appearance of the classical neurological symptoms. In another study, decreased cytokine production was observed in the blood cells of FXTAS female carriers and in the splenocytes of *FMR1* knockin mice, and this effect was associated with increased CGG repeat length [104]. These findings suggest that the role of toxic RNA and the immune system in the pathogenesis of FXS and FXTAS is more complex and requires further studies.

4. Therapeutic Strategies

Although no causal therapy is currently available and only symptomatic treatment is offered to patients, many therapeutic approaches are being tested to reverse or slow down the progression of the disease. These approaches vary depending on the target, strategy used, delivery method, and experimental model. Taking advantage of the knowledge concerning the pathogenic mechanisms of triplet repeat expansion diseases, the most direct therapeutic strategies target toxic transcript and protein. Antisense oligonucleotides and RNA interference triggers, such as short interfering RNA (siRNA), vector-based short hairpin RNA (shRNA), and artificial miRNA (shmiR), are widely used to silence the expression of mutant and normal/mutant genes in an allele-selective or non-allele-selective strategy, respectively (described in [105–107]). The main issues being explored concern the dose, delivery, and distribution of the therapeutic molecules in the brain, duration of silencing effect, and safety issues [108–110]. In a preclinical study, McBride et al. demonstrated that artificial miRNA molecules delivered into the brain of a human non-primate model with the use of a viral vector (AAV2/1) were safe and effective [111]. Partial non-allele-specific reduction of *HTT* expression (45%) in the putamen of rhesus macaque is well tolerated until 6 weeks after injection. No signs of local or peripheral inflammation were observed by analysis of reactive microglia or proinflammatory cytokine expression (IL-1β, TNF-α), which would be predicted to increase if HTT reduction induced neural toxicity. As peripheral tissues are also affected in HD, systemic delivery of artificial miRNAs (AAV serotype 9) targeting mutant huntingtin was recently proposed [112]. RNA interference triggers significantly reduced mutant HTT expression in multiple brain regions and peripheral tissues, thus preventing atrophy and inclusion formation in key brain regions, as well as weight loss in transgenic mice with HD.

Targeting inflammatory pathways and modulating their sensor-transducer-effector functions might be effective in preventing disease progression rather than in reversing the existing pathology. As immune effects manifest both in the CNS and periphery and because there is currently a lack of clarity as to the starting point of the immune cascade, anti-inflammatory therapies will have to target both destinations. On the other hand, suppressing innate immune function may act as a double-edged sword by creating a risk of infections. Thus, selective and fine-tuned therapeutic approaches are required.

Various drugs have been tested to inhibit inflammation pathways in neurodegenerative diseases. Antiapoptotic therapeutic strategies tested in mouse models of polyQ diseases include the inhibition of caspase function [113], inhibition of mitochondrial release of the cytochrome complex, which acts as an activator of the apoptotic pathway [114], and modulation of the initiation of the apoptotic signal [115]. Promising results were obtained with the use of minocycline, which is a second-generation tetracycline that inhibits microglia activation and acts as a caspase inhibitor, thus modulating apoptosis. Therapeutic approaches using minocycline in mouse models of polyQ diseases are described in detail in a review by Switonski et al. [116]. Caspase 1 inhibition by minocycline slows degeneration and disease progression in mouse models of HD [40]. In another study, minocycline delayed disease progression in a R6/2 mouse model of HD by inhibiting caspase-1 and caspase-3 mRNA upregulation and decreasing inducible nitric oxide synthetase activity [42]. However, subsequent studies did not confirm these results and showed no change in survival or even the toxicity of minocycline at higher doses [117, 118].

There are also reports, mostly for HD, describing promising results with the use of cytokine inhibitors. Activation of cannabinoid receptor 2 (CB_2) decreases inflammatory responses and the production of proinflammatory cytokines and is protective in mouse models of neurodegenerative diseases, including multiple sclerosis, ALS, and Parkinson's disease. CB_2 receptor levels are elevated in postmortem HD brains and mice models [119, 120], and treatment of the R6/2 HD mouse model with a CB_2 receptor agonist suppresses neurodegeneration by regulating the IL-6 level in the blood [120]. This effect was even observed in the late stages of the disease and was further confirmed using IL-6-neutralizing antibodies. These findings support the link between peripheral immune cell signaling and neurodegeneration in HD [120]. Surprisingly, elevated cytokine levels may be normalized by silencing mutant huntingtin using RNAi. The authors demonstrated that mutant huntingtin induces the transcriptional changes and NFκB pathway dysregulation that results in elevated cytokine levels. After treating human primary macrophages (LPS stimulated) with anti-HTT-siRNA the huntingtin level decreased, and the IL-6, IL-8, and TNF-α levels were significantly reduced [51].

Another study demonstrated that transplantation of wild-type bone marrow cells into HD mice ubiquitously expressing full-length huntingtin (YAC128 and BACHD

mice) normalizes the elevated levels of serum cytokines and chemokines, including IL-6, IL-10, CXCL1, and IFNγ [36]. Furthermore, peripheral administration of a kynurenine 3-monooxygenase (KMO) inhibitor decreases microglial activation, extends life span and improves the phenotype of HD mice [121].

Although immune system activation is probably not a main pathogenic factor in TREDs, a number of studies show that it may be a good therapeutic target. The tested drugs improve the phenotypes of treated animals by normalizing cytokine levels, slowing neurodegeneration and disease progression, and extending life span, which bodes well for results in human trials.

5. Summary

It seems that the inflammation observed in repeat expansion diseases may actively influence the progression of the disease. However, the normal functions of proteins in polyQ diseases may modulate the immune response because these proteins are involved in various processes, including transcription regulation, ubiquitin-mediated proteolysis, alternative splicing, and chromatin remodeling. These different functions might explain the different results obtained for specific polyQ diseases. The results presented in this review indicate that both toxic factors, that is, protein and RNA, may act to trigger inflammatory pathways and neurodegeneration. Further understanding of the role of inflammation in the pathogenesis of TREDs may allow for the design of better therapeutic approaches, slowing disease progression and improving the life of patients.

Conflict of Interests

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

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Review Article

Relationship between Gingival Inflammation and Pregnancy

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An increase in the prevalence and severity of gingival inflammation during pregnancy has been reported since the 1960s. Though the etiology is not fully known, it is believed that increasing plasma sex steroid hormone levels during pregnancy have a dramatic effect on the periodontium. Current works of research have shown that estrogen and progesterone increasing during pregnancy are supposed to be responsible for gingivitis progression. This review is focused not only on epidemiological studies, but also on the effects of progesterone and estrogen on the change of subgingival microbiota and immunologic physiological mediators in periodontal tissue (gingiva and periodontal ligament), which provides current information about the effects of pregnancy on gingival inflammation.

1. Introduction

Periodontal health in pregnant women has become a field of research since the 1960s, resulting in a flurry of studies to focus on it [1]. Gingival inflammation associated with pregnancy has been initiated by dental plaque and exacerbated by endogenous steroid hormones [2]. Meanwhile, the bidirectional interaction between systemic conditions and periodontal status has been taken more seriously into consideration with the proposition of periodontal medicine since the middle 1990s [3]. Although it is mandatory to exclude the effects of previously existing periodontal inflammation and dental plaque in order to explore the sole effect of pregnancy on periodontal health, the works of research in this regard have rarely been performed. This narrative review summarizes the current status of epidemiological and mechanistic studies on the changes of periodontium during pregnancy, especially the normal periodontium in order to elucidate the effect of pregnancy on the progress of gingival inflammation.

2. Epidemiological Studies

2.1. Prevalence. An increase in the prevalence and severity of gingival inflammation during pregnancy without plaque association has been reported since the early 1960s [1, 4, 5]. Clinically, preexisting gingivitis or periodontitis in pregnant women would be worsening dramatically. The periodontal changes are characterized by increasing periodontal probing depths, bleeding upon probing or mechanical stimulation, and gingival crevicular fluid flow, which disappears postpartum [6]. In previous studies, it appears that gingival inflammation shows prevalence from 30% to 100% when pregnancy occurs [7]. Meanwhile, some cross-sectional research showed that the percentage of pregnant women with gingivitis was 89% in Ghana, 86.2% in Thailand, and 47% in Brazil [8–10]. This variation may reflect the different populations studied and their characteristics, as well as the differences in definitions of periodontal disease between studies [8].

2.2. Periodontal Changes during Pregnancy. In accordance with previous studies [1, 4–7], recent cross-sectional and longitudinal studies have further confirmed and extended the association between pregnancy and gingival condition in many cultural and ethnic groups. In 2000, a group of researchers reported the findings of the study including 47 pregnant women and 47 nonpregnant women who served as matched controls in a rural population of Sri Lankans [11]. The periodontal status of the pregnant women was evaluated in the first, second, and third trimester of pregnancy and

the final examination was at three months postpartum. The authors found that although the plaque levels remained unchanged, the gingival index (GI) of pregnant women was significantly increased and peaked in the third trimester but dropped at 3 months postpartum [11]. The results were consistent with the findings of another cohort study in 2003 consisting of 200 pregnant women and 200 nonpregnant controls in Jordan [12]. In this study, it was reported that pregnant women had significantly higher GI and periodontal pocket depth (PPD) with similar plaque index (PI) compared with nonpregnant women. The clinical parameters (PPD and GI) increased in parallel with the increase in the stage of pregnancy, which reached the maximum at the eighth month [12]. In another companion study with a smaller sample size of 19 pregnant women, bleeding on probing (BOP) decreased from 41.2% at the twelfth week of pregnancy to 26.6% postpartum without any active periodontal therapy [13].

In addition to periodontal clinical parameters as above, clinical attachment level (CAL) measurements were also detected in the recent studies mentioned above. From these studies, the increased inflammation was detected in the gingival region rather than in other periodontal sites, indicating that pregnancy only has reversible effect on the gingiva without inducing periodontal attachment loss. It could be speculated that periodontal attachment loss requires a chronic inflammatory state of the gingiva lasting longer than pregnancy when the gingival changes occur [14]. However, this speculation remains to be proved. The recent studies observing the periodontal condition of women taking combined oral contraceptives (progesterone and estradiol) for at least 1 year have not reached an agreed conclusion about the change of CAL [15–17]. Some results showed that attachment loss was significantly greater in the users of combined oral contraceptives (COC) compared to the nonusers [15, 18]. The others found no difference in CAL between women taking COC and controls [16, 17]. One of the possible explanations for the discrepancy was that these study designs were partly different [19]. More experiments with oral contraceptives and long-term studies are necessary for answering this issue.

Recent studies further confirmed that gingivitis associated with pregnancy seemed to be dependent on, but unrelated to, the amount of dental plaque accumulation [20]. It seemed that good oral hygiene in pregnancy was able to partially neutralize hormonal effect [21]. Although, as it is well known, periodontal diseases have been considered to be microorganisms initiated, whether pregnancy's influence on gingival tissue might be independent or pregnancy by itself would cause new gingivitis has been proposed. Two most recent cohort studies were performed according to this proposal. Differed from those studies described above, these studies included the healthy periodontium without any gingival inflammation and excellent oral hygiene marked with fairly low plaque index in the subject criteria. One of these studies followed 48 pregnant Spanish women with healthy periodontium and examined their periodontal index in the first, second, and third trimesters and at 3 months postpartum. Despite maintaining fairly low Pl values, the pregnant women showed an increase in GI which maintained high levels in the third trimester and then decreased at 3 months postpartum [22]. In the other longitudinal study, the authors described the development of gingival inflammation in 30 periodontally healthy pregnant women with good oral hygiene in Finland. They found that the increase in gingival inflammation evaluated by BOP and the number of deep periodontal pockets (PPD $\geqslant$ 4 mm) in pregnant women was not related to dental plaque simultaneously between the first and second trimesters, followed by a decrease afterwards [23]. These two studies tried to wipe off the effects of previously existing gingival inflammation and dental plaque accumulation on the progress of pregnancy gingival inflammation. From these two studies, the increase in inflammatory changes of gingiva was mainly induced by pregnancy. The results further confirmed the possibly negative influence of pregnancy on periodontal situation. However, it is clear that it is difficult to keep the teeth without any plaque. Thus, the most persuading and powerful study should be based on plaque-free experimental animal models.

No matter whether the plaque levels remained unchanged or low, the concept that a progressive increase in gingival inflammation without periodontal attachment loss during pregnancy and apparent decrease following parturition is strengthened by these data from most studies. However, there are still a few works of research denying the association between pregnancy and gingival inflammation. Miyazaki et al. observed that there was no difference in periodontal status between pregnant and nonpregnant women in a study using the CPITN index to assess the periodontal conditions of 2424 pregnant and 1565 nonpregnant women. In addition, to observe that 95% of the pregnant women and 96% of the nonpregnant women had some signs of periodontal disease, the authors also noticed that pregnant women even had a healthier periodontal condition; that is, the number of sextants with healthy periodontal tissues was higher, the percentage of people having deep pockets (6 mm or deeper) was lower, and the need for prophylaxis was lower in pregnant than in nonpregnant women [24]. The difference of populations, the criteria for defining healthy periodontal condition, the clinical measurements used, and the numbers of teeth examined may complicate the results of these observations. Similarly, Jonsson and his colleagues found that none of the periodontal parameters for the pregnant females differed significantly from those of nonpregnant females. These parameters showed no significant correlation with the progression of pregnancy [25]. Since the findings were based on a small sample size of 9–14 subjects, there is the limitation in this study.

3. Mechanistic Studies

3.1. Estrogen, Progesterone, and Their Receptors. The exact mechanisms for the onset of the greater gingival inflammation during pregnancy have not yet been clearly described. Since the 1970s, the obvious increase in circulating levels of estrogen and progesterone was considered to have a dramatic effect on the periodontium throughout pregnancy and be correlated with this clinical feature [26]. The principal estrogen in plasma is estradiol, which is produced by the

ovary and the placenta. The principal progestin in female is progesterone, secreted by the corpus luteum, placenta, and the adrenal cortex [7]. During pregnancy, both of them are elevated due to continuous production by the corpus luteum at the beginning and the placenta afterward. By the end of the third trimester, progesterone and estrogen reach the peak plasma levels of 100 and 6 ng/mL, respectively, which are 10 and 30 times the levels observed during the menstrual cycle [27]. In animal models, the physiological effect of estrogen on gingiva was also observed [28]. When the serum estrogen concentrations in baboons were suppressed below 100 pg/mL by the administration of aromatase inhibitor, gingival enlargement developed. The gingiva recovered clinically when estradiol was added. The results indicated that estrogen profoundly affects physiologic events in the gingiva, including cellular proliferation and differentiation, whether directly or indirectly. Another report showed that the estrogen level determined the level of gingival margin inflammation developing against microbial plaque [29], when detecting 30 pregnant and 24 nonpregnant females. From above, both too low and too high estrogen levels have harmful effect on the gingiva.

Studies that investigated the impact of sex steroids on the periodontium are supported by the following observations. Localization of estrogen receptor (ER) and progesterone receptor (PgR) has been reported in the human periodontium, demonstrating that the periodontal tissues are the target tissues for these hormones [30]. Also, in earlier reports, ER was found in the periodontium of human, including gingival and periodontal ligament [30, 31]. However, using polymerase chain reaction analysis, Parkar et al. did not detect the expression of ER in any of the periodontal or gingival tissue samples [32]. The discrepancy was explained by the authors with the lack of specificity of the techniques used in previous experiments. In addition, the receptor subtypes were not specially examined in earlier reports [7].

Recent studies have further demonstrated the localization and subtypes of estrogen and progesterone in periodontium. Kawahara and Shimazu have reported that human GFs expressed poor ER-α signal but chiefly expressed ER-β. This was speculated to be the first description of the ER subtype in gingival component cells by the authors [33]. Jönsson and colleagues in their serial studies confirmed the ER subtypes in periodontal tissue [34]. ER-β immunoreactivity was observed in the nuclei of about 40% of cultured human PDLCs, while no ER-α immunoreactivity was detected, suggesting that estrogen influences the functional properties of periodontal ligament cells preferentially through ER-β. According to the authors, this was the first report revealing that ER-β is expressed in human PDLCs [34]. Recently, it was further suggested that ER-β localize not only in nuclei but also in mitochondria of human PDLCs, demonstrating that estrogen, probably via ER-β, influences mitochondrial function and energy metabolism in human PDLCs [35]. In addition, Välimaa et al. reported that gingival epithelial cells in healthy gingiva expressed the ER-β protein [36]. Nebel et al. further found that ER-β was located not only in nuclei of epithelial cells in all layers of the gingival epithelium, but also in cells of the lamina propria [37]. It could be concluded that ER-β was the predominant ER in periodontium, implying that the effects of estrogen on gingival tissues were mediated by ER-β [37].

However, the discrepancy exists in the expression of PgR. Jönsson et al. found that no PgR was expressed in human PDLCs [38]. Kawahara and Shimazu reported that human GFs expressed low PgR expression [33]. In a recent study in China, the authors detected the expression of PgR in human PDLCs by reverse transcriptase-polymerase chain reaction and immunocytochemistry, which showed that the PgR was expressed in human PDLCs at the gene and protein levels [39]. The staining methods and procedures, cell source, age of donors, and menstrual cycle stage might explain the discrepancies between the results. Taken collectively, it is clear that the periodontium is a target tissue for estrogen and progesterone, although the presence of PgR has not been conclusively demonstrated in these tissues.

Periodontium is a unique structure composed of two fibrous (gingival and periodontal ligament) and two mineralized (cementum and alveolar bone) tissues [7]. For the reason that pregnancy probably has an effect only on the gingiva and has no permanent effects on periodontal attachment, meantime, the effect of female sex hormones on periodontal ligament and tooth supporting alveolar bone has rarely been investigated [40]; this paper mainly focuses on the impact of progesterone and estrogen on two fibrous tissues (gingival and periodontal ligament) and a review of the impact of hormones on alveolar bone is not given here.

3.2. Alterations in Subgingival Microbiota. It is widely agreed that the majority of tissue damage in gingivitis and initial periodontal lesions occurs via an inflammatory response of the host to the presence of microbes, their structural and metabolic products, and the products of affected tissues themselves [41]. Pregnancy-associated gingivitis is no exception. It has been suggested that estrogen and progesterone can modulate the putative periodontal pathogens, the immune system in the gingiva, the specific cells in the periodontium, and the gingival vasculature [7, 8]. Recent studies were mainly performed to investigate the influence of pregnancy on microbial organisms and host response factors related to pregnancy gingivitis formation.

Periodontium acts as a reservoir of subgingival bacteria. Changes in the subgingival microbiota have been proposed as a potential mechanism for exacerbated gingival inflammation during pregnancy. In this regard, it should be kept in mind that there are three classic works of research in the early eighties of the last century. In one longitudinal study of 20 pregnant women, Kornman and Loesche were the first to report statistically significant increases in the levels of *Bacteroides intermedius* during the second trimester, with a reduction during the third trimester and after delivery. The marked increase in the proportion of the bacteria seemed to be associated with increased serum levels of progesterone or estrogens which substituted for the naphthaquinone requirement of the pathogens and thus acted as a growth factor for the bacteria [6]. In their following research in vitro, both estradiol and progesterone were involved in the fumarate reductase system of subspecies of *Bacteroides intermedius* and therefore

appeared to have potential to alter the subgingival microbial ecology by directly influencing the metabolic pathways of these pathogens [42]. Also, in one cross-sectional study, Jensen et al. reported a 55-fold greater level of *Bacteroides* species during pregnancy over nonpregnancy and 16-fold increase in those taking contraceptives over the control group [43]. Not all the early studies corroborated these findings. As shown in an early assessment, Jonsson et al. found no difference in the levels of *Bacteroides intermedia* between pregnant and nonpregnant controls or any correlation with the progression of the pregnancy [26]. Jonsson's findings led to the speculation that the increase in *Bacteroides intermedia* during the second trimester of pregnancy may actually be independent of estrogens or progesterone and occur for other reasons [8]. Similarly, the small sample size was the limitation of this study.

With the taxonomic evolution of *Bacteroides* species and the development of the molecular method, recent research provided new information on alterations in subgingival microbiota. In the open cohort study, Carrillo-De-Albornoz et al. reported that the worsening gingival inflammation was associated with the presence of subgingival *Porphyromonas gingivalis* and *Prevotella intermedia*, which were positively correlated with maternal hormone levels during pregnancy [44]. However, the proportions of the subgingival periodontal pathogens did not differ throughout pregnancy, although significant differences were found for all the pathogens after delivery [44]. Based on a small sample of pregnant women, Adriaens and coworkers reported the changes in subgingival microflora by DNA-DNA hybridization for 37 species and found that the quantities of *Porphyromonas gingivalis* and *Tannerella forsythia* at the 12th week of pregnancy were associated with gingivitis measured by BOP. No differences in the levels for any of the 37 bacterial species were found between 12th and 28th weeks of pregnancy, although a decrease in 17 of 37 species was found between the 12th week and postpartum, including *Prevotella intermedia* [45].

Many studies mentioned above have employed subgingival bacteria plaque as samples, including those from paper points or curettes. In other recent studies, there is another kind of sample available for measuring the number of oral bacteria, which is saliva sample. According to Umeda et al., whole saliva samples have been reported to contain subgingival periodontopathogens and thus represent an excellent alternative to sampling individual periodontal pockets, which is superior to taking periodontal pocket samples to detect *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens*, and *Treponema denticola* in the oral cavity. It might be that whole saliva samples simply contain higher concentrations of the bacteria than a periodontal pocket sample suspended in 0.4 mL water to be detected by PCR [46]. A recent cross-sectional case-control study by Yokoyama et al. used unstimulated saliva of pregnant women to detect periodontopathogens, including *Prevotella intermedia*, *Campylobacter rectus*, *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans,* and *Fusobacterium nucleatum*. The results showed that *Campylobacter rectus* tended to be higher in pregnant women than in nonpregnant women. The level of *Campylobacter rectus* was positively correlated with the estradiol concentration in the pregnant women [47]. The authors explained the reason of the growth of *Campylobacter rectus* as formate enhancement from the growth of *Prevotella intermedia* that was stimulated by direct interaction of female sex hormones on the fumarate reductase system. Also, another study showed that the growth of *Campylobacter rectus* was significantly enhanced by incorporating either estradiol or progesterone in human gingival fibroblasts (HGF) [48]. However, the authors failed to find that *Prevotella intermedia* were related to signs of gingival inflammation or estradiol concentrations in the saliva, which was not corroborated in other studies [25, 43–45]. This discrepancy could be due to different types of samples (unstimulated saliva compared with subgingival plaque) used and the occurrence rate of *Prevotella intermedia* which seemed to be slightly higher in subgingival sites than in unstimulated saliva [47]. The previous study suggested that the stimulation by masticating a piece of paraffin may increase the outflow of gingival crevicular fluid from the periodontal pocket, which loose the attached microorganisms or clumps of microorganisms from oral biofilms into salivary sediment and then may artificially increase the concentration of components in the saliva [49]. However, there is different opinion about this point. Gürsoy et al. considered that the collected and stimulated saliva contained a higher proportion of glandular saliva, diluting the concentration of gingiva-derived components [50]. This opinion was also proved by their serial longitudinal study [51]. The authors collected subgingival plaque and stimulated saliva samples from periodontally healthy Finnish women and examined them for the presence of *Prevotella intermedia*. In the saliva samples, the proportions of salivary *Prevotella intermedia* did not differ significantly either within the subject group or between the two groups. In subgingival plaque, the level of *Prevotella intermedia* increased transiently twice in the pregnant group, reaching the highest peaks during the second trimester, although the differences were not significant.

It should be noted that the bacteria known as *Fusobacterium nucleatum* were referred to in some aforementioned studies. As an opportunistic oral bacterium, it is associated with various forms of periodontal diseases, including gingivitis. Recently, *Fusobacterium nucleatum* has been gaining increasing attention because of its association with adverse pregnancy outcomes. It is capable of invading not only gingival epithelial cells, gingival fibroblasts, and periodontal ligament fibroblasts, but also other different types of human cells [52, 53]. Unlike other periodontal pathogens, translocation of *Fusobacterium nucleatum* in the acute infection model is organ-specific, that is, only in the placenta, likely due to the immune suppression in the placenta [54]. The recent report of a term stillbirth caused by oral *Fusobacterium nucleatum* provided the first human evidence that the bacteria originated from the mother's subgingival plaque and translocated to the placenta and fetus, causing acute inflammation leading to the fetal demise [55]. Some other authors also focused on the comparison of *Fusobacterium nucleatum* in their works of research. In the two cross-sectional studies aforementioned, no differences were noted in *Fusobacterium* species between pregnant and nonpregnant

women [43, 47]. Yokoyama's research further found the correlation between *Fusobacterium nucleatum* and the parameters such as estradiol concentrations and sites (PD = 4 mm), though they found female sex hormones did not promote the growth of *Fusobacterium nucleatum* in their previous in vitro study [47, 48]. Therefore, it was hypothesized that the increased number of PD = 4 mm sites in the pregnant women may have the growth of *Fusobacterium nucleatum*. However, this hypothesis was not consistent with their early findings that both the pregnant and nonpregnant women were comparable in terms of the level of *Fusobacterium nucleatum* [47]. In Adriaens et al.'s longitudinal study, no changes in *Fusobacterium nucleatum naviforme* and *Fusobacterium nucleatum polymorphum* occurred between 12th and 28th weeks of pregnancy. However, both of them decreased greatly at 4 to 6 weeks postpartum. BOP at 12th week is associated with higher counts of *Fusobacterium nucleatum naviforme* and *Fusobacterium nucleatum polymorphum* [45].

Taken together, there is no definite evidence linking increased concentrations of estrogen or progesterone during pregnancy with certain periodontal pathogens. Most works of research focused on *Bacteroides* species have equivocal results, in spite of different methods and different nomenclatures. Studies are needed to further elucidate the change of subgingival microbial profile of pregnant women.

3.3. Changes in Host Immunoinflammatory Response. Immunological changes have long been considered to be, at least in part, responsible for periodontal conditions observed during pregnancy [6]. In the various immune mechanisms in the process of gingival inflammation, polymorphonuclear leukocytes (PMNs) are the primary effector cells and appear to play a major role. When stimulated by bacterial pathogens, host cells release proinflammatory cytokines as a part of the immune response. These cytokines recruit PMNs to the site of infection, releasing a variety of biologically active products, such as chemokines, proteolytic enzymes, cytokines, and reactive oxygen species (ROS) [56, 57], and thus indirectly contribute to increase of gingival inflammation. PMNs have been considered to be protective in periodontal disease [58]. It is generally agreed that the damage to periodontal tissue may be aggravated by depressed function of PMNs [59]. During pregnancy, some degree of immunosuppression was reported, which minimizes the risk of fetal rejection [60]. Increased concentrations of female sex hormones may modulate the function and activity of PMNs. Impaired neutrophil functions have been observed throughout pregnancy and are considered to be linked to an increased susceptibility to inflammation [61–64]. Furthermore, human GFs and PDLCs, which are active participants in the oral immune defense system, far from being primarily supporting cells, may potentially produce chemokine signals, proteinases, and cytokines when exposed to suboptimal concentrations of stimuli or to relevant inflammatory cytokines, which associated with periodontal disease [65–68]. Accordingly, the data about the alteration in chemotaxis, cytokines, enzymes, and antioxidant secreted from PMNs, human GFs, or PDLCs in response to the inflammatory stimuli during pregnancy are reviewed in this chapter.

3.3.1. Chemotaxis. In an in vitro study, Miyagi et al. found that progesterone significantly enhanced the chemotaxis of PMNs at a concentration of 200 ng/mL and low concentrations of estradiol reduced it at 0.4 ng/mL which is the most effective concentration, while estradiol and progesterone did not alter chemotaxis of monocytes at any concentration tested [59]. In C. A. Lapp's and D. F. Lapp's recent in vitro study, the chemokines produced by human GFs in response to interleukin-1β (IL-1β) were significantly inhibited by medroxyprogesterone acetate (MPA) [65]. More recently, Nebel and coworkers investigated the effects of estrogen on the production of chemokines from PDLCs treated with lipopolysaccharide (LPS) and found that a physiological concentration of the endogenous estrogen (100 nm 17β-estradiol, which was the same concentration of E2 observed in plasma during pregnancy) differentially regulated chemokine expression in human PDL cells. The results showed that estrogen induced downregulation of chemokine ligand 3 (CCL3) mRNA and upregulation of chemokine ligand 5 (CCL5) gene activity in PDLCs while the expression of chemokine ligand 2 (CCL2) was unaffected by estrogen [68].

3.3.2. Cytokines. The hormonal modulation of effects on cytokines in periodontium has been studied extensively. In Miyagi et al.'s following serial in vitro studies, they concluded that monocytes probably played a role in gingival inflammation more through their release of a variety of cytokines than through their migration to the inflamed lesion. Prostaglandin (PG) E2 by LPS-stimulated human monocytes was enhanced by progesterone at both 2.0 and 20 ng/mL and was reduced by estradiol at 0.4 ng/mL but enhanced at 20 ng/mL. IL-1 was also shown to be inhibited by estradiol and progesterone in a dose-dependent manner [69, 70]. Recently, Yokoyama et al. found that production of interleukin-6 (IL-6) and interleukin-8 (IL-8) by human GFs was enhanced significantly by the stimulation with both estrogen and progesterone at high concentrations comparable to those found in plasma of pregnant women in their study, which suggested that the capacity of female sex hormones to enhance cytokines production by human GFs has the potential to contribute to periodontal disease progression during pregnancy [48]. However, an in vitro study by Lapp et al. has shown that sex hormones had an inhibitory effect on the secretion of IL-6 production by human GFs in response to IL-1 and high levels of progesterone during pregnancy affected the development of localized inflammation by reducing the production of IL-6 [71]. Another in vitro study has also shown that sex hormones at physiological concentrations (E2 of 10^{-9} to 10^{-7} M) had an inhibitory effect on the secretion of proinflammatory cytokines, including tumor necrosis factor-α (TNF-α), IL-1β, and IL-6 by human PDLCs treated with *E. coli* LPS [72]. Smith et al. also found that TNF-α levels in blood neutrophils decreased during the menstrual cycle when estrogen and progesterone concentrations were elevated, supporting a potential anti-inflammatory effect of ovarian hormones on neutrophils [73]. These studies suggested an anti-inflammatory effect of sex hormones at high levels in vitro. However, Jönsson et al.

did not find that LPS-induced IL-6 production by human PDLCs was reversed by a physiologically high concentration of E_2 (100 nM) in human PDLCs, suggesting that estrogen did not exert an anti-inflammatory effect [74]. The in vitro studies mentioned above focused on the effect of sexual hormones on cytokines in periodontal tissue were under the challenge of bacteria. Due to different concentration of ovarian hormones and different experimental protocol, the results were inconsistent.

Despite numerous in vitro studies evaluating the hormonal modulation of effects on cytokines in periodontium, only a few human studies have investigated the change of local proinflammatory mediators in pregnant patients until now [13, 75–77]. In Figuero's cohort study [16], the salivary sexual hormones and gingival crevicular fluid (GCF) levels of a panel of cytokines in samples collected from 48 pregnant women with healthy periodontium were assessed. They found that the levels of IL-1β and PGE_2 showed no significant changes during pregnancy, though their concentrations were higher than those found in nonpregnant women. Exacerbated gingival inflammation during pregnancy could not be associated with changes in PGE2 or IL-1β. But, as reported by the authors, the high incidence of dropouts and the lack of homogeneity between the groups might be the limitations of their study [16]. This result corroborated the findings of one cohort study with only 19 pregnant women by Bieri et al., who also found no significant differences in the expression of IL-1α, IL-1β, IL-8, and TNF-α in GCF between week 12 and postpartum, interpreting that the changes in gingival inflammation indicated by BOP may only be weakly associated with the expression of these selected cytokines in GCF during pregnancy [13]. However, the periodontium of the patients in the study was not defined to healthy periodontium before pregnancy as in the former study. Additionally, some cross-sectional studies also found that some proinflammatory mediators may not be associated with gingival inflammation during pregnancy. Otenio et al. found no differences in the expression levels of IL-1β, IL-6, and TNF-α in pregnant women with and without periodontal disease in comparison with expression of the same genes in nonpregnant women with and without periodontal disease, suggesting that periodontal disease is not influenced by pregnancy [77]. Interestingly, the authors found an apparent reduction in the expression of IL-6 in pregnant women with periodontal disease compared to that in pregnant women without periodontal disease, which is in agreement with the previous in vitro study mentioned above that reported that high levels of progesterone during pregnancy had an inhibitory effect on the secretion of IL-6 by human GFs in response to IL-1 [71].

Similar to the changes of GCF cytokine levels during pregnancy obtained from various works of research, some results were also reported in recent cohort studies evaluating GCF levels of cytokines in the menstrual cycle of periodontally healthy women. In a longitudinal study with 18 periodontally healthy premenopausal women exhibiting stable menstrual cycles, Markou and coworkers found that only IL-6 GCF levels were significantly different between ovulation and progesterone peak, and the subclinical increase of IL-6 at progesterone peak was not accompanied by clinical changes in the periodontium [78]. This result is partly consistent with Becerik et al.'s research among periodontally healthy subjects. The levels of inflammatory markers in GCF were similar in different phases of the menstrual cycle, though the patients had elevated gingival inflammation measured by BOP in ovulation (OV) and menstruation (ME) compared to premenstrual (PM) phases [79]. Inconsistent results existed in Baser et al.'s research, which evaluated the IL-1β and TNF-α levels in GCF during the menstrual cycle among pregnant women with excellent plaque control. The study showed that IL-1β levels in GCF and BOP scores increased significantly from the menstruation day to the predominant progesterone secretion day [80]. These discrepancies can be partially explained by differences in patient selection criteria and time point of clinical sampling [79].

Matrix metalloproteinases (MMPs) are involved in periodontal destruction. However, their role in pregnancy gingivitis is not well studied. In 2010, Gürsoy and coworkers first demonstrated the relationship between the changes of neutrophilic enzymes in saliva and GCF and periodontal status during pregnancy and postpartum in their longitudinal study series [50]. Results showed that a significant reduction of paraffin-stimulated salivary MMPs and tissue inhibitor of matrix metalloproteinase- (TIMP-) 1 expression occurred, despite the increased inflammation and microbial shift towards anaerobes. The increased gingival inflammation was not reflected by the enzymes examined in GCF. MMP-8 and PMN elastase levels of GCF stayed steadily at low levels during pregnancy, despite increasing BOP and PD scores. Their results are supported by some in vitro studies. Lapp et al. showed that progesterone may control and reduce local production of MMPs by cultured human GFs in response to interleukin-1 [81]. Smith et al. also found that MMP-9 levels in blood neutrophils decreased during the menstrual cycle when estrogen and progesterone concentrations were elevated [73]. The reduction of proteinase concentrations in local tissues, including saliva and GCF, may show impairment of neutrophil functions during pregnancy, which may partially explain induced or enhanced susceptibility to gingivitis during pregnancy. In addition, these findings could explain, at least in part, the reason that pregnancy gingivitis itself does not predispose or proceed to periodontitis.

3.3.3. Oxidative Stress. Oxidative stress is a mediator through which immune response in periodontium and pregnancy may be linked. Pregnancy is inherently a state of oxidative stress arising from the increased metabolic activity in placental mitochondria and production of reactive oxygen species (ROS), mainly that of superoxide anion (O^{2-}). Meanwhile, scavenging power of antioxidants is reduced [82]. Oxidative stress also plays a significant role in the pathology of periodontal diseases [83]. Imbalance between oxidative stress and antioxidants may play a role in the pathogenesis of periodontitis. Individuals with periodontal disease display high levels of local and systemic biomarkers of oxidative stress [84, 85]. Subjects with worse periodontal health tend to have greater oxidative injury [86]. Recently, the possible relationship among maternal periodontal condition, maternal oxidative stress, and pregnancy has been the subject of

several studies. Hickman and colleagues, in a large prospective cohort of healthy pregnant women, examined whether maternal periodontal disease was associated with oxidative stress measured by serum 8-isoprostane. Results indicated that the presence of moderate to severe periodontal disease was significantly associated with increased maternal serum 8-isoprostane, suggesting that maternal periodontal disease was associated with higher oxidative stress during pregnancy [87]. In their earlier report with the same study population, they first reported that periodontal disease and preeclampsia may be linked through maternal systemic oxidative stress measured by serum 8-isoprostane [88]. This may explain their early report in 2008. They found that maternal periodontal disease with systemic inflammation measured by C-reactive protein was associated with an increased risk for preeclampsia [89].

On the other hand, the antioxidant capacity of saliva and gingival crevicular fluid contributes largely to the protection of periodontium against oxidative stress [90]. However, relatively few studies have focused on the change of antioxidant capacity in periodontium during pregnancy. In 2009, Akalin and collaborators, in their longitudinal study, first investigated the periodontal status and antioxidant (AO) defenses during pregnancy. Serum and GCF total AO capacity and superoxide dismutase (SOD) enzyme concentrations were compared among the pregnant patients with chronic periodontitis (CP), pregnant patients with gingivitis (PG), periodontally healthy pregnant women (P-controls), nonpregnant women with CP, and nonpregnant periodontally healthy women. The results showed that systemic and local GCF AO levels decreased in pregnancy and periodontitis, and AO defense reached the lowest level in the last phase of pregnancy, whereas periodontal status deteriorated. The same occurred with SOD. Notably, in periodontally healthy pregnant women, compared to pregnant women with periodontal disease, AO and SOD levels in GCF were higher at the beginning of the pregnancy, but the difference in the third trimester was not statistically significant, suggesting that the GCF AO levels decline in pregnancy was influenced more by pregnancy than by periodontal inflammation, indicating that pregnancy may be a risk factor for the inflammation of periodontium [91]. However, a cross-sectional study performed on a group of pregnant women with or without diabetes has shown some different findings. In this study, Surdacka and colleagues collected unstimulated whole mixed saliva and evaluated the antioxidant system measured by catalase activity. Compared with the healthy individuals, pregnant women with diabetes were found to have markedly increased plaque formation and gingival and periodontal status, as well as increased salivary antioxidant capacity and proinflammatory cytokine levels, which indicated the ongoing inflammatory reaction. These parameters did not seem to correlate with healthy pregnant women. The authors speculated that infection could be taken as a source of oxidative stress that triggered an increase in salivary antioxidant defense [92]. The possible explanation for the disparity between the two studies is the differences in the length of the study period, the mediator measured, and the health status of the study subjects collected. In patients with long-term disease and systemic complications, it is unclear whether oxidative stress is causative for or is a result of these conditions.

Totally, the changes of chemotaxis, cytokines, enzymes, and antioxidants in periodontium during pregnancy are still unclear, regardless whether they are from GF, PDLC, or PMNs. It is speculated that the sexual hormones may exert both anti-inflammatory and proinflammatory effects on the periodontium in a dose-dependent manner. Thus, the gingiva in pregnancy is rendered less efficient at resisting the inflammatory challenges produced by bacteria. At the same time, gingivitis in pregnancy is limited and does not predispose or proceed to periodontitis.

3.4. Influences on Cells of the Periodontium. The function of cells in periodontal tissue may be affected by estrogen and progesterone. In an early report, sex steroid hormones have been shown to directly and indirectly exert influence on cellular proliferation, differentiation, and growth in gingiva [6]. In Mariotti's recent study, cellular proliferation and the number of cells entering the S-phase of the cell cycle were significantly increased in the cultures of human premenopausal gingival fibroblasts stimulated by physiologic concentrations of estradiol (1 nM), while both collagen and noncollagen protein productions were reduced [93]. Nebel et al. found that estrogen attenuated proliferation of human gingival epithelial cells monitored by measuring DNA synthesis at high (500 nM and 10 μM) but not low (10 nM) concentrations of estradiol, suggesting a concentration-dependent mechanism [37]. The effects of E2 on hPDL cells were also studied. In recent research by Mamalis, a significant increase in hPDL cell proliferation occurred after estradiol stimulation (100 nM), while cell proliferation did not change after blocking ER-β by the short interfering RNA (siRNA) technique. However, collagen synthesis remained unaffected by estradiol stimulation in both stable transfected and nontransfected cells [94]. This observation confirms the results of the previous study that failed to show that estrogen at physiological concentrations (100 nM or lower) mediated significant alterations in collagen synthesis of periodontal ligament cell [38]. However, the physiological concentration (100 nM) of E2 was found to enhance DNA synthesis in human breast cancer MCF-7 cells, suggesting that the effects of estrogen on collagen synthesis are cell/tissue specific [38]. In summary, the data presented here suggest that there is no stimulatory effect of estrogen on the relative amount of collagen synthesized by gingival fibroblasts, PDL cells, or gingival epithelial cells. Also, the stimulatory effects of estrogen on gingival cellular proliferation exist in a concentration-dependent manner.

Due to the uncertainty of location of progesterone receptor in periodontal tissues, the effect of progesterone on cells of the periodontium is far from being determined. There is insufficient information available concerning this regard. Though in low levels, PgR was reported in human GFs, suggesting that progesterone should have an effect on their function [33]. In an in vitro study, an inhibitory effect of progesterone on the proliferation rate of human GFs was observed. Progesterone at concentrations of 50 and 100 μg/mL significantly reduced cellular growth in both

cultures derived from a healthy and a diabetic (type II) individual, therefore partly explaining the unfavorable effects of hormonal changes during pregnancy on the gingival tissue [95]. Yuan et al. suggested that progesterone stimulated the proliferation and differentiation of the human PDLCs by PgR [39]. However, Jönsson et al. implied that progesterone does not have a direct effect on PDLCs function; for no nuclear PgR, immunoreactivity was observed in PDLCs [38].

4. Conclusion

Based on the data described above, the connection between increased plasma levels of pregnancy hormones and a decline in periodontal health status exists. In addition, the influence of sex hormones can be minimized with good plaque control. From above, it can be assumed that the fluctuation in estrogen and progesterone levels during pregnancy exerts the influence of subgingival microbiota and a spectrum of inflammatory responses in gingival tissues through the changes of chemotaxis, cytokines, enzymes, and antioxidants from PMNs, GFs, and PDLCs and thus indirectly contributes to increased gingival inflammation. The mechanisms responsible for these changes are not fully known. Thus, further works of research are needed to fully elucidate the exact molecular mechanism linking periodontal condition with pregnancy.

Conflict of Interests

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

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Research Article

Blockade of the JNK Signalling as a Rational Therapeutic Approach to Modulate the Early and Late Steps of the Inflammatory Cascade in Polymicrobial Sepsis

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Cecal ligation and puncture (CLP) is an experimental polymicrobial sepsis induced systemic inflammation that leads to acute organ failure. Aim of our study was to evaluate the effects of SP600125, a specific c-Jun NH_2-terminal kinase (JNK) inhibitor, to modulate the early and late steps of the inflammatory cascade in a murine model of CLP-induced sepsis. CB57BL/6J mice were subjected to CLP or sham operation. Animals were randomized to receive either SP600125 (15 mg/kg) or its vehicle intraperitoneally 1 hour after surgery and repeat treatment every 24 hours. To evaluate survival, a group of animals was monitored every 24 hours for 120 hours. Two other animals were sacrificed 4 or 18 hours after surgical procedures; lung and liver samples were collected for biomolecular and histopathologic analysis. The expression of p-JNK, p-ERK, TNF-α, HMGB-1, NF-κB, Ras, Rho, Caspase 3, Bcl-2, and Bax was evaluated in lung and liver samples; SP600125 improved survival, reduced CLP induced activation of JNK, NF-κB, TNF-α, and HMGB-1, inhibited proapoptotic pathway, preserved Bcl-2 expression, and reduced histologic damage in both lung and liver of septic mice. SP600125 protects against CLP induced sepsis by blocking JNK signalling; therefore, it can be considered a therapeutic approach in human sepsis.

1. Introduction

Sepsis and systemic inflammatory response syndrome (SIRS) are systemic reactions to different inflammatory stimuli such as infection, burns, and trauma [1]. A strict regulation of the inflammatory process is necessary in order to mantain a correct balance between protective or tissue-damaging inflammatory response. If the inflammatory reaction becomes unregulated, systemic and excessive activation of innate immunity results in SIRS or sepsis.

The incidence of sepsis in North America is of 3 cases per 1000 persons, with an estimated increase rate of 1.5% per year. The mortality rate associated with sepsis (40–60%) is so high as to make sepsis the leading cause of death in noncoronary intensive units and one of the major burdens for the healthcare systems throughout the world [2].

Although it has been proposed more than 30 years ago, as experimental model for sepsis induction [3], the CLP model has been considered to be the gold-standard model of sepsis [4]. Sepsis, together with hypotension, acute respiratory distress syndrome (ARDS), hepatic failure, disseminated intravascular coagulation, and organ dysfunction, is associated with a poor prognosis. These alterations occur first in the lung and then in the liver [5].

Bacterial proliferation, endotoxin production, and exotoxin are able to induce an overexpression of pro-inflammatory madiators by macrophages, monocytes, endothelial cells, and neutrophils, thus leading to tissue injuries and organs failure [5, 6].

The NF-κB transcription factor system is known to control the expression of a number of genes involved in the innate immune response of the body against infection and inflammation. Genes responsible for immunoreceptors, cytokines, chemokines, and apoptosis are all modulated by this important family of transcription factors [7]. NF-κB activity is reported to be impaired in chronic inflammation [8]. Recently, we showed that inhibition of NF-κB succeeded in maintaining the balance between pro- and anti-inflammatory cytokines in vivo in a model of polymicrobial sepsis [6]. Phosphorylation of NF-κB and thus transcription of proinflammatory mediators are promoted by the activation of various mitogen-activated protein kinases (MAPKs). MAPKs, such as ERK1/2 and JNK, in turn are activated by bacterial products, cytokines, and chemokines [6–9]. Indeed, JNK is a crucial mediator involved in the activation of proinflammatory cytokines and apoptosis in different cells [9–11]. During septic shock, proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 are dramatically increased to block the infection and tissue damage [6, 12–15]. Other late mediators of inflammation such as HMGB-1 have been involved in septic shock. In fact, it has been showed that suppressing the HMGB-1 activity exert positive effects in experimental sepsis [16].

Previous in vitro experiments demonstrated that SP600125 acts as MAPKs inhibitor, exhibiting a greater selectivity for all the 3 isoforms of JNK (JNK-1, JNK-2, and JNK-3) rather than for the other kinases; indeed, SP600125 inhibit JNK at a lower concentration then those required to inhibit ERK and p38 (IC50 0.04 μM vs > 10 μM), as previously reported [16]. Furthermore, the dose of 15 mg/kg was described as being able to block the expression of TNF-α in a murine model of endotoxin-induced inflammation [16]. Moreover, Bennett et al. showed that the JNK inhibitor significantly reduces the inflammatory response in a model of peritonitis induced lung damage [16, 17]. In light of these considerations, we hypothesized that inhibition of JNK signalling might improve systemic sepsis.

Therefore, aim of our study was to investigate the efficacy and the molecular mechanism of SP600125 in this murine model of polymicrobial sepsis.

2. Materials and Methods

2.1. Animals, Experimental Procedure, and Treatments. All procedures complied with the standards for the care and use of animal subjects, as stated in the Guide for the Care and Use of Laboratory Animals, and were approved by the Committee on Animal Health and Care of Messina University. The 5-week-old male C57BL/6J mice (Charles River, Calco, LC, Italy), used for this study, had free access to a standard diet and tap water.

They were maintained on a 12-hour light/dark cycle at 21°C.

Cecal ligation and puncture (CLP) was performed in C57BL/6J mice as previously described [18].

The animals (n = 35) were randomized in three groups, respectively, Sham (n = 7), CLP (n = 14), and CLP + SP600125 (n = 14); moreover, both CLP and CLP + SP600125 groups were further parted in two other subgroups of seven animals each and sacrificed, respectively, 4 h and 18 h after the treatment. Additionally, 40 animals were also randomized in Sham (n = 10), CLP (n = 15), and CLP + SP600125 (n = 15) and monitored for 120 hours for mortality assessment.

Particularly, mice were anesthetized with ether, and a midline incision was made below the diaphragm to expose the cecum. The cecum was ligated at the colon juncture with a 4-0 silk ligature suture without interrupting intestinal continuity. The cecum was punctured once with a 22-gauge needle. The cecum was returned to the abdomen, and the incision was closed in layers with a 4-0 silk ligature suture. After the procedure, the animals were fluid-resuscitated with sterile saline (1 mL) injected subcutaneously (sc). Sham controls were subjected to the same procedures as were those with CLP without ligation and puncture of the cecum. Shams were treated with SP600125 or vehicle. Animals were randomised to receive either SP600125 (15 mg/kg i.p.) or its vehicle (1 mL/kg of a 10% DMSO/NaCl solution) 1 hour after CLP procedure.

2.2. Sample Collection. Samples of liver and lung were collected at both time points (4 h and 18 h) to perform the molecular analysis. At 18 h were also collected specimens of the same tissues to perform histopathologic evaluation.

2.3. Isolation of Total Proteins and Western Blot Analysis. After removal, samples of lung and liver were homogenized in 1 mL lysis buffer (25 mM Tris/HCl, pH 7.4, 1.0 mM ethylene glycol tetraacetic acid, 1.0 mM ethylenediamine tetraacetic acid, 0.5 mM phenylmethyl sulfonylfluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin A, and 10 μL/mL NP-40). The homogenate was subjected to centrifugation at 15.000 rpm for 15 minutes. The concentration of total proteins was determined by using the Bio-Rad protein-assay kit (Milan, Italy). The supernatant was collected, mixed with Laemmli sample buffer, and stored at −20°C until analysis.

Western blot analysis was carried out in lung and liver samples to determine p-JNK (Thr183 and Tyr185), p-ERK1/2 (Thr202 and Tyr204), p-NF-κB p65 (Ser536), TNF-α, BAX, Bcl-2, HMGB-1, Rho, and Ras levels, as previously described [18]. Equal loading of protein was determined on stripped blots with β-actin. Primary antibodies were purchased from Cell Signaling Technologies (p-NF-κB, p-ERK1/2, p-JNK, β-actin; Danvers, MA, USA), Abcam (Cambridge, MA, USA; HMGB-1, Rho and Ras), Bio-vision (Milpitas, CA, USA; BAX and Bcl-2), and Millipore (Billerica, MA, USA; TNF-α). Secondary, peroxidase conjugated, antibodies were obtained from Thermo Fisher Scientific (Waltham, MA, USA). The protein signals were evidenced by the Enhanced Chemiluminescence (ECL) system and quantified by scanning densitometry by using a bio-image analysis system (Bio-Profil

Celbio, Milan, Italy). Results were expressed as integrated intensity compared with those of control normal animals measured within the same batch.

2.4. Histological Evaluation. For light microscopy, lung and liver tissues were rapidly removed and fixed in 10% buffered formalin. Subsequently, they were embedded in paraffin, cut, and stained with hematoxylin and eosin (H&E). Assessment of tissue changes was carried out by an experienced pathologist who was blinded to the treatments. The histological study of liver sections was based on the following parameters: infiltration of inflammatory cells, steatosis, necrosis, and ballooning degeneration. The parameters considered for scoring lung damage were infiltration of inflammatory cells, vascular congestion, and interstitial edema. All parameters were evaluated by the following score scale of values: 0, absent; 1, mild; 2, moderate; and 3, severe.

3. Results

3.1. Effects of SP600125 on CLP Induced Mortality. In order to assess the impact of treatment on sepsis-induced mortality, C57BL/6 mice subjected to CLP or sham operation were treated 1 hour after the surgical procedures with SP600125 (15 mg/kg/i.p.) or vehicle. The treatment was repeated every 24 hrs. All the CLP animals were fluid resuscitated *via* administration of sterile 0.9% NaCl saline solution (1 mL/mouse). Animal survival was monitored for up to 120 hours. CLP-induced sepsis in mice produced a significantly higher mortality compared with sham animals (Figure 1). SP600125 administration was able to increase the survival rate in treated animals and reduced mortality in CLP mice (Figure 1).

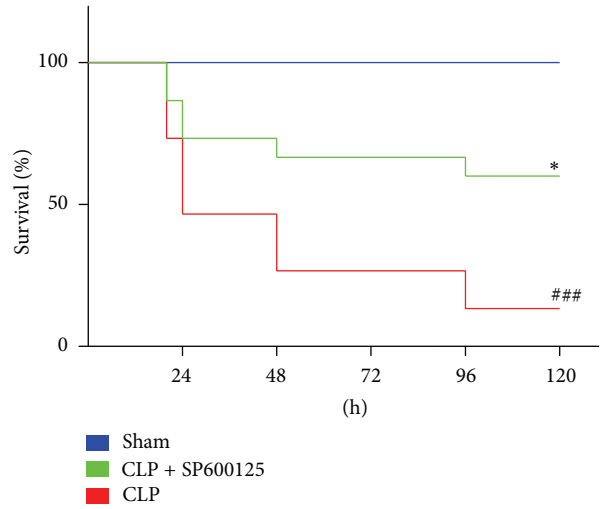

Figure 1

3.2. Effects of SP600125 Treatment on Early p-ERK1/2 and p-JNK Expression. In order to evaluate the effectiveness of MAPKs blockade, we assessed the levels of both and p-JNK in lung and liver 4 hrs after the surgical procedures. As shown in Figure 2, CLP determined an activation of both ERK1/2 and JNK signalling, resulting in a strong increase in phosphorilation of both the proteins in lung and liver of CLP mice. This confirms that MAPKs signalling is an early event in the inflammatory cascade during polymicrobial sepsis. Treatment with SP600125 prevented the phosphorilation and the activation of both ERK1/2 and JNK in both lung and liver when compared with untreated CLP animals (Figure 2). Indeed the inhibitory effect on p-JNK was greater than that on p-ERK, thus confirming that SP600125 is more specific inhibitor of JNK.

3.3. Effects of SP600125 Treatment on Early NF-κB and Caspase 3 Expression. NF-κB, one of the main transcriptional factors involved in inflammatory and immune response, is known to be activated by MAPKs; when deregulated and/or robustly activated, it also primes apoptotic cell death. Therefore, we evaluated the levels of p-NFκB to assess the successful blocking of either MAPKs signalling or the extrinsic apoptotic pathway investigated by the means of caspase 3.

Our results clearly showed (Figure 3) that CLP significantly increases the p-NFκB levels in lung and liver samples, 4 hrs after the surgical procedure, confirming the role of this transcription factor in the early events of the inflammatory cascade. By contrast SP600125 caused, through the inhibition on ERK1/2 and JNK proteins, a significant reduction in NFκB phosphorilation in both lung and liver (Figure 3). In addition the treatment produced a significant reduction in the expression of caspase 3 protein in lung and liver, thus preventing the activation of the apoptotic signalling (Figure 3).

3.4. Effects of SP600125 Treatment on Early RHO and RAS Expression. The early signalling culminating in MAPKs activation involves the priming of the Rho and Ras protein. Rho and Ras proteins were overexpressed in CLP animals compared with sham mice (Figure 4) 4 hrs after the surgical procedures. Treatment with SP600125 did not affect Rho and Ras overexpression (Figure 4), thus confirming the specificity of SP600125 on MAPKs.

3.5. Effects of SP600125 Treatment on Late TNF-α and HMGB-1 Expression. TNF-α plays a key role in CLP-induced sepsis; therefore we evaluated the expression of this proinflammatory cytokine 18 hours after the CLP procedure. TNF-α expression was significantly enhanced in the lung and liver of CLP animals treated with vehicle compared with sham ones (Figure 5). SP600125 treatment significantly reduced TNF-α levels in the lung and liver of CLP mice (Figure 5). Furthermore, considering the proapoptotic role of HMGB-1, we measured this late cytokine in both lung and liver tissue 18 h after the CLP procedure. Administration of SP600125 reduces the expression of this late cytokine protein in both lung and liver tissue of CLP animals (Figure 5).

3.6. Effects of SP600125 Treatment on the Late BAX and BCL-2 Expression. Eighteen hours after CLP, BAX levels

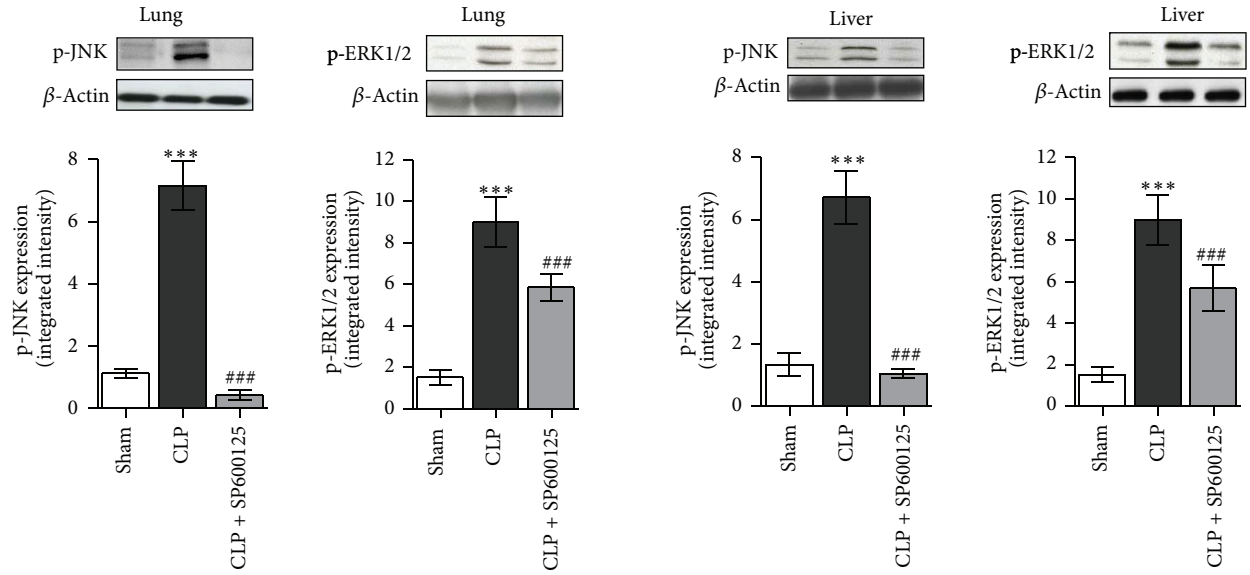

Figure 2

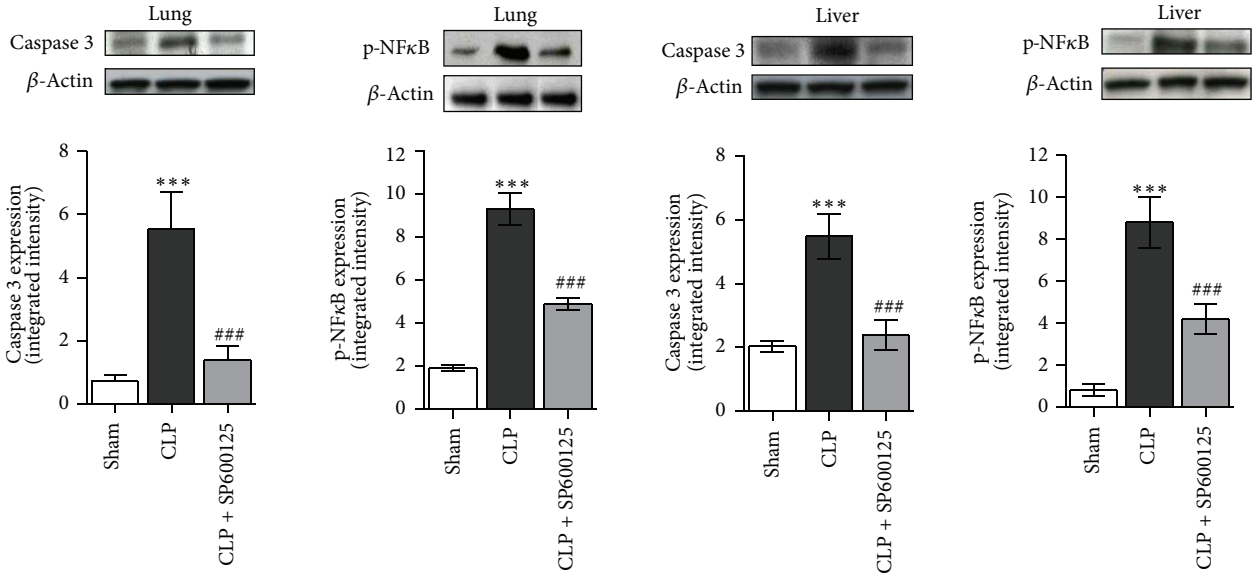

Figure 3

were significantly enhanced while Bcl-2 protein significantly reduced in both lung and liver (Figure 6). Treatment with SP600125 decreased BAX expression and enhanced BCL-2, thus suggesting the specific JNK inhibitor blunts the activation of proapoptotic signal in polymicrobial sepsis.

3.7. Effects of SP600125 on Histopathology Features. As shown in Figure 7 and in Table 1, CLP caused significant changes in the architecture of both lung and liver. In the lung of CLP animals an increased inflammatory infiltrate was observed as well as a consistent augmentation of both the oedematous and

Table 1: SP600125 effects on lung histologic damage in CLP mice. * * * is for $P < 0.0001$.

	Parameters	Sham	CLP	CLP + SP600125
Lung	Inflammatory infiltrate	0	2.31 ± 0.41	$1.28 \pm 0.23^{***}$
	Vascular congestion	0	2 ± 0.18	$1.1 \pm 0.37^{***}$
	Interstitial edema	0	2.12 ± 0.53	$1.32 \pm 0.44^{***}$
Liver	Inflammatory infiltrate	0	2.1 ± 0.39	$1.3 \pm 0.33^{***}$
	Steatosis	0	2.89 ± 0.56	$1.56 \pm 0.26^{***}$
	Necrosis	0	2.2 ± 0.47	$0.9 \pm 0.21^{***}$
	Ballooning degeneration	0	2.76 ± 0.47	$1.58 \pm 0.48^{***}$

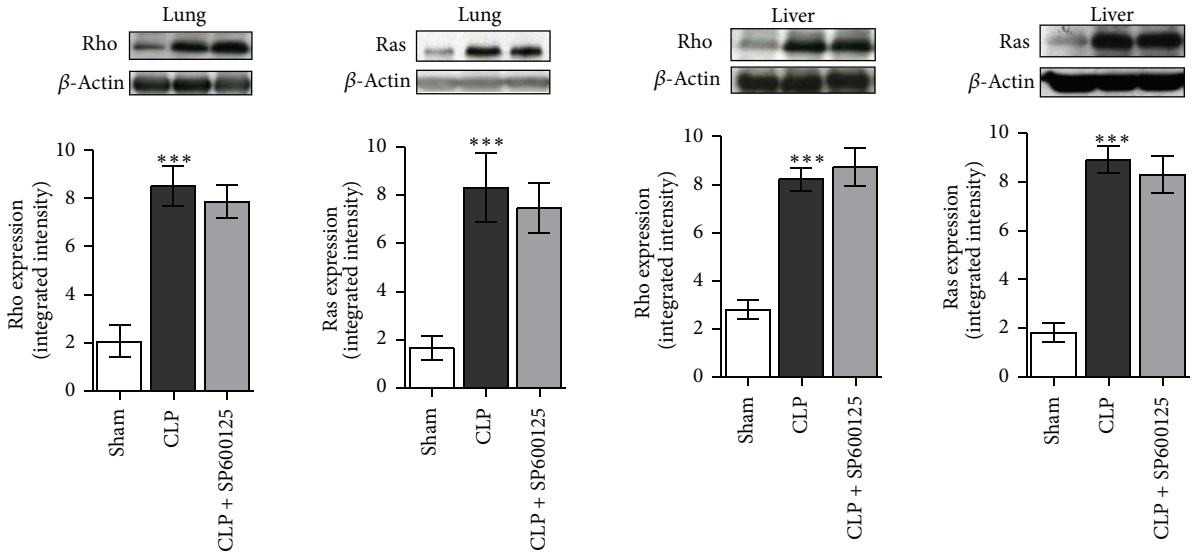

Figure 4

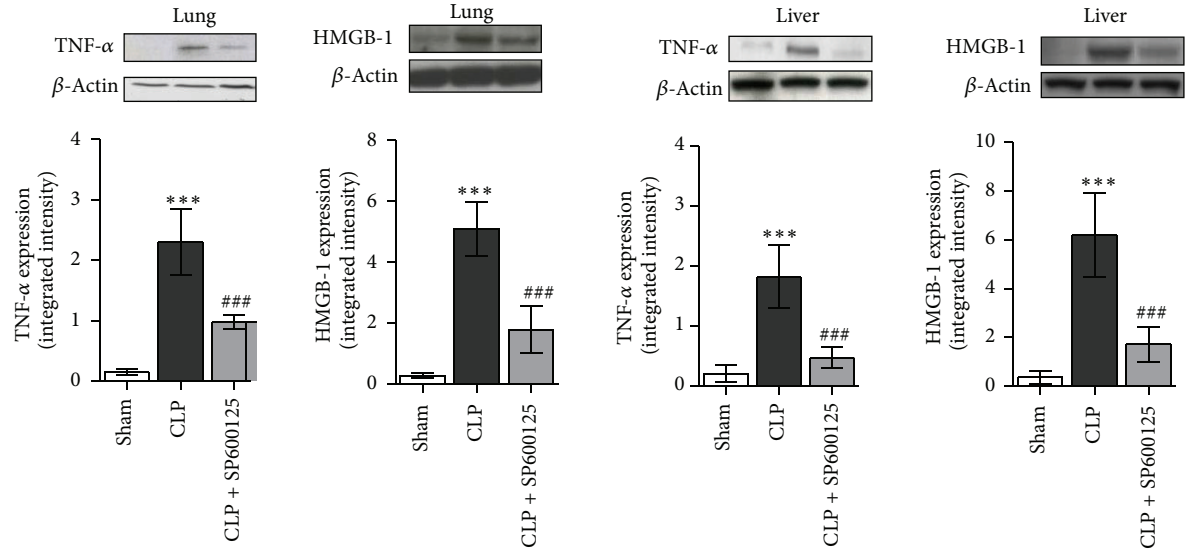

Figure 5

hemorrhagic areas when compared with sham animals (Figures 7(a) and 7(b) and Table 1). By contrast specimens from CLP treated animals showed reduced edema, inflammation, and vascular congestion (Figure 7(c) and Table 1).

CLP caused a liver damage characterized by a diffuse inflammatory infiltrate, relevant steatosis, necrosis, and also ballooning degenerated areas (Figures 7(e) and 7(d) and Table 1). SP600125 caused a reduction in steatosis and necrosis (Figures 7(f) and 7(e) and Table 1) and decreased the damage induced by CLP.

4. Discussion

The inflammatory cascade primed by CLP-induced polymicrobial sepsis is known to be correlated with an overwhelmed production and secretion of proinflammatory cytokines that could lead to several pathological consequences, such as accumulation of leukocytes, apoptotic cell death, and necrosis, finally causing multiple organ failure.

SP600125 has been shown to inhibit the MAP-kinase JNK and to partially antagonize ERK1/2, by a not specific and indirect action [16]. Both kinases are majors players involved in mediating and transferring the early inflammatory stimulus from cell membrane to the nucleus [9, 10]. Therefore the blockade of this early step represents a rationale therapeutic approach to limit the pathological inflammatory cascade during septic states.

In this study we showed that the pharmacological inhibition of JNK, induced 1 hour after the CLP procedure *via* a repeated SP600125 administration (every 24 hours), was able to achieve a significant reduction in the inflammatory process and to cause a marked improvement in survival of treated

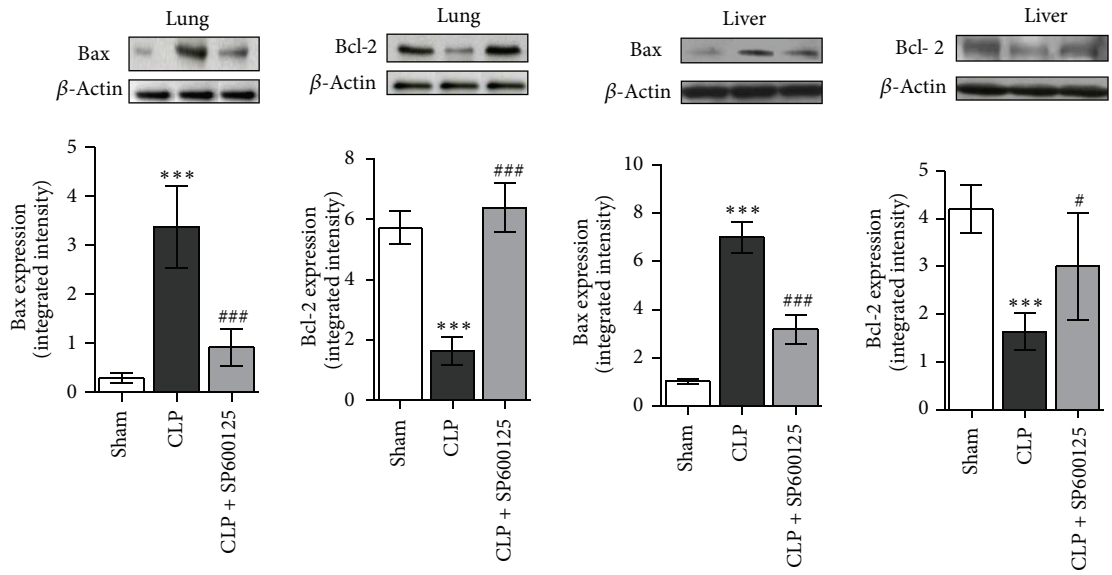

Figure 6

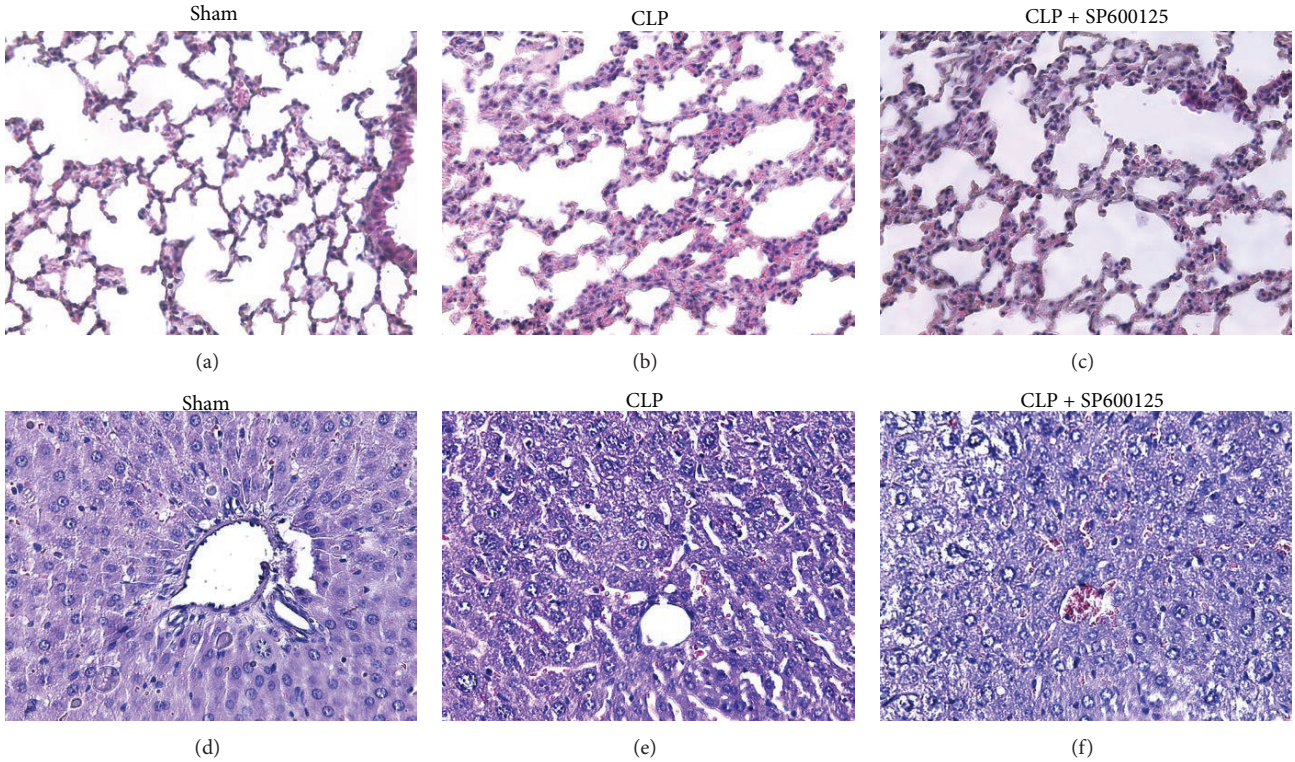

Figure 7

mice compared with untreated animals (Figure 1). This result is of particular interest: in fact in a clinical setting the improvement in the survival may allow creating a therapeutic window in which the underlying antibiotic drugs are more likely to reach the therapeutic efficacy.

Cecum ligation and puncture is an experimental model of polymicrobial sepsis that allows dissecting out the early and late components of the inflammatory cascade. Therefore we used this experimental paradigm to investigate the precise molecular pathways that are targeted by SP600125. Our hypothesis was that the drug may block the early and late steps of the inflammatory cascade by inhibiting JNK kinase: indeed we were able to demonstrate that SP600125 blunts JNK activation in both lung and liver. However CLP also triggered

the activation of Rho and RAS proteins, two additional components of the proinflammatory pathway, which might lead in turn to JNK and ERK1/2 phosphorilation, with consequent activation of NF-κB.

To investigate this molecular pathway we studied the effects of SP600125 on these two proteins and we found that the drug did not change the expression of Rho and RAS, thus ruling out the hypothesis of an interference with these two "actors" of the inflammatory cascade.

The net results of the early steps of the molecular cascade are an increased production of TNF-α and HMGB-1, two cytokines responsibles, at the initial stage of the inflammatory process, for the increase in leukocytes infiltrate and edema, and at later stage apoptosis and necrosis that may culminate in systemic organ failure.

These complex sequelae of events are evidenced by our experiments. Blocking the up-stream signalling mediated by JNK kinase was able to downregulate the TNF-α levels in both lung and liver tissues, thus producing an improvement in the histological features of both these organs as confirmed by the histopathologic evaluations (Figure 7(f)). Moreover, SP600125 treatment caused the same degree of downregulation in the proinflammatory protein HMGB-1 and in the proapoptotic caspase 3 and BAX together with an upregulation of Bcl-2, a well-known antiapoptotic mediator.

These molecular findings paralleled with the histologic evaluations carried out on liver and lung samples, which depict a general restoring of the normal architectural characteristics of both tissues; in fact we observed a regression of inflammatory infiltrate as well as a reduction of both interstitial edema and vascular congestion in the lung. Furthermore a more well-structured parenchyma, with partial reorganization of hepatic lobule architecture, a general reduction of both steatotic and necrotic areas, and a regression of the ballooning degeneration phenomenon were observed in the liver.

In conclusion our findings clearly suggest that the blockade of the JNK mediated signalling may represent an innovative and effective approach in the management of polymicrobial sepsis.

Conflict of Interests

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

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Research Article

Interleukin-18 Increases TLR4 and Mannose Receptor Expression and Modulates Cytokine Production in Human Monocytes

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Interleukin-18 is a proinflammatory cytokine belonging to the interleukin-1 family of cytokines. This cytokine exerts many unique biological and immunological effects. To explore the role of IL-18 in inflammatory innate immune responses, we investigated its impact on expression of two toll-like receptors (TLR2 and TLR4) and mannose receptor (MR) by human peripheral blood monocytes and its effect on TNF-α, IL-12, IL-15, and IL-10 production. Monocytes from healthy donors were stimulated or not with IL-18 for 18 h, and then the TLR2, TLR4, and MR expression and intracellular TNF-α, IL-12, and IL-10 production were assessed by flow cytometry and the levels of TNF-α, IL-12, IL-15, and IL-10 in culture supernatants were measured by ELISA. IL-18 treatment was able to increase TLR4 and MR expression by monocytes. The production of TNF-α and IL-10 was also increased by cytokine treatment. However, IL-18 was unable to induce neither IL-12 nor IL-15 production by these cells. Taken together, these results show an important role of IL-18 on the early phase of inflammatory response by promoting the expression of some pattern recognition receptors (PRRs) that are important during the microbe recognition phase and by inducing some important cytokines such as TNF-α and IL-10.

1. Introduction

Interleukin-18 (IL-18) belongs to the fourth member of the IL-1 family and is produced by a wide variety of cells including macrophages, dendritic cells (DCs), neutrophils, adipocytes, Kupffer cells, microglial cells, and certain neurons in the brain. This cytokine presents many unique biological effects, including pleiotropic, multifunctional, and proinflammatory actions [1–4].

Like IL-1β, the prototype member of the family, IL-18 secretion does not happen via endoplasmic reticulum and Golgi apparatus. The cytokine is produced as a leaderless and biologically inactive 24 kDa precursor protein called pro-IL-18, which is cleaved by IL-1β converting enzyme, called caspase-1, to produce 18 kDa mature and biologically active cytokine [3, 5, 6]. Caspase-1 is presented in an inactive 45 kDa precursor form whose activation requires assembly of multiunit complexes involving certain nucleotide-binding and oligomerization domain- (NOD-) like proteins, called inflammasomes, that are responsible for recruiting and activating caspase-1 precursor molecules [7–9]. So, an increased production of biologically active IL-18 requires two distinct stimuli: one increases IL-18 gene expression at mRNA and protein levels and usually comes from recognition of

pathogen products by a pattern recognition receptor (PRR); the second signal causes inflammasome assembly, caspase-1 activation, and secretion of mature IL-18 [10–12].

IL-18 was initially described as an IFN-γ-inducing factor that upregulates the IL-12Rβ subunit on T cells and has generally been considered a Th1 type cytokine [13, 14]. However, depending on the context of stimulation, the cytokine microenvironment, and genetic predisposition, IL-18 can promote a Th1 or Th2 response [15]. The IL-18Rα membrane protein is responsible for ligand binding and TIR domains present in the cytoplasmic tails of the receptor chains transduce signals in target cells, which involves MyD88- and TRAF6-dependent pathways to activate NF-κB and JNK cascades [1, 2, 4, 16]. Although the pleiotropic effects of IL-18 show an important role in the modulation of Th2 cytokines, when acting independently of the action of IL-12 [15], its main function would be to participate in inflammatory response by inducing production of several proinflammatory cytokines and chemokines including TNF-α, IL-8, IL-1β, MIP-1α, NO, MMP, CXCL8, CXCL9, and CXXL10 from a variety of human cells [3, 4, 17].

The innate immune response is initiated through the activation of pattern recognition receptors (PRRs) by pattern-associated molecular patterns or PAMPs and endogenous molecules produced by injured tissue. These receptors regulate many aspects of innate immunity and determine the polarization and function of adaptive immunity [18–21], but they are also involved in the maintenance of tissue homeostasis by regulating tissue repair and regeneration [10, 20, 22]. TLRs are the most extensively studied recognition sensors that participate in the initiation of inflammation [20]. TLR2 recognizes peptidoglycan and lipoteichoic acids of Gram-positive bacteria. Besides, TLR2 is involved in the recognition of other bacterial components such as lipoprotein/lipopeptides, lipoarabinomannan, phenol-soluble modulin, porins, and glycolipids [23]. However, TLR4 can recognize lipopolysaccharide, heat shock proteins, flavolipin, mannan, fibrinogen, taxol, glycoinositolphospholipids, retroviral envelope protein, hyaluronic acid, and fibronectin [24].

Tissue-resident macrophages express all TLRs (except TLR3) and are highly responsive to their agonist [20]. In these cells, TLRs are important for each stage of phagocytosis, ranging from engulfment of invading pathogens to antigen processing and presentation of antigenic peptides. TLRs also lead to the production of cytokines such as tumor necrosis factor- (TNF-) α and interleukin- (IL-) 1β, and to the release of chemokines that induce endothelial cell activation and drive inflammatory cell recruitment, regulate the generation of vasoactive lipids and reactive oxygen species [20, 21, 25, 26]. In addition, TLR activation regulates the expression of major histocompatibility complex (MHC) molecules and costimulatory molecules [27] and induces the release of IL-12 and IL-10, cytokines which differentially alert DCs to polarize naive T cells and activate specific adaptive immunity [28].

The mannose receptor (MR, CD206) is a member of the MR family, which is a subgroup of the C-type lectin superfamily that comprises transmembrane and soluble proteins such as selectins and collectins and can bind terminal mannose, fucose, or N-acetyl glucosamine and consequently recognizes a wide variety of ligands, including several bacterial, viral, and fungal pathogens [29]. Thus, MR is considered a PRR and pathogens recognized by this receptor include *Candida albicans*, *Leishmania*, *Mycobacterium tuberculosis*, HIV, *Pneumocystis carinii*, dengue virus, and selected strains of *Klebsiella pneumonia*, *Cryptococcus neoformans,* and *Streptococcus pneumoniae* [29].

Despite its role on resistance of infections, high levels of IL-18 has been related to the pathogenesis of several disorders and diseases, such as Chronic kidney disease (CKD) [30], Atherosclerosis [31–33], Sickle cell anemia (SCA) [34], Acute Myocardial Infarction and Heart Failure [35–37], polycystic ovary syndrome [38], Severe Traumatic Brain Injury [39]; Chronic Obstructive Pulmonary Disease [40], hepatitis C [41], Autoimmune Hepatitis [42], and mainly sepsis, due Melioidosis, an infection caused by the gram-negative bacillus Burkholderia pseudomallei (formerly Pseudomonas) [43]. Therefore, the effect of IL-18 on PRRs expression and cytokines production could account for the severity of the inflammatory response observed in these diseases, particularly in sepsis.

Thus, the present study was designed to better elucidate the role of IL-18 on the expression of some PRRs such as TLR2, TLR4, and MR by human monocytes isolated from peripheral blood, and its effect on TNF-α, IL-12, IL-15, and IL-10 production by these cells, once IL-18 is involved in the development of various diseases as mentioned above. The results presented herein demonstrate a clear role of IL-18 in directly modulating TLR4 and MR expression and TNF-α and IL-10 production by these cells.

2. Subjects and Methods

2.1. Donors. Fifteen healthy blood donors from the Faculdade de Medicina de Botucatu (FMB), UNESP, Brasil (age range 20–50 years), were included in this study. The Research Ethics Committee approved the study, and informed consent was obtained from all the subjects (2513/07).

2.2. Monocyte Isolation. Heparinized venous blood was obtained from healthy adults. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation at 400 g for 30 min on Ficoll-Paque Plus (density (d) = 1.077) (GE Healthcare Bio-Sciences AB, Uppsala). Briefly, heparinized blood was mixed with an equal volume of RPMI-1640 tissue culture medium (Sigma-Aldrich, St. Louis, USA), and samples were layered over 10 mL of Ficoll-Paque Plus in a 50 mL conical plastic centrifuge tube. After centrifugation at 400 g for 30 min at room temperature, the interface layer of PBMC was harvested and washed twice with RPMI-1640 tissue culture medium (Sigma-Aldrich). The PBMC suspension was stained with neutral red (0.02%) which is incorporated by monocytes and allows their identification and counting in a hemocytometer chamber. After counting, the mononuclear cell suspension was adjusted to 1×10^6 monocytes/mL in RPMI-1640 (Sigma-Aldrich) containing 10% heat-inactivated fetal calf serum (Complete Tissue Culture Medium—CTCM), dispensed into

1000 μL/well in 24-well flat-bottom plates (TPP, Trasadingen, Switzerland) and used for flow cytometry analysis and for cytokine production. After incubation of cultures for 1 h at 37°C in 5% CO_2, nonadherent cells were removed by aspiration and each well was rinsed twice with RPMI-1640. This procedure resulted in cultures with more than 95% of monocytes. The resulting monocyte cultures were treated or not with IL-18 (MBL, Medical & Biological Laboratories Co. Ltda), 100 ng/mL, for 18 h at 37°C in 5% CO_2. In some cocultures, anti-IL-18 (MBL), 0.5 μg/mL, was used before the IL-18 treatment, to block IL-18 effects. Control groups with negative isotype control were also tested.

2.3. Flow Cytometry. For CD14, TLR2, TLR4, and MR expression, adherent monocytes were detached from wells by putting the plate on ice and using HyQTase Cell Detachment Solution (HyClone Laboratories Inc., Logan, UT, USA). After that, cells were put into polystyrene tubes for cytometric analysis (BD Labware, Franklin Lakes, NJ USA) and were washed and incubated with mouse anti-human CD14-PE/Cy7, mouse anti-human CD206-APC (MR), mouse anti-human TLR2-FITC, and mouse anti-human TLR4-PE (all from BioLegend, Inc., San Diego, CA) according to the instructions of the manufacturer. Nonspecific signals were calculated and attenuated by isotype control (BioLegend) tubes. After incubation for 20 min at room temperature in the dark, cells were washed and a fixative solution consisting of 5% formaldehyde in buffer (Becton Dickinson, San Jose, CA) was added; then cells were analyzed. Control experiments showed that HyQTase Cell Detachment Solution did not affect cell viability nor altered the expression of all receptors evaluated (data not shown).

For TNF-α, IL-10, and IL-12 intracellular analyses, monocyte cultures were pretreated with Brefeldin A Solution (BioLegend), six hours prior to harvest. Afterwards, detached monocytes were distributed into polystyrene tubes for cytometric analysis (BD Labware). Cells were washed and incubated with mouse anti-human CD14-PE/Cy7 (BioLegend), according to the manufacturer's instructions. Next, the permeabilization and staining procedures were conducted using a Cell Permeabilization Kit FIX&PERM (ADG, AN DER GRUB Bio Research GMBH, Kaumberg, Austria). Cells were stained with rat anti-human IL-10-PE, mouse anti-human IL-12/IL-23 p40-FITC, and mouse anti-human TNF-α-APC (all from BioLegend). Nonspecific signals were calculated and attenuated by isotype control (BioLegend) tubes. After incubation for 20 min at room temperature in the dark, cells were washed and a fixative solution consisting of 5% formaldehyde in buffer (Becton Dickinson) was added; then cells were analyzed.

For both, cells were analyzed with a FACSCalibur flow cytometer (Becton Dickinson). Data (an average of 10,000 events per sample) were analyzed with the software CELL QUEST (Cell Quest Software).

2.4. Measurement of Cytokines. After IL-18 treatment, monocyte culture supernatants were separated from cell debris by centrifugation at 1000 g for 15 min and stored at −80°C. The TNF-α, IL-10, IL-12, and IL-15 concentrations were measured by capture ELISA using BD OptEIA human ELISA Set (BD Biosciences, Franklin Lakes, NJ, USA). IL-18 concentrations were measured by human IL-18 ELISA Kit (MBL). ELISA was performed according to the manufacturer's protocols. Cytokine concentrations were determined with reference to a standard curve for serial twofold dilutions of recombinant cytokines. Absorbance values were measured at 492 nm using a micro-ELISA reader (MD 5000; Dynatech Laboratories).

2.5. Statistical Analysis. Data were analyzed statistically using GraphPad Prism software (GraphPad Prism 5.0, San Diego, CA). The results were compared by Friedman test, followed by Dunn's Multiple Comparison Test, with the significance level set at $P < 0.05$.

3. Results and Discussion

The innate immune system promptly responds to the invasion of microbes and acts as the first line of defense, whereby innate immune cells such as macrophages or DCs play a central role in the production of proinflammatory cytokines and nitric oxide after recognition of pathogen [44]. This response is triggered by PRRs that interact with pathogen structures and send signals to the host cell.

To better understand the role of IL-18 in the expression of TLR2, TLR4, and MR by purified $CD14^+$ monocytes, cells were treated with IL-18 and subsequently analyzed by flow cytometry. The results showed that IL-18 was able to increase TLR4 and MR expression by $CD14^+$ monocytes (Figure 1). However, the cytokine treatment did not affect TLR2 expression (Figure 1(d)). The blocking of IL-18 with specific neutralizing antibody showed a reversal on the TLR4 and MR expression results, as shown in Figures 1(e) and 1(f). The treatment with negative isotype control did not affect the response of monocytes (data not shown). These are new data that support the autocrine role of IL-18 by identifying an important direct modulation of TLR4 and MR on human monocytes by this cytokine, seeing that purified human monocytes treated with this cytokine presented higher expression of TLR4 and MR than control cells, whereas the blocking of IL-18 with anti-IL18 reversed this effect.

TLR-2 and TLR-4 are constitutively expressed by various cell members of the immune system including macrophages, neutrophils, and DCs (reviewed [20]). The expression of TLR-2 and TLR-4 is tightly regulated by several proinflammatory cytokines. But until now, the role of IL-18 in the expression of PRRs is not completely understood, and studies have reported an indirect effect of IL-18 on these cells via Th1 activation. Radstake et al. [45] showed that TLR-2 and TLR-4 are expressed in synovial tissue of patients with rheumatoid arthritis, with clinically active disease, and these expressions were associated with the levels of both IL-12 and IL-18. However, IL-12 and IL-18 treatment *in vitro* did not affect the expression of TLR-2 or TLR-4 on purified monocytes. An upregulation of TLR-2 and TLR-4 was just seen when PBMC

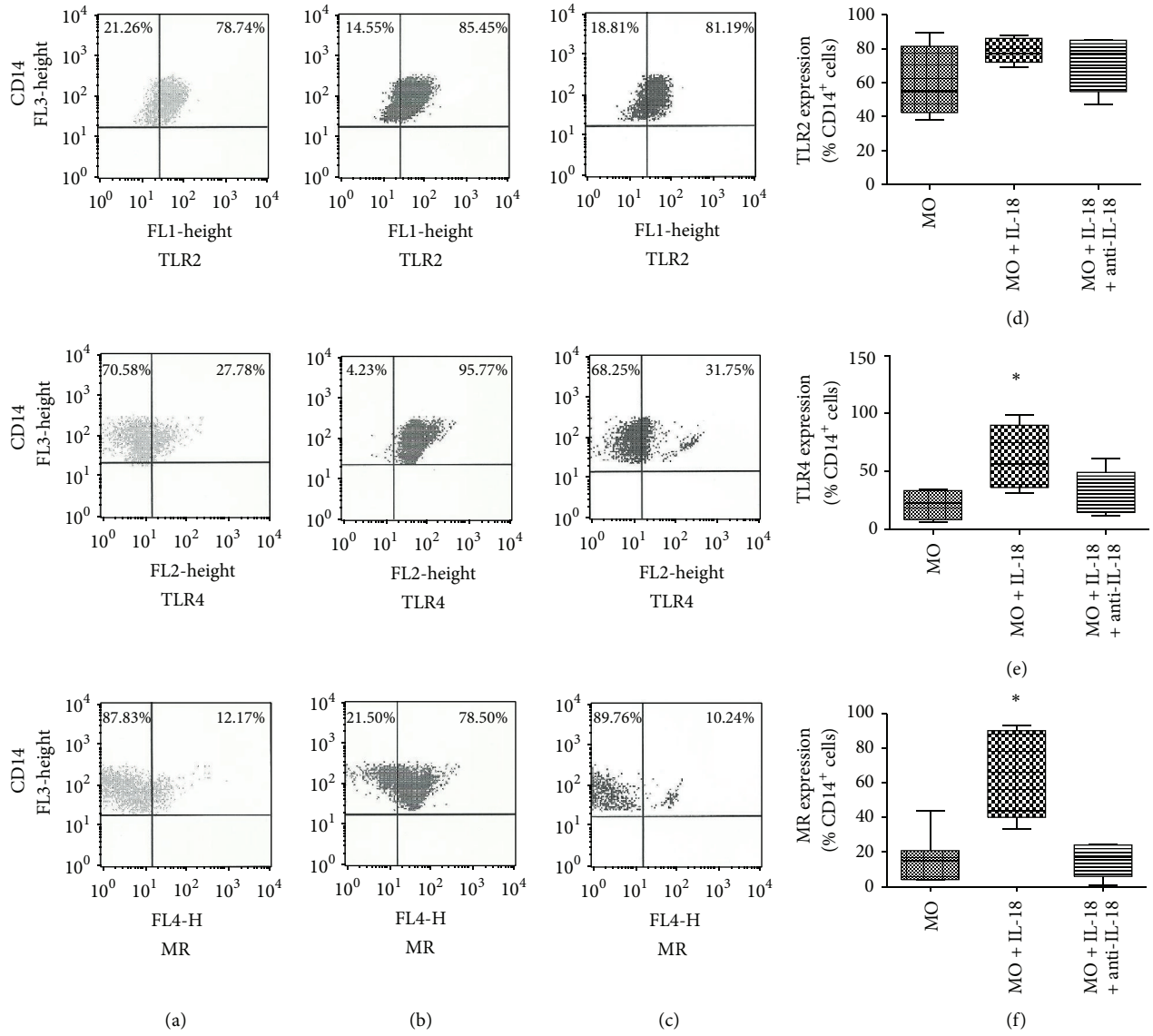

Figure 1: TLR2, TLR4 and MR expression in $CD14^+$ monocytes. $CD14^+$ monocytes (column (a)) were treated with IL-18 (100 ng/mL) (column (b)), or IL-18 (100 ng/mL) plus anti-IL-18 (0,5 μg/mL) (column (c)), for 18 hours and evaluated by flow cytometry. Box-and-whisker plot showing data distribution of 15 healthy subjects tested for TLR2 (d), TLR4 (e), and MR (f). Horizontal lines represent the median values; boxes represent the 25th to 75th percentiles and vertical lines the 10th to 90th percentiles. *Statistical significance between groups is indicated ($P < 0.05 \times$ other groups).

were treated with IL-18. This effect was inhibited by the blocking of IFN-γ, thus showing an indirect role of IL-18 on TLR2 and TLR4 expression via induction of IFN-γ by T cells [45].

This increased expression of TLR4 by IL-18 that was detected in this study could promote a series of events, after pathogen recognition, trigging the production of cytokines. After ligand binding, TLRs dimerize and undergo conformational changes, which are required for the recruitment of adaptor molecules, via their TIR domains. These adaptor molecules, namely, MyD88, Mal (MyD88 adapter-like)/TIRAP (TIR-domain-containing adaptor protein), TRIF (Toll-receptor-associated activator of interferon), TRAM (TRIF-related adaptor molecule), and SARM (sterile α and armadillo motifs), contribute to the specificity of individual responses to pathogens. Each TLR can mediate a tailored response in association with different combinations of these adaptors. Two major pathways can be activated by TLRs; the MyD88-dependent pathway results in the activation of NF-κB and activating protein-1 (AP-1), regulating the transcription, mRNA stability, and translation of numerous proinflammatory cytokine genes, such as TNF-α, IL-6, IL-12, and IFNs, while the TRIF-dependent pathway results in the activation of type I interferons (IFNs) [46].

Thus, this direct effect of IL-18 on the increase of TLR4 expression could account on several inflammatory diseases. One of the most important disease, that presents high levels

of IL-18 (more than 10,000 pg/mL) is sepsis [43]. It was observed that patients with severe Gram-negative infection (Melioidosis) had elevated levels of IFN-γ, IL-18, IL-12p40, and IL-15 on admission, with significantly higher levels in blood culture-positive [43]. It was also observed that IFN-γ production by whole blood stimulated with heat-killed *Burkholderia pseudomallei* was inhibited by anti-IL-12 treatment more than anti-IL-18 or anti-IL-15, and the effect of anti-IL-12 was further enhanced by anti-IL-18 treatment, suggesting that, during Gram-negative sepsis, IFN-γ production is controlled at least in part by endogenous IL-18, IL-12, and IL-15 [43]. Puren et al. [47] in a previous study evaluated a simple 24 h human whole blood culture that was treated with IL-18 in different concentrations, plus low concentration of LPS, showing that only IL-18 did not induce IFN-γ production. However, the combination of LPS plus increasing concentrations of IL-18 (0.625–10 nM) resulted in an increased IFN-γ production in a dependant manner. The combination, however, was independent of the concentration of LPS. It was also detected that cultures treated with IL-18 + LPS showed an increased production of IL-6, IL-8, and TNF-α, and LPS-induced TNF-α production was potentiated by IL-18 [47]. Recently, it was also demonstrated that the exposure of RAW264.7 cells to LPS/ATP triggered the activation of caspase-1 and the cleavage of interleukin-(IL-) 1β, as well as the release of other cytokines, such as IL-18 and IL-33 [48]. Thus, once demonstrated that LPS/ATP triggered the activation of caspase-1 with release of IL-18 [48], as well as the identification of IL-18 effect on TLR4 expression, this could also explain the systemic activation of cells, and amplification of the response observed in sepsis.

Another important PRR is the MR (CD206), a type I transmembrane protein that possesses eight extracellular CTLDs and a short cytoplasmic tail which lacks classical signaling motifs; it is expressed by macrophages, some DCs, and a variety of other cells and tissues [29, 49, 50]. The MR has been shown to induce a variety of cellular responses, but the molecular mechanisms responsible for transducing the intracellular signals from this receptor are unclear. The recognition of microorganisms by this receptor has been shown to promote the production of a number of cytokines such as TNF-α, GM-CSF, IL-12, IL-8, IL-6, and IL-1β, although there is also evidence that the MR can inhibit the production of certain cytokines, including TNF-α [29, 50–52]. A mechanism that could account for the negative effect of MR ligation on proinflammatory cytokine production is the upregulation of IRAK-M (an inhibitor of TLR signaling that blocks the dissociation of IRAK-1 and IRAK-4 from MyD88), since this regulator could be induced by treatment with the MR ligand mannan [53]. Rajaram et al. [54] reported that virulent *Mycobacterium tuberculosis* and mannose-capped lipoarabinomannan induce the expression of nuclear receptor/transcriptional factor PPARγ (peroxisome proliferator-activated receptor γ) in human macrophages and that this upregulation of PPARγ expression was mediated by the MR. The induction of this new pathway serves as a negative regulator of macrophage activation by altering the expression of many inflammatory genes [54–56], modulating macrophage differentiation and activation through transrepression of the transcription factors NF-κB, AP-1, and STAT [57–61], and attenuating the respiratory burst [62]. These attributes have important implications for the control of infections. But although the MR plays a clear role in homeostasis, its role in antimicrobial immunity remains unclear [50]. Besides that, MR has been considered an important marker of M2 macrophages, mainly in adipose tissue macrophages (ATMs) [63], and recent studies showed increase of IL-18 expression in subcutaneous and abdominal adipose tissues of obese subjects or with metabolic syndrome, and in monocyte-derived macrophage cultures exposed to hyperglycaemia [64, 65]. Study also showed that circulating levels of IL-18 were higher in obese subjects [64]. Now, it was showed that leptin stimulates caspase-1 activity in monocytes and that leptin-induced IL-18 secretion is dependent on caspase-1 activity suggesting a signalling pathway between leptin and the inflammasome in these cells [66]. The authors suggested that leptin-stimulated IL-18 could be explained by a secondary effect of the upregulation of other cytokines, such as TNF-α, as having been described by other studies [67], once leptin had no direct effect on monocyte TNF-α secretion [68]. Confirming these results, Esser et al. [69] showed that the metabolically unhealthy obese phenotype seems to be associated with an increased activation of the NLPR3 inflammasome in macrophages infiltrating visceral adipose tissue. Thus, our results suggest that IL-18 production could account for MR expression and induce ATMs into alternative M2 macrophages.

Knowing the ability of IL-18 to induce either Th1 or Th2 responses [15], we also tested the capacity of IL-18 to induce some pro- or anti-inflammatory cytokines by $CD14^+$ monocytes. The IL-18 treatment induced an increase in TNF-α (Figures 2(a) and 2(b)) and IL-10 (Figures 2(c) and 2(d)) levels by $CD14^+$ monocytes. The productions of intracellular IL-12 (Figure 2(e)) and IL-15 (Figure 2(f)) were not induced by IL-18. The IL-12 levels in culture supernatant of $CD14^+$ monocyte controls and in that treated with IL-18 were undetected by ELISA, while IL-15 levels did not differ between groups. The blocking of IL-18 with specific neutralizing antibody reversed the effect of IL-18 on TNF-α and IL-10 production by these cells. The treatment with negative isotype control did not affect the production of the quantified cytokines (data not shown). Then, our results showed an important role of IL-18 in increasing the endogenous production of IL-10 and TNF-α by monocytes.

TNF-α is known to induce proinflammatory activities through various cell types including mononuclear and polymorphonuclear phagocytes, in which it is responsible for the activation of cytocidal systems and plays a major role in host defense [70–72]. Takahashi et al. [73] demonstrated the increased production of TNF-alpha, IL-12, and IFN-γ by PBMC treated with IL-18. These results were correlated with the upregulation of ICAM-1, B7.2, and CD40 expression on monocytes. Blocking the engagement of these adhesion molecules by antibodies against ICAM-1 and B7.2 reduced the cytokine production by IL-18-treated PBMC [74, 75]. The authors suggested that IL-18 induces cytokine production through upregulation of adhesion molecule expression on monocytes [73]. But now, our results show a direct effect of

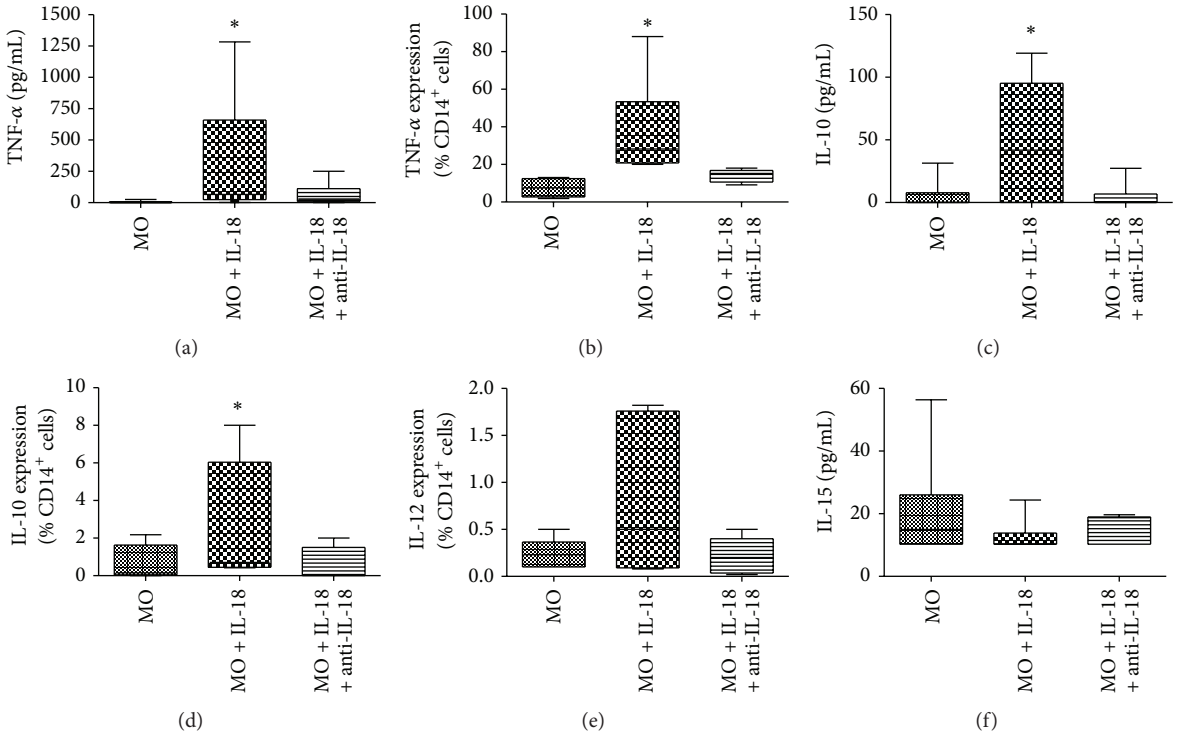

Figure 2: Production of TNF-α ((a) and (b)), IL-10 ((c) and (d)), IL-12 (e), and IL-15 (f) in $CD14^+$ monocytes treated with IL-18 (100 ng/mL) or IL-18 (100 ng/mL) plus anti-IL-18 (0,5 μg/mL), for 18 hours in culture supernatants, evaluated by ELISA ((a), (c), and (f)), and intracellular staining, by flow cytometry ((b), (d), and (e)). Box-and-whisker plot showing data distribution of 15 healthy subjects tested. Horizontal lines represent the median values; boxes represent the 25th to 75th percentiles and vertical lines the 10th to 90th percentiles. *Statistical significance between groups is indicated ($P < 0.05$ × other groups).

IL-18 on purified monocytes by inducing TNF-α production that together with effects shown on the TLR4 receptor could further compromise the response mainly in sepsis.

On the contrary, IL-10 is a cytokine produced by $CD4^+$ T helper type 2 (TH_2) cells, $CD8^+$ T cells, monocytes, macrophages, and B cells. It was first described as an inhibitor of activation and cytokine production by TH_1 cells [76]. However, IL-10 suppresses the activity of T and NK cells indirectly, via monocyte and macrophage inhibition, and is considered a macrophage deactivation factor [76]. This IL-10 effect may occur mainly by influencing macrophage recruitment, viability, morphology, phagocytosis, the production of cytokines and expression of their receptors such as the major histocompatibility complex and costimulatory molecules, antigen presentation, generation of reactive oxygen and nitrogen intermediates, and the killing of microbes and tumor cells [76, 77]. Studies suggest that IL-10, beyond acting on monocytes/macrophages and lymphocytes, may also exert an important regulatory action on neutrophil functions [78, 79].

We would like to point out that, in this study, these new very interesting results regarding the direct effect of IL-18 on human monocytes by inducing both TNF-α and IL-10 production and MR expression could indicate that IL-18 participates on the induction of both classically (M1) and alternatively (M2) activated macrophages, once after IL-18 treatment, cultured cells presented higher TNF-α, IL-10 production and MR expression. M1 macrophages are characterized by high microbicidal capacity and secretion of proinflammatory cytokines such as TNF-α, while M2 macrophages present high expression of mannose, galactose, and scavenging receptors, more phagocytic activity, and a phenotype characterized by high expression of IL-10 and low expression of IL-12 [80, 81]. Further investigations are being conducted in our laboratory to better elucidate these mechanisms.

4. Conclusions

In conclusion, our findings showed that IL-18 affects TLR4 and MR expression on human monocytes, and TNF-α and IL-10 production by these cells. Taken together, these results implies that this cytokine may also play an important role in the initiation of innate immune responses, participating in severity or resolution of infections and inflammatory diseases, since monocytes and macrophages are the main components of this response.

Conflict of Interests

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

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Review Article

Role of IL-38 and Its Related Cytokines in Inflammation

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Interleukin- (IL-) 38 is a recently discovered cytokine and is the tenth member of the IL-1 cytokine family. IL-38 shares structural features with IL-1 receptor antagonist (IL-1Ra) and IL-36Ra. IL-36R is the specific receptor of IL-38, a partial receptor antagonist of IL-36. IL-38 inhibits the production of T-cell cytokines IL-17 and IL-22. IL-38 also inhibits the production of IL-8 induced by IL-36γ, thus inhibiting inflammatory responses. IL-38-related cytokines, including IL-1Ra and IL-36Ra, are involved in the regulation of inflammation and immune responses. The study of IL-38 and IL-38-related cytokines might provide new insights for developing anti-inflammatory treatments in the near future.

1. Introduction

Our understanding of the interleukin-1 family (IL-1F) has recently expanded to encompass 11 members: IL-1F1-IL-1F11 [1]. These cytokines are also termed IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-18, IL-36Ra, IL-36α, IL-37, IL-36β, IL-36γ, IL-38, and IL-33, respectively [2] (Table 1). These protein molecules play a prominent role in inflammation and immune responses, acting as the first line of defense against invasive pathogenic microorganisms and physical damage. IL-38 is a novel member of the IL-1F identified in 2001 by a unique high throughput cDNA screening approach taking advantage of a set of oligonucleotide probes to hybridize successive arrays of human cDNAs from various tissues [3, 4]. The former name of IL-38 is IL-1HY2, similar to IL-36Ra (IL-1HY1). IL-38 is the 10th member of IL-1F and its receptor is termed IL-1 receptor-related protein 2 (IL-1Rrp2, IL-36R). IL-38 is an IL-36 antagonist and functions as a typical receptor antagonist similar to IL-1Ra and IL-36Ra [5]. IL-38 reduces inflammation by preventing the binding of agonist receptor ligands to IL-36R, a specific receptor of IL-38.

2. Biological Characteristics of IL-38

The novel IL-1-like gene, *IL-38*, is located in the IL-1 family cluster (except *IL-18* and *IL-33*) on human chromosome 2q13-14.1 near the *IL-1Ra* gene (*IL-1RN*) and *IL-36Ra* gene (*IL-36RN*) [6]. The *IL-38* gene is located 49,479 bp upstream from *IL-1RN* on the same DNA strand [7]. *IL-38* shares high sequence homology with *IL-1Ra* and *IL-36Ra*. The primary translated product is an IL-38 precursor, 152 amino acids in length and with 16.9 kD molecular mass. Sequence analysis indicated that the IL-38 protein shares 41% homology with IL-1Ra and 43% homology with IL-36Ra [4, 7] and lower homology (14–30%) with IL-1β and other IL-1 family proteins. In mammalian Chinese hamster ovary cells, recombinant IL-38 protein was synthesized into two forms, a major form at 25 kD and a minor form at 17 kD. Lin et al. [3] suggested that the major form of IL-38 might be a result of posttranslational protein modifications, such as phosphorylation. However, studies have shown that the IL-38 protein lacks N-glycosylation and O-glycosylation consensus sites in Chinese hamster ovary cells [3]. As is typical of the IL-1 family, including IL-36Ra, IL-36α, IL-36β, and IL-36γ, IL-38 lacks a signal peptide and caspase-1 consensus cleavage site [3, 7]. Furthermore, the natural N terminus for IL-38 is still unclear [7]. Using the multiple alignment sequence profile-based searching method (PSI-BLAST), an automated sequence and structure searching procedure (high throughput modeling), and a fold recognition method (SeqFold), three-dimensional structural models of IL-38 were predicted. The IL-38 structural model displays a 12-β-stranded trefoil

Table 1: IL-1 family members [10, 11].

Cytokine	Family name	Receptor	Coreceptor	Property
IL-1α	IL-1F1	IL-1RI	IL-1RAcp	Proinflammatory
IL-1β	IL-1F2	IL-1RI	IL-1RAcp	Proinflammatory
IL-1Ra	IL-1F3	IL-1RI	NA	Antagonist for IL-1α, IL-1β
IL-18	IL-1F4	IL-18Rα	IL-18Rβ	Proinflammatory
IL-36Ra	IL-1F5	IL-36R	NA	Antagonist for IL-36α, IL-36β, and IL-36γ
IL-36α	IL-1F6	IL-36R	IL-1RAcp	Proinflammatory
IL-37	IL-1F7	IL-18Rα?	Unknown	Anti-inflammatory, transcription regulating factor [12]
IL-36β	IL-1F8	IL-36R	IL-1RAcp	Proinflammatory
IL-36γ	IL-1F9	IL-36R	IL-1RAcp	Proinflammatory
IL-38	IL-1F10	IL-36R	Unknown	Antagonist for IL-36α, IL-36β, and IL-36γ
IL-33	IL-1F11	ST2	IL-1RAcp	Proinflammatory, transcription regulating factor [13]

NA: not applicable; ?: requires confirmation.

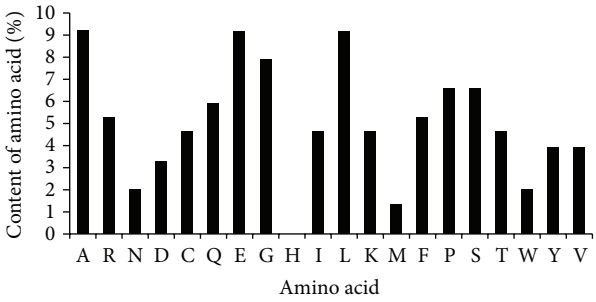

Figure 1: Amino acid composition of human IL-38 protein.

structure and shares similarity with the crystal structure of IL-1Ra and IL-1β [3, 8]. The characteristics of IL-38 provide further evidence that it belongs to the IL-1F. In addition, IL-38 was categorized in the IL-36 subfamily according to the length of its precursor. Thus, IL-38 likely also belongs to this subfamily because it has the ability to bind to the common receptor IL-36R [2, 9].

To date, no reports have described the components and properties of IL-38. We adopted the ProtParam tool [14, 15] application to analyze the amino acid composition of IL-38, which consists of 19 amino acids. A (alanine), E (glutamic acid), and L (leucine) were the most prevalent amino acids (9.2%) in IL-38, followed by G (glycine, 7.9%), P (proline, 6.6%), and S (serine, 6.6%). Only H (histidine) was not present in IL-38 (Figure 1). The molecular weight of IL-38 is 16.9 kD and is consistent with a previous study, as predicted by ProtParam tool. IL-38 has a half-life of 7 h, an isoelectric point (pI) of 4.94, and the molecular formula of $C_{757}H_{1164}N_{198}O_{226}S_9$, as analyzed by SOPMA (self-optimized prediction method from alignment) [15, 16]. Moreover, the second structure of IL-38 was composed of a β-turn, random coil, β-sheet, and α-helix. Analysis further indicated that the random coil and β-sheet were uniformly distributed in the protein chain.

By multitissue first-strand cDNA PCR analysis, IL-38 mRNA was measured in a range of tissues, including heart, placenta, fetal liver, skin, spleen, thymus, and tonsil. IL-38 was expressed mostly in the skin and in proliferating B cells of the tonsil [3]. However, in nonimmune tissues, such as human heart and placenta, IL-38 was present at low levels, similar to other IL-1F members [7]. Some IL-1F members are constitutively produced, whereas others have inducible expression, being rapidly induced by bacteria or inflammatory mediators [7, 17, 18]. The expression type of IL-38 is currently unknown and requires further research.

3. Receptor and Signaling Pathway of IL-38

In 2001, it was speculated that IL-38 acted as an IL-1 receptor antagonist because of its amino acid homology to the naturally occurring IL-1Ra and the observation that IL-38 could bind to the soluble IL-1 receptor type I (IL-1RI). IL-1RI was once considered a receptor for IL-38 [3, 5]. However, the binding affinity of recombinant IL-38 is significantly lower than that of IL-1Ra and IL-1β. Recently, researchers doubted whether IL-1RI was a receptor for IL-38. IL-1Rrp2 was regarded as an IL-38-specific receptor and was also called IL-36R. In a report by van de Veerdonk [5], the combining capacity between IL-38 and IL-1RI, IL-36R, IL-18R, and IL-1R accessory proteins (IL-1RAP, IL-1RAcP) was compared, in the presence of increasing concentrations of IL-38. IL-38 bound to IL-36R but did not bind to the other immobilized receptors. Furthermore, IL-38 binding to immobilized IL-36R was comparable to IL-36Ra binding to the same receptor. It was observed that increasing the concentration of IL-38 resulted in increased optical density, reaching a plateau at 16.7 μg/mL, a higher value than that obtained for IL-36Ra. Based on the binding studies, these data suggest IL-38 could act by blocking the IL-36R pathway.

The most recently identified IL-1 family members are widely expressed in inflammatory cells. These cytokines combine with the cell-surface receptor IL-1R and induce downstream signaling, including downstream nuclear transcripts such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1). Furthermore, as a feedback and adjustment mechanism, these signaling molecules induced the expression of cyclooxygenase, nitric oxide synthase, and other inflammatory mediators to promote the development of inflammation

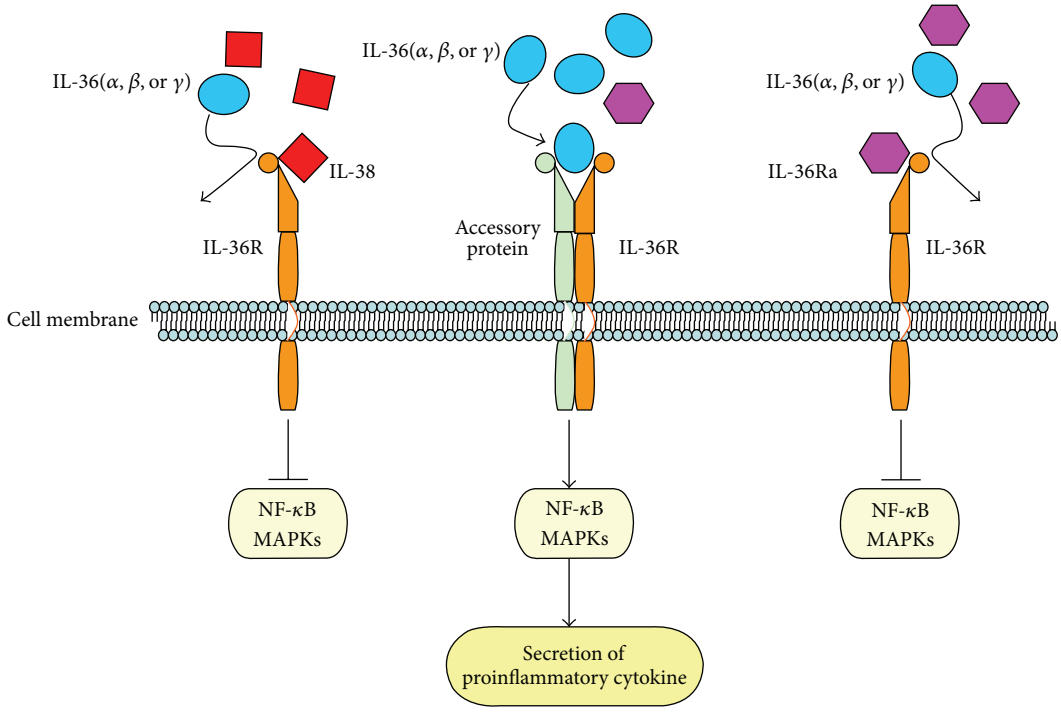

Figure 2: Receptor and signaling pathway of IL-38.

[19, 20]. In line with the characteristics of IL-38 and the homology of IL-38 and IL-36Ra, it can be concluded that IL-38 has a role in inflammatory disease by IL-36Ra pathway-related molecules (Figure 2). The biological function of IL-38 is to inhibit IL-36 cytokine (IL-36α, IL-36β, and IL-36γ) binding to IL-36R, similar to IL-36Ra. According to its activity as a receptor antagonist, IL-38 may have an anti-inflammatory function. IL-38 might also be related to IL-1R and IL-18R signaling pathways, although there is no evidence regarding its role in these specific signaling pathways.

4. Biological Activity of IL-38 and Related Cytokines

Because of its homology with other IL-1F members, IL-38 is thought to have the biological activity of IL-1F members. IL-1 cytokines are primarily proinflammatory cytokines as they stimulate the expression of genes associated with inflammation and immunological diseases. IL-1α or IL-1β binds to its primary receptor IL-1RI, which recruits a second receptor subunit, IL-1RAcP. Formation of the receptor heterodimer induces biological responses typically involving the activation of NF-κB and mitogen-activated protein kinase (MAPK) pathways [21]. IL-1F6 (IL-36α), IL-1F8 (IL-36β), and IL-1F9 (IL-36γ) also activate NF-κB and MAPKs similar to IL-1. Therefore, most molecules involved in IL-1F-induced signaling, such as cytokines, chemokines, adhesion molecules, and enzymes, are mediators of inflammatory diseases [22, 23].

4.1. IL-1Ra. IL-1Ra (IL-1F3) is the receptor antagonist of IL-1, a protein composed of two major subunits, IL-1α and IL-1β [24]. IL-1Ra is synthesized and released in response to the same stimuli that lead to IL-1 production. IL-1Ra, a potent anti-inflammatory cytokine, competitively inhibits stimulation by inflammatory mediators by binding to IL-1R1 and preventing the recruitment of IL-1RAcP. IL-1Ra is associated with severe autoimmune and inflammatory diseases such as periodontitis [25], vaginitis [26], non-Hodgkin's lymphoma [27], gastric cancer [28, 29], osteoarthritis [30], precancerous lesions [31], and inflammatory bowel diseases [32]. Deficiency of the IL-1-receptor antagonist (DIRA), caused by mutations in *IL1RN*, can lead to an autosomal recessive autoinflammatory disease. DIRA allows unopposed actions of IL-1, resulting in life-threatening excessive systemic IL-1-mediated inflammation with skin and bone involvement [33]. A study by Korthagen et al. aimed to elucidate the influence of polymorphisms in *IL1RN* on idiopathic pulmonary fibrosis (IPF) susceptibility and mRNA expression. Polymorphisms of *IL1RN* manifested as a variable number tandem repeat (VNTR), which affected IL-1Ra mRNA expression, suggest that lower levels of IL-1Ra predispose to developing IPF [34]. In addition, *IL-1RA* VNTR may be associated with Parkinson's disease risk [35]. *IL1RN* may be a future novel therapeutic target with high specificity, low toxicity, and side effects for the treatment of specific diseases. It is important to enhance our understanding of *IL1RN* functions, such as its interaction with other genes and the influence of environmental factors on its production, to develop treatments with reduced side effects [34].

In addition, IL1Ra-deficient (IL-1Ra−/−) mice, good animal models for experimental studies, spontaneously develop several inflammatory diseases, resembling arthritis [36], aortitis [37], intervertebral disc degeneration [38], and psoriasis

in humans due to unopposed excess IL-1 signaling. The current knowledge also suggested that IL-1Ra, an endogenous inhibitor of IL-1, related to alcoholic steatohepatitis [39], liver damage [40], lung damage [41], fat mass [42], and aqueous-deficient dry eye in autoimmune diseases [43].

4.2. IL-36Ra. IL-36Ra (IL-1F5) shares 44% homology with IL-1Ra and is an antagonist of IL-36α, IL-36β, and IL-36γ. IL-36Ra has a β-stranded trefoil structure, similar to other family members of IL-1, with a conserved hydrophobic core. IL-36Ra binds to IL-1Rrp2 and has biological effects on immune cells. The mechanism of IL-36Ra antagonism is analogous to IL-1Ra by forming a functional signaling complex. IL-36Ra protein starting at Val-2 is fully active and inhibits IL-36α, IL-36β, and IL-36γ. The A-X-Asp motif is conserved in all IL-1F members at the N-terminal, where Val-2 of IL-36Ra lies in this 9-amino acid conserved sequence. All four IL-36 cytokines lack a conventional signal sequence and function as extracellular cytokines although it is unclear how they are secreted [44]. In addition, IL-36Ra antagonist activity requires removal of the N-terminal methionine present in the primary translation product [45]. More extensive N-terminal amino truncation of IL-36α, IL-36β, and IL-36γ can dramatically increase their specific activities [45].

IL-36Ra plays a key role in innate and adaptive immunity by stimulating helper T-cell responses and it is associated with many inflammatory diseases. Recessive homozygous mutations in *IL36RN* are the major cause for the development of generalized pustular psoriasis [46–49]. Mutations in *IL36RN*, including premature termination codon mutations, frameshift mutations, and substitutions of amino acid lead to incorrect folding of the IL-36Ra protein. These mutations inhibit the activity of IL-36Ra, thus failing to antagonize IL-36 signaling pathways and inducing inflammatory skin disease due to the high levels of IL-36α, IL-36β, and IL-36γ. Excessive IL-36 levels in the skin of mice lead to symptoms to human psoriasis. However, IL-36Ra-deficient mice develop the more serious pustular psoriasis. Therefore, treatment with a combination of IL-36Ra and IL-36R might improve psoriasis by inhibiting IL-36 stimulation and might be an ideal treatment strategy for inflammation of human skin. IL-36Ra functions as an anti-inflammatory cytokine in the brain [50] and enhances the hippocampal expression of IL-4. This is a consequence of its interaction with the orphan receptor, single Ig IL-1R-related molecule (SIGIRR)/TIR8. Collectively, *in vitro* IL-4 mRNA and protein expression in glia induced by the interaction of IL-36Ra and SIGIRR/TIR8 play a critical role in its anti-inflammatory properties [5]. IL-36 cytokines also have a significant association in the pathogenesis of rheumatoid arthritis [9, 51, 52], inflammatory lung diseases [53, 54], obesity [55], bile duct occlusion disorder, and chronic glomerulonephritis [56]. Data strongly suggest that IL-36Ra might be a useful treatment for IL-36-related diseases.

4.3. IL-38. In recent years, scholars identified a novel $CD4^+$ T-cell subtype, which was different from T-helper 1 (Th1) and Th2 cells. These cells were named Th17 cells due to the expression of IL-17 and these discoveries have improved our understanding of inflammatory processes. Th17 cells are different from natural T-cell precursors, and the mature cells secrete a variety of cytokines such as IL-17 and IL-22 [57, 58]. Th17 plays an important role in a variety of autoimmune diseases and has an independent regulatory mechanism for their differentiation and development. Th17 is associated with the pathogenesis of systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, psoriasis, inflammatory bowel disease, and autoimmune thyroid diseases [44]. Previous studies demonstrated the functions of IL-38 and Th17 cells by blocking the IL-1R, IL-18R, and IL-36R pathways [5]. These data suggested that the influence of IL-38 on Th17 cells was similar to blocking IL-1R and IL-36R pathways, which suppressed IL-17 and IL-22 secretion. Consistent with binding data and the suppression of IL-17 and IL-22, we suggest that IL-38 has similar biological effects on Th17 cells.

IL-38 gene polymorphisms are associated with psoriatic arthritis (PsA), ankylosing spondylitis (AS) [59–61], and cardiovascular disease [62], suggesting that IL-38 is strongly correlated with these inflammatory diseases. The frequencies of Th17 cells are significantly increased in the peripheral blood of patients with PsA and AS [8, 63–68]. In addition, the number of Th17 cells and serum IL-17 levels were strongly related to systemic disease activity both at the onset and during disease progression of PsA and AS [68]. IL-38 reduced the expression of *C. albicans*-induced IL-17 and IL-22 from peripheral blood mononuclear cells (PBMCs) by reducing the stimulation of proinflammatory cytokines in the tissues. A recent study reported that low concentrations of IL-38 were more effective than higher concentrations in inhibiting IL-17 and IL-22 production because higher concentrations modestly increased IL-22 [5].

Similar to IL-36Ra, blocking IL-38 suppressed *C. albicans*-induced Th17 cytokine production [5, 69]. Both IL-38 and IL-36Ra inhibited the production of IL-17 and IL-22 by specifically binding to the cell surface-specific protein receptor IL-36R. However, neither IL-38 nor IL-36R functions as a classic receptor antagonist. In PBMCs, the dose-response suppression of IL-38 and IL-36Ra by IL-36γ-derived IL-8 was not similar to that of IL-1Ra. Neutrophils and T cells in inflammatory tissues are attracted by IL-8, a chemokine. IL-38 decreased the production of proinflammatory cytokines similar to IL-36Ra [5, 70]. In contrast, IL-38 and IL-36Ra have parallel effects on the production of lipopolysaccharide-induced IL-6 from dendritic cells (DCs), inducing a twofold increase [5]. IL-6 has two adverse effects on immune cells: IL-6 is proinflammatory but also suppresses inflammation in tissues injured by burns or other damages.

IL-1Ra and IL-38 have a comparable dose-effect regarding their antagonist activities and function as classic receptor antagonists; the higher the concentration of IL-22, the stronger its inhibition. Compared with IL-1Ra and IL-38, IL-36Ra does not behave as a typical receptor antagonist. IL-38 and IL-36Ra function as antagonists at high concentrations, but at low concentrations, they inhibit the binding of coreceptors. Thus, IL-38 and IL-36Ra are defined as partial receptor antagonists, although they mimic the effects of IL-1Ra on the production of inflammatory cytokines.

5. Future Perspectives

IL-1 and most related family members are primarily proinflammatory cytokines that induce the expression of genes associated with inflammatory diseases. Only IL-37 acts as an anti-inflammatory cytokine. The binding of IL-1Ra and IL-36Ra to their receptor reduces inflammation by blocking the binding of receptor ligands. The production of fungal-induced IL-17, IL-22, and IL-36γ-derived IL-8 was decreased by IL-38, which may play an important anti-inflammatory role in inflammatory diseases. Many articles have demonstrated that IL-1Ra and IL-36Ra are associated with arthritis and psoriasis, respectively. In addition, IL-38 can specifically bind to IL-36R, similar to IL-36Ra. IL-36 cytokine has significant *in vivo* effects on DCs and T cells in human immune responses via its role in the differentiation of inflammatory Th1 cells [70–72].

In conclusion, the current knowledge supports the concept that IL-38 may be closely associated with IL-36-mediated inflammatory diseases. Thus far, the IL-1 receptor antagonist anakinra, the soluble decoy receptor rilonacept, and the neutralizing monoclonal anti-IL-1β antibody canakinumab have been approved as IL-1-targeting agents for the treatment of specific diseases. Another study demonstrated the beneficial use of a monoclonal antibody directed against the IL-1 receptor and a neutralizing anti-IL-1α antibody in clinical trials [73]. The IL-38-related signaling pathway is poorly understood and requires further study. Furthermore, the function and mechanism of IL-38-related diseases remain elusive and awaits elucidation. The increasing knowledge of the mechanisms that regulate chronic inflammatory conditions such as rheumatoid arthritis may provide a potential strategy for the development of anti-inflammatory treatments for autoimmune diseases and establish a theoretical basis for clinical trials and drug development.

Conflict of Interests

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

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Letter to the Editor

Comment on "The Effect of a Community-Based, Primary Health Care Exercise Program on Inflammatory Biomarkers and Hormone Levels"

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We read with great interest the recent paper by C. B. Papini et al. in which the authors examined "impact of a community-based exercise program in primary care on inflammatory biomarkers and hormone levels" in the 1-year quasiexperimental study [1]. The authors very clearly discussed the relation between exercise and inflammation. They concluded that community-based exercise program can result in a decrease or maintenance of inflammatory biomarkers after 1 year, and it has the potential to be a viable public health approach for chronic disease prevention. This study displayed that public health exercise intervention delivered in low-income communities has the potential to use a beneficial effect and improve or maintain inflammatory biomarkers profiles, supporting the prevention of chronic diseases.

The authors did not discuss exclusion criteria in this paper. However it is well established that any type of systemic inflammation, autoimmune disorders, and malignant or chronic illnesses may affect inflammatory biomarkers and hormone levels [2]. Also obesity is related to elevated serum levels of some inflammatory markers, such as leptin, TNF-α, and CRP [3, 4]. Because of high prevalence of these conditions, we believe that these situations may have a role in the results of the paper by C. B. Papini et al. [1].

In our opinion, future clinical studies assessment of considering these conditions may be helpful for exact results. We hope that bearing in mind these conditions would add to the value of the well-written paper of C. B. Papini et al. [1].

Conflict of Interests

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

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Clinical Study

Infliximab Preferentially Induces Clinical Remission and Mucosal Healing in Short Course Crohn's Disease with Luminal Lesions through Balancing Abnormal Immune Response in Gut Mucosa

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This study was undertaken to evaluate the efficacy of infliximab (IFX) in treatment of Crohn's disease (CD) patients. 106 CD patients were undergoing treatment with IFX from five hospitals in Shanghai, China. Clinical remission to IFX induction therapy was defined as Crohn's disease activity index (CDAI) < 150. Clinical response was assessed by a decrease in CDAI ≥ 70, and the failure as a CDAI was not significantly changed or increased. Ten weeks after therapy, 61 (57.5%) patients achieved clinical remission, 17 (16.0%) had clinical response, and the remaining 28 (26.4%) were failed. In remission group, significant changes were observed in CDAI, the Simple Endoscopic Score for Crohn's Disease (SES-CD), and serum indexes. Patients with short disease duration (22.2 ± 23.2 months) and luminal lesions showed better effects compared to those with long disease duration (71.0 ± 58.2 months) or stricturing and penetrating lesions. IFX markedly downregulated Th1/Th17-mediated immune response but promoted IL-25 production in intestinal mucosa from remission group. No serious adverse events occurred to terminate treatment. Taken together, our studies demonstrated that IFX is efficacious and safe in inducing clinical remission, promoting mucosal healing, and downregulating Th1/Th17-mediated immune response in short course CD patients with luminal lesions.

1. Introduction

Crohn's disease (CD) is a chronic relapsing and remitting inflammatory disorder of any part of the gastrointestinal tract. To date, the incidence and prevalence of CD are increasing in China [1, 2]. Although accumulating evidence indicates that CD is the consequence of a dysregulation of innate and adaptive immune responses to commensal enteric bacteria in a genetically susceptible host, the etiology of the disease still remains elusive [3, 4]. It has been shown that T helper cell (Th) 1 related proinflammatory cytokines (e.g., TNF, IFN-γ) and Th17-associated cytokines (e.g., IL-17A, IL-21, and IL-23) are significantly increased, but IL-25 is markedly decreased in the inflamed mucosa of CD patients [5–7]. Traditional treatments, including 5-aminosalicylates, enteral nutrition, corticosteroids, and immunosuppressive agents, have unsatisfied clinical outcomes in some patients, and some may rely on corticosteroids or increase their risk of developing steroid-related adverse effects on the basis of a long-term treatment. Therefore, it is imperative that other therapeutic options are considered. In recent years, although several biologic drugs, best represented by anti-TNF mAb

(infliximab, IFX), have been developed in inducing remission in CD patients, their advent has revolutionized the disease treatment [8].

IFX is a monoclonal IgG1 antibody targeted against anti-tumor necrosis factor (TNF), which is composed of a human constant region IgG1 light chain that accounts for nearly 75% of the antibody and a mouse variable region (25%). The main mechanism of IFX is to neutralize the biological activity of TNF by binding with high affinity to the soluble and transmembrane forms of TNF and to inhibit binding of TNF with its receptors (p55/p75 subunits) [9], which came up with a new approach for the treatment of active CD with very inspiring results in the field of efficacy and safety [10]. Moreover, other mechanisms also play a role, ever not fully understood, in affecting barrier function, ADCC activation, lymphocyte apoptosis, mucosal angiogenesis, and regulating inflammatory cytokines in intestinal mucosa [11].

IFX is the first biologic agent approved for the treatment of CD. Previous work has shown that IFX is more effective than placebo in randomized controlled trials at inducing remission of active CD, maintaining remission of the disease, and promoting mucosal healing and fistula closures in CD patients [9, 12–14]. Moreover, evidences have also demonstrated that IFX therapy has striking response and remission rates, decreases CD-related hospitalization and the rate of surgery, improves the quality of life, and reduces the costs of care for CD patients without an increase in side reactions [15].

In this study we found that IFX was effective in inducing clinical remission and promoting intestinal mucosal healing in CD patients, particularly in those short course patients with luminal lesions, while the failure of IFX therapy was frequently observed in CD patients with long disease duration or stricturing and penetrating lesions. Furthermore, IFX could markedly suppress Th1/Th17-associated proinflammatory cytokine production and upregulate IL-25 expression in inflamed mucosa of CD patients.

2. Materials and Methods

2.1. Ethics Statement. These retrospective studies were approved by the Shanghai Tenth People's Hospital of Tongji University, Shanghai, China; Ruijin Hospital of Shanghai Jiaotong University, Shanghai, China; Xinhua Hospital of Shanghai Jiaotong University, Shanghai, China; Zhongshan Hospital of Fudan University, Shanghai, China; and East Hospital of Tongji University, Shanghai, China, from December 2009 to October 2013. The Institutional Review Board and Ethics Committee at each study center approved the protocol, and all patients provided written informed consent. All authors had access to the study data and had reviewed and approved the final paper. Eligible patients had an established diagnosis.

2.2. Patient and Sample Collection. One hundred and six patients with CD who had been treated with IFX were recruited in five university hospitals in Shanghai, China. The diagnosis of CD was based on conventional clinical features and radiological and endoscopic features, and finally confirmed by histological examination of ileal and colonic biopsies [16]. Cases were determined according to the Montreal classification system [17]. They were all naive to biological agent therapy and received IFX at a dose of 5 mg/kg body weight at weeks 0, 2, and 6 as an IFX induction regimen. It was administered by a two-hour intravenous infusion. The transfusion reaction was monitored at the same time. Laboratory parameters such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), hemoglobin (Hb), and albumin (Alb), as well as clinical data such as Crohn's disease activity index (CDAI, calculated as defined by Best et al. [18] which was filled by physicians), adverse reactions, and occurrence of complications, were monitored at time of registration and at each follow-up visit. Colonoscopy was performed prior to and 10 weeks after initial treatment of IFX, and mucosal ulceration status was assessed by the Simple Endoscopic Score for Crohn's Disease (SES-CD) score 0 to 3 of every five ileocolonic segments under ileocolonoscopy. Endoscopic remission was determined as a SES-CD score of 0 to 2 [19]. Intestinal biopsies were taken at sites of active inflammation adjacent to ulcerations for histology and analysis of the mRNA levels of Th1 related cytokines (TNF, IFN-γ), Th17 related cytokines (IL-17A, IL-21, IL-23p19, and RORC), IL-25, and IL-10, which were associated with CD pathogenesis by quantitative real-time polymerase chain reaction (PCR).

2.3. Definition of the Efficacy of IFX. The clinical efficacy of IFX in our study was evaluated at week 10 after initial administration, the point at which patients were followed up for sustaining treatment of IFX. Clinical remission was defined as a CDAI score of <150 points, and clinical response as a decrease in the CDAI score ≥ 70 points at the evaluation time point compared to the baseline index. The failure category included all the remaining patients, whose CDAI was not significantly changed or increased [16, 18, 20].

2.4. Evaluation of Safety. Any adverse events happening during the treatment were recorded, including infusion reactions or adverse events believed to be associated with IFX. Infusion reaction was defined as any adverse event occurring during an infusion or within 1 to 2 hours after the infusion like fever, chills, primarily chest pain, dyspnea, pruritus, and urticaria. Anaphylaxis might occur at any time during IFX infusion. Severe adverse events were defined as serious adverse events resulting in death, life threatening, requiring hospitalization, or persistent or significant disability.

2.5. Quantitative Real-Time PCR. Total RNA was extracted from the fresh-frozen biopsies using the RNeasy Kit (Invitrogen Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. Total RNA quantity and quality isolated from each sample were assessed using a NanoVue spectrophotometer (GE Healthcare, Piscataway, NJ, USA), with a 260/280 ratio of >1.8 and 28S/18S ratio of >1.4 for the majority of the samples. The cDNA was synthesized with SYBR PrimeScript RT reagent kit (TaKaRa, Dalian, China)

according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed in the ABI prism 7900 HT sequence detector (Applied Biosystems, Foster City, CA, USA) using SYBR green methodology. β-Actin was used as the endogenous reference gene. All primers were synthesized and purchased from Sheng Gong BioTech (Shanghai, China) and used according to standard methodologies. All PCR reactions were run in triplicate and performed with 40 cycles using the following conditions: 95°C for 1 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 30 sec. The relative target gene expression levels were calculated as a ratio relative to the β-actin reference mRNA. Quantitative real-time PCR analysis was carried out using the $2^{-\Delta\Delta Ct}$ method [21–23].

2.6. Statistical Analysis. Data are expressed as mean ± standard deviation or percentage. The baseline characteristics of the patients classified into clinical remission, clinical response, and failure groups were estimated by a simple descriptive analysis and the χ^2 test. Parameters including CDAI, CRP, ESR, Hb, Alb, SES-CD, and inflammatory cytokines were compared with Student's t-test for quantitative variables. Statistical analysis was performed using SPSS Statistics version 16.0 (SPSS, Chicago, IL, USA). A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Baseline Demographics. As shown in Table 1, a total of 106 CD patients (77 males and 29 females) were recruited in our study to receive IFX therapy with CDAI ranging from 153 to 519. The mean age of diagnosis was 27 years old (13–78 years old) and mean duration was 38.68 months (0.5–192 months) before starting treatment. Eighty-five (80.2%) patients were diagnosed from 17 to 40 years of age (A2); 66 (62.3%) of them had ileocolonic disease (L3, both ileal and colonic involvement) and no patients had isolated upper gastrointestinal disease (L4). Forty-nine (46.2%) had an initial nonstricturing and nonpenetrating behavior of diseases (B1). Perianal diseases were present at 32 (30.2%) patients before treatment, and 28 of them had perianal fistula. Only 19 (17.9%) of the patients were current smokers. Twelve patients (11.3%) had prior bowel resection for CD. There were 49 (46.2%) never receiving traditional therapy such as corticosteroids or immunosuppressive agents called "top-down strategy," while the so-called "step-up strategy" is based on the traditional therapeutic approach of CD, progressive intensified course of treatment, as the disease severity increases. Both the "top-down strategy" and the "step-up strategy" patients received combination of immunosuppressive agents (e.g., azathioprine), which was most commonly used in each referred center. For the fistulae patients routine antibiotics (metronidazole and/or ciprofloxacin) were also administrated.

3.2. Efficacy of IFX in the Treatment of CD. This study included 106 active CD patients with a median CDAI score of 223 (range from 153 to 519) prior to starting therapy. Significant decrease was observed in the mean values of CDAI when compared with data at baseline and after treatment (233.0 ± 69.1 versus 148.5 ± 80.2; $P < 0.05$), as well as the levels of serum CRP, ESR, Hb, Alb, and SES-CD ($P < 0.05$). Based on the change of CDAI, 61 (57.5%) participants achieved clinical remission with CDAI below 150, another 17 (16.0%) did not squeeze into "clinical remission," but they were up to the standard "clinical response" with a decrease of CDAI ≥ 70, but ≥150. The remaining 28 patients (26.4%) were unfortunately classified as failure to IFX with both CDAI ≥ 150 and a decrease of CDAI ≤ 70 or an increase of CDAI from the the baseline.

Table 2 shows the demographic database of three groups throughout the study period. Twenty-eight patients in failure group were composed of 18 (64.3%) "step-up strategy" and 10 (35.7%) "top-down strategy" patients. There were no apparent differences in age of diagnosis or sex, but patients in failure group had a significantly longer duration of disease compared with those in remission group ($P < 0.01$). A statistical difference was also observed in patients with three kinds of disease behaviors (B1, B2, and B3) according to the Montreal classification by chi-square test, showing that IFX appeared to be more effective in CD patients with B1 behavior than in those with B2 and B3 behaviors ($P < 0.01$). Moreover, no differences were observed among CD patients according to disease locations ($P > 0.05$).

Table 1: Characteristics of patients with CD in our study.

Gender (M/F)	77/29
Mean duration of disease before IFX in months	38.7 ± 42.9
Mean age of diagnosis in yrs	27.2 ± 9.7
A1 (≤16 yrs)	10 (9.4%)
A2 (17–40 yrs)	85 (80.2%)
A3 (>40 yrs)	11 (10.4%)
Location	
L1 (ileum only)	18 (17.0%)
L2 (colon only)	22 (20.8%)
L3 (ileocolonic)	66 (62.3%)
L4*	0
Behavior	
B1 (nonstricturing, nonpenetrating)	49 (46.2%)
B2 (stricturing)	29 (27.4%)
B3 (penetrating)	28 (26.4%)
$P^{\#}$	32 (30.2%)
Fistula	
Perianal	28 (26.4%)
Enterocutaneous	3 (4.8%)
Enterovaginal	3 (2.8%)
Intestinal	12 (11.3%)
Current smoker	19 (17.9%)
Previous CD-related abdominal surgery	12 (11.3%)
Step-up/top-down strategy	57/49

*A modifier that can be added to L1–L3 when concomitant upper gastrointestinal disease is present.
#Added to B1–B3 when concomitant perianal disease is present.

Table 2: Demographic databases of patients in clinical remission, clinical response, and failure after IFX induction therapy.

Variable	Remission group ($n = 61$)	Response group ($n = 17$)	Failure group ($n = 28$)	P value
Age of diagnosis, yrs	27.3 ± 8.5	25.6 ± 9.4	28.0 ± 12.4	0.72
A1 (≤16)	5	2	3	
A2 (17–40)	50	13	22	0.99
A3 (>40)	6	2	3	
Gender, male/female	46/15	10/7	21/7	0.38
Disease duration, mths	22.2 ± 23.2 (0.5–96)	44.6 ± 37.9 (3–147.6)	71.0 ± 58.2 (2–192)	<0.0001
Location				
L1, *n* (%)	11 (18.0)	4 (23.5)	3 (10.7)	
L2, *n* (%)	7 (11.5)	7 (41.2)	8 (28.6)	0.03
L3, *n* (%)	43 (70.5)	6 (35.3)	17 (60.7)	
Behavior				
B1, *n* (%)	38 (62.3)	5 (29.4)	6 (21.4)	
B2, *n* (%)	15 (24.6)	6 (35.3)	8 (28.6)	0.0007
B3, *n* (%)	8 (13.1)	6 (35.3)	14 (50.0)	
Current smokers, *n* (%)	8 (13.1)	4 (23.5)	7 (25)	0.32
Step-up strategy, *n* (%)	26 (42.6)	13 (76.4)	18 (64.3)	0.02
Top-down strategy, *n* (%)	35 (57.4)	4 (23.6)	10 (35.7)	

Figure 1 shows the changes of active indices of CD patients from three groups described above. All serum indices were significantly improved at week 10 after the commencement of treatment ($P < 0.05$). Serum indices such as CRP and ESR were markedly decreased in remission group than in clinical response and failure groups ($P < 0.05$). Likewise, serum Hb and Alb were remarkably increased in remission group than in clinical response and failure groups ($P < 0.01$). In contrast, no significant changes were observed in failure group during the study period.

3.3. Mucosal Healing after IFX Therapy. To evaluate intestinal mucosal healing after IFX therapy, all patients underwent endoscopy before and 10 weeks after IFX induction therapy. As shown in Figures 1 and 2, SES-CD was found to be significantly decreased 10 weeks after IFX therapy compared with that before therapy in all patients (13.6 ± 7.7 versus 7.75 ± 8.7; $P < 0.05$). Surprisingly, the mean values of SES-CD from CD patients in remission group were markedly decreased 10 weeks after TNF administration compared to those before IFX treatment (7.7 ± 7.0 versus 2.9 ± 5.3; $P < 0.01$). Of note, 28 patients (26.4%) got endoscopic remission, 20 patients (18.9%) were in deep remission (both SES-CD ≤ 2 and CDAI < 150), 7 (6.6%) were from clinical response group, and the only 1 left was from failure group.

3.4. Cytokines Profiles after IFX Therapy. Intestinal biopsies were taken from 53 patients including 25 in remission group, 15 in response group, and 13 in failure group. As shown in Table 3, the mRNA levels of Th1-associated cytokines (TNF, IFN-γ) and Th17-associated cytokines (IL-17A, IL-21, IL-23p19, and RORC) were found to be markedly decreased 10 weeks after IFX therapy as compared with those before IFX treatment in remission group ($P < 0.005$), but IL-25 was significantly increased ($P < 0.005$) as compared with that before IFX treatment. Likewise the active indices of CD, no significant changes were observed in the failure group before and 10 weeks after IFX treatment. IL-10 was found to be not statistically significant in all three groups. These data suggest that IFX induces mucosal healing through downregulating Th1/Th17-associated cytokines and promoting IL-25 production, thus balancing abnormal immune responses in intestinal mucosa.

3.5. Outcome of Fistula. Table 4 shows the outcomes of fistulae after IFX therapy which were estimated by endoscopic or magnetic resonance imaging (MRI). In our study, 46 CD patients were recorded to have fistulae including perianal, enterocutaneous, enterovaginal, and intestinal fistulae. The efficacy of IFX therapy in the CD patients with fistulae was observed to have a significant difference by chi-square test, showing that the percentage of fistulae closure/improvement in remission and response groups (75.0%) was significantly higher than that in failure group (11.1%) after IFX therapy ($P < 0.01$). It was worth noting that 17 patients (60.7%) with perianal fistula had good response to IFX therapy, showing closure/improvement of fistula. However, among 11 patients (39.3%) with IFX therapy failure, the majority of participants (63.6%) were from the failure group. Interestingly, poor efficacy was observed in CD patients with enterovaginal and intestinal fistulae, particularly from the failure group. Notably, three patients with enterocutaneous fistulae recovered entirely.

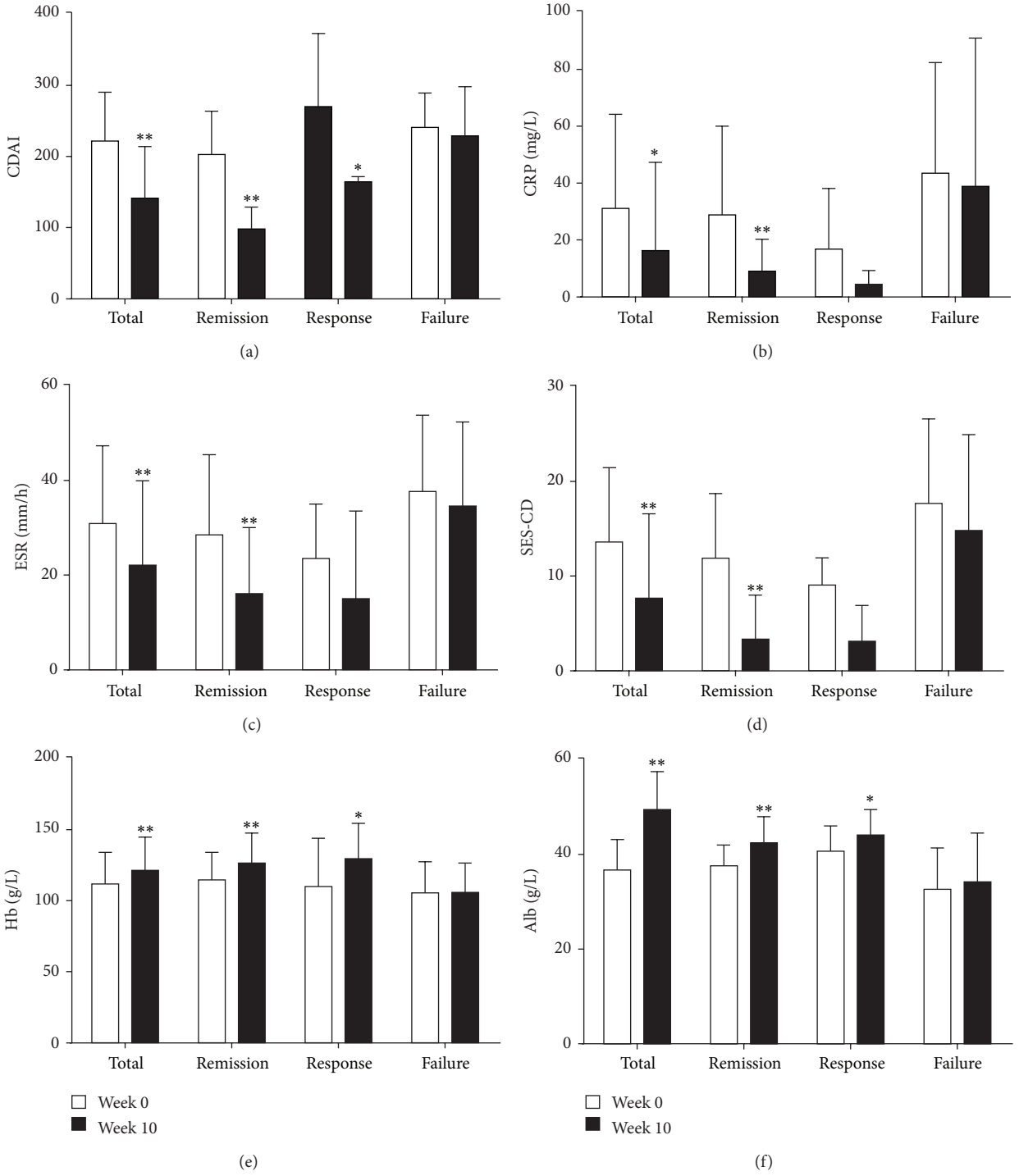

Figure 1: IFX therapy induces clinical remission and promotes intestinal mucosal healing in CD patients. The changes of CDAI (a), CRP (b), ESR (c), SES-CD (d), Hb (e), and Alb (f) were analyzed at weeks 0 and 10, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$ versus values before IFX therapy.

3.6. Adverse Effects. Twenty-four patients (22.6%) experienced adverse events, and none of them had serious adverse event and discontinued therapy during the induction phase (Table 5). The most common reactions were rash or fever belonging to infusion reactions, which were observed in 8 episodes and 7 cases at or after the injection site separately while another 2 patients developed mild elevations of ALT (85 and 72 U/L, resp., normal reference value: 0–40 U/L) after first IFX injection and showed no symptoms. Patients who suffered from adverse events were successfully treated with

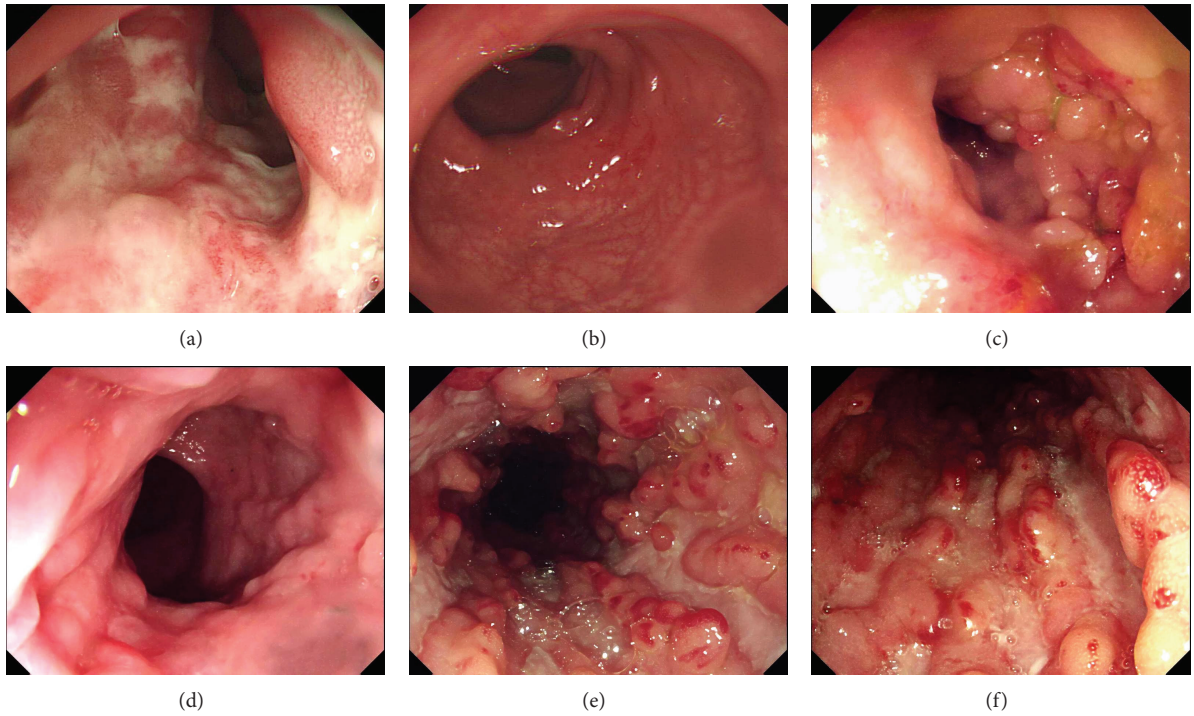

Figure 2: IFX therapy promotes intestinal mucosal healing in CD patients. Representative endoscopic photographs are demonstrated from a patient in remission group (a, b), a patient from response group (c, d), and a patient from failure group (e, f) before (a, c, and e) and 10 weeks after IFX treatment (b, d, and f).

conventional medical therapy and it had no effect on the subsequent progress. Taken together, IFX therapy was well tolerated in active CD patients even though it was a short-term follow-up.

4. Discussion

The treatment of CD is aimed at inducing remission, maintaining remission, preventing relapses, controlling complications, restoring intestinal physiological function, and postponing surgical intervention. IFX has been reported to bring about expectations with the main mechanism of blocking the role of TNF in the treatment of IBD. In the current study, we reported the efficacy of IFX in inducing clinical remission and promoting mucosal healing in active CD patients from multicenter of China. We demonstrated a response to IFX induction therapy in 73.6% and intestinal mucosa benefit achieving endoscopic remission under endoscopic examination in 26.8% CD patients. In addition, 57.5% of CD patients got CDAI scores < 150 at the last time point of follow-up, indicative of remission. Furthermore, proinflammatory cytokines (e.g., TNF, IFN-γ, IL-17A, IL-21, and IL-23) were markedly downregulated, while expression of IL-25 was highly upregulated in inflamed mucosa of CD patients after IFX treatment in remission group. These data indicate that short course CD patients with luminal lesions without serious complications preferentially achieve clinical remission or response.

Previous work has indicated that early treatment with IFX is usually associated with better response, reducing intestinal mucosal lesions, preventing disease progression, and avoiding complications by comparing three groups of CD patients with disease duration of 2 weeks, 2 months, and 7.5 years, respectively [12, 20, 24–27]. Study by Johnson et al. [28] also reported that early treatment could reduce tissue fibrosis by histological scoring of fibrosis at day 21 from the *Salmonella typhimurium* mouse model of intestinal fibrosis. In our study, we observed that patients with shorter duration of disease accounted for the most in clinical remission by IFX administration compared with parallel with longer disease period. Furthermore, our study also showed that the patients with luminal inflammatory disease had significantly better clinical benefit than those with stricturing or penetrating disease, consistent with the previously reported [29]. It likely suggests that patients with severe diseases such as stricture or penetration may need alternative therapeutic approaches, such as surgical or endoscopic interventions.

In our study, the indices reflecting CD activities (e.g., CRP, ESR, Hb, Alb, and SES-CD) were significantly improved in remission group after IFX therapy (Figure 1), consistent with previous reports [30, 31]. Hb and Alb as the parameters reflecting the nutritional status were apparently improved after IFX treatment. It is hypothesized that IFX therapy could restore intestinal epithelial barrier, leading to enhanced absorption of nutrients and iron. In addition, patients with perianal fistulae have better outcomes than intestinal fistulae. IFX is reported to block physiological binding of TNF and

Table 3: Changes of inflammatory cytokines in inflamed mucosa of CD patients before and 10 weeks after IFX treatment.

		Remission group ($n = 25$)	Response group ($n = 15$)	Failure group ($n = 13$)
IFN-γ	Week 0	112.6 ± 34.5	118.9 ± 32.1	117.2 ± 30.4
	Week 10	32.5 ± 11.2*	64.7 ± 21.6*	109.2 ± 32.6
TNF	Week 0	62.3 ± 12.5	63.8 ± 13.2	61.8 ± 15.8
	Week 10	11.5 ± 4.5*	40.1 ± 8.9+	58.9 ± 14.7
IL-10	Week 0	8.5 ± 3.6	9.8 ± 3.9	9.2 ± 3.5
	Week 10	12.8 ± 2.9	12.5 ± 4.1	8.9 ± 3.0
IL-17A	Week 0	35.8 ± 10.5	34.6 ± 9.8	36.5 ± 8.7
	Week 10	7.6 ± 3.2*	18.8 ± 5.2*	36.7 ± 10.1
IL-21	Week 0	21.3 ± 6.3	20.1 ± 7.2	21.4 ± 6.1
	Week 10	3.6 ± 1.2*	11.9 ± 3.5*	19.8 ± 5.9
IL-23p19	Week 0	24.5 ± 6.8	25.5 ± 5.4	26.9 ± 5.7
	Week 10	5.8 ± 2.1*	12.8 ± 4.3+	24.5 ± 6.6
IL-25	Week 0	8.2 ± 3.2	9.8 ± 3.6	7.9 ± 3.5
	Week 10	26.9 ± 8.3*	15.6 ± 4.8	8.8 ± 2.9
RORC	Week 0	26.1 ± 7.0	29.4 ± 8.8	25.8 ± 7.3
	Week 10	7.6 ± 3.5*	16.7 ± 6.7*	23.4 ± 5.7

* $P < 0.005$; + $P < 0.05$ versus values before initial therapy with IFX.

Table 4: Efficacy of IFX therapy on fistula of CD patients.

Variable	n	Remission group (failed/total)	Response group (failed/total)	Failure group (failed/total)
Perianal	28	3/15	1/5	7/8
Enterocutaneous	3	0/2	0/1	0/0
Enterovaginal	3	0/1	1/1	1/1
Intestinal[a]	12	0/1	2/2	8/9
Total[b]	46	3/19	4/9	16/18

[a]Two patients with intestinal fistula also had a perianal fistula.
[b]$P < 0.01$ by chi-square test.

Table 5: Adverse effects during IFX therapy in CD patients.

Description of the events	Number of patients (%)
Any adverse events	24 (22.6%)
Any serious adverse events	0
Infusion reactions	22 (20.8%)
Fever or chills	7 (6.6%)
Primarily chest pain	0
Dyspnea	0
Myalgia	4 (3.8%)
Pruritus/rash	8 (7.5%)
Nausea/vomiting	3 (2.8%)
Anaphylaxis	0
Seizures	0
Hypotension	0
Infections	0
Hepatotoxicity	2 (1.9%)
Lupus-like syndrome	0
Psoriasiform rash	0
Deaths	0

reduce tissue inflammation [32]. We suspect that perianal fistulae do not have more chances to intimately contact intestinal flora leading to local inflammatory response, while intestinal fistulae contact intestinal flora continuously, even if TNF is suppressed. Moreover, other proinflammatory mediators may be also sustained. For these patients with a failure of IFX therapy, the more frequencies of complex or aggressive disease phenotypes are present, and other therapeutic approaches (e.g., surgery, novel biological agents) may be warranted.

Our data have shown that IFX therapy could down-regulate Th1/Th17-associated proinflammatory cytokines and promote IL-25 production, consistent with previous work showing that serum level of IL-23 was significantly decreased in rheumatoid arthritis patients treated with IFX [33]. Even though IL-25 is structurally related with Th17 cytokine family, it is not only necessary for the induction of Th2-mediated immune response [34], but also required for the generation of innate type cells which may produce Th2-associated cytokines at the initiation of an adaptive Th2-mediated response [35]. Our recent work has proved that IL-25 markedly suppresses IBD $CD4^+$ T-cell activation to produce proinflammatory cytokines [31]. Taken together, the induction of clinical remission and promotion of intestinal mucosal healing in active CD patients by IFX may be owing to

downregulating the expression of proinflammatory cytokines (e.g., IL-17A, IL-21, and IL-23).

Previous works have shown that the CD patients who encountered failure or infusion reactions may be related to the formation of antibodies to IFX (ATI) [36, 37]. Furthermore, a direct relationship between the failure to IFX and the presence of ATIs has been confirmed in serum samples levels during IFX induction phase [38]. Therefore, it is necessary to monitor serum ATI concentrations, which can be used to predict response or infusion reactions. It is also helpful to adjust the dosage, particularly the trough level of IFX, for individual administration to improve efficacy.

Several mild side effects have been observed during IFX infusion, such as fever, chill, rash, pruritus, and dyspnea. No severe adverse events were seen in our study, which is different from earlier reports of infections, malignancy, and deaths [37, 39, 40]. The reactions are attributed to systemic immune response, susceptibility genetic background, or environment factors.

5. Conclusions

In summary, our experience presented here details the efficiency of IFX in the management of CD patients from multicentral study. Patients are more likely to achieve clinical benefit if they have luminal inflammatory disease and short disease duration. IFX downregulates Th1/Th17-associated proinflammatory cytokines and upregulates IL-25 expression in intestinal mucosa. We have also demonstrated a good safety profile with IFX, albeit during a short follow-up period. However, the effect on intestinal fistulae patients was far from satisfactory.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

L. Yu and X. Yang contributed equally to this work. L. Yu and X. Yang carried out study concept and design, statistical analysis, and paper writing and revision; L. Xia, J. Zhong, W. Ge, J. Wu, H. Liu, and F. Liu carried out acquisition of data and analysis and interpretation of data; Z. Liu carried out study concept and design, paper revision, funding provided, and administrative, technical, and material support.

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Research Article

Macadamia Oil Supplementation Attenuates Inflammation and Adipocyte Hypertrophy in Obese Mice

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Excess of saturated fatty acids in the diet has been associated with obesity, leading to systemic disruption of insulin signaling, glucose intolerance, and inflammation. Macadamia oil administration has been shown to improve lipid profile in humans. We evaluated the effect of macadamia oil supplementation on insulin sensitivity, inflammation, lipid profile, and adipocyte size in high-fat diet (HF) induced obesity in mice. C57BL/6 male mice (8 weeks) were divided into four groups: (a) control diet (CD), (b) HF, (c) CD supplemented with macadamia oil by gavage at 2 g/Kg of body weight, three times per week, for 12 weeks (CD + MO), and (d) HF diet supplemented with macadamia oil (HF + MO). CD and HF mice were supplemented with water. HF mice showed hypercholesterolemia and decreased insulin sensitivity as also previously shown. HF induced inflammation in adipose tissue and peritoneal macrophages, as well as adipocyte hypertrophy. Macadamia oil supplementation attenuated hypertrophy of adipocytes and inflammation in the adipose tissue and macrophages.

1. Introduction

The role of a diet with a higher content of unsaturated fatty acids, in place or concomitant to a diet with high content of lipids, has been appointed as an effective strategy to control metabolic disorders [1]. Monounsaturated fatty acids (MUFA) rich diet has been reported to decrease plasma total cholesterol and LDL-cholesterol and increase HDL-cholesterol levels [2–5]. Moreover, when saturated fatty acids are replaced by MUFA in the diet of obese women, levels of inflammatory markers decrease, including IL-6 and visfatin in serum [6]. Macadamia nut oil is rich in monounsaturated fatty acids, containing approximately 65% of oleic acid (C18:1) and 18% palmitoleic acid (C16:1) of the total content of fatty

acids [7]. Macadamia oil is the main source of palmitoleic acid in the human diet. Some studies have shown that diet rich in macadamia can improve the lipid profile [2, 8–10], but to date there is no studies on the effect of supplementation of macadamia oil on adipocyte hypertrophy and inflammation.

In 2008, Cao and colleagues [11] showed that mice deficient in lipid chaperones aP2 and mal1 present increased levels of palmitoleic acid in serum. Elevated levels of circulating palmitoleic acid restored sensitivity of insulin in liver and skeletal muscle, hepatosteatosis, and hyperglycemia, generated by high-fat diet. With this, the authors named this fatty acid as a lipokine, since palmitoleic acid has a hormonal-like effect [11].

The administration of high-fat diet in C57BL6 mice induces metabolic perturbations similar to those observed in humans. In fact, consumption of the high levels of saturated fatty acids is associated with overweight, visceral obesity, inflammation, dyslipidemia, and insulin resistance, in skeletal muscle, liver, and adipose tissue [12–17]. Saturated FFA promotes inflammation by interaction with toll-like receptor 4 (TLR4), activating NFκB, JNK, and AP-1 pathways [18, 19].

A low grade inflammation is established with increase in plasma levels of IL-6, IL-1β, prostaglandins, TNF-α, and leptin and decrease in the production and secretion of adiponectin, IL-10, and IL-4 [20, 21]. The increase in local inflammation is potentiated by the recruitment of macrophages to adipose tissue and polarization of M2 macrophages (macrophages type 2) to M1 macrophages (macrophages type 1) [16, 22, 23].

The aim of our study was to evaluate the effect of macadamia oil supplementation, rich in MUFA (palmitoleic and oleic acids), on adipose tissue and peritoneal macrophages inflammation in mice fed a balanced diet or high-fat diet rich in saturated fatty acids. We measured glucose uptake (2-6 deoxyglucose uptake) and mRNA content of proteins (GLUT-4; IRS-1) involved in insulin signaling in soleus muscle. The contents of IL-10, IL-6, TNF-α, and IL-1β in peritoneal macrophages and adipose tissue were also determined. The adipocyte size was also evaluated.

2. Materials and Methods

2.1. Animals. All experiments were performed according to protocols approved by the Animal Care and Use Committee of the Institute of Biomedical Sciences, University of São Paulo. C57BL/6 male mice (8 weeks old) were used in this study. Animals were housed with light-dark cycle of 12-12 h and temperature of 23 ± 2°C. Animals were divided into four groups: (a) control diet (CD), (b) high-fat diet (HFD), (c) control diet supplemented with macadamia nut oil (Vital Âtman, Uchoa, SP, Brazil) (CD + MO), and (d) high-fat diet supplemented with macadamia oil (HF + MO). Control groups were run concomitantly. The oil composition is shown in Table 1. During the first 4 weeks preceding the induction of obesity by HFD, all groups were *ad libitum* fed a control diet (76% carbohydrates, 9% fat, and 15% proteins). Similar protocol has been used in our previous studies [24, 25]. CD + MO and HF + MO were supplemented by oral gavage at 2 g per Kg of body weight, three times per week, during 12 weeks. This dosage of oil was chosen based on previous studies from our group using different oils with no signs of hepatic toxicity [24]. CD and HF diet received water at the same dose.

Table 1: Fatty acid composition of macadamia oil.

Fatty acid	%
C12:0 lauric acid	0.09
C14:0 myristic acid	0.82
C16:0 palmitic acid	8.45
C16:1n7 palmitoleic acid	19.11
C17:0 heptadecanoic acid	0.28
C16:2n4 9,12-hexadecadienoic acid	0.02
C16:3n4 6,9,12-hexadecatrienoic acid	0.06
C18:0 stearic acid	3.90
C18:1n9 oleic acid	56.35
C18:1n7 vaccenic acid	3.09
C18:2n6 linoleic acid (LA)	1.35
C18:3n3 linolenic acid (ALA)	0.12
C20:0 arachidic acid	2.79
C20:1n9 gondoic acid	2.18
C20:1n11 gadoleic acid	0.12
C22:0 behenic acid	0.75
C22:1n9 erucic acid	0.22
C22:5n3 eicosapentaenoic acid	0.30
SFA	16.08
MUFA	80.01
PUFA	1.83
PUFA n3	0.42
PUFA n6	1.35
n3/n6	0.31

SFA = saturated fatty acids, sum of C12:0, C14:0, C16:0, C17:0, C18:0, C20:0, and C22:0; MUFA = monounsaturated fatty acids, sum of C16:1, C18:1n7, C18:1n9, C20:1n9, C20:1n11, and C22:1n9; PUFA = polyunsaturated fatty acids, sum of C16:3n4, C18:2n6, C18:3n3, and C22:5n3; PUFA n3 = sum of C18:3n3 and C22:5n3; PUFA n6 = C18:2n6.

2.2. Serum Parameters Analysis. Serum triacylglycerol, total cholesterol, LDL-cholesterol, and HDL-cholesterol were determined by colorimetric assays (Labtest Diagnostics, Lagoa Santa, MG, Brazil). Serum glucose and insulin were measured using LABTEST colorimetric assay and radioimmunoassay (Millipore, Billerica, MA, USA), respectively, as described by Masi et al. (2012) [24]. The HOMA index was determined by calculating fasting serum insulin (μU/mL) × fasting plasma glucose (mmol L^{-1})/22.5. Leptin and adiponectin were measured using the protocol of the manufacturing R&D system.

2.3. GTT and ITT. Glucose tolerance test (GTT) and insulin tolerance test (ITT) were carried out in all groups after 6 h fasting at the end of the 10th and 11th weeks of treatment, respectively.

The methodologies used for GTT and ITT were similar to that described by Masi et al. (2012) [24].

2.4. Insulin Responsiveness in Incubated Soleus Muscle. Animals were euthanized on CO_2 chamber and soleus muscles rapidly and carefully isolated and weighed (8–10 mg). This protocol was described in [24, 25].

Table 2: Primer sequences of the genes studies for real-time PCR.

Primer name	Forward	Reverse
RPL-19	5-AGC CTG TGA CTG CCA TTC-3	5-ACC CTT CCT CTT CCC TAT GC-3
GLUT-4	5-CAT TCC CTG GTT CAT TGT GG-3	5-GAA GAC GTA AGG ACC CAT AGC-3
IRS-1	5-CTC AGT CCC AAC CAT AAC CAG-3	5-TCC AAA GGG CAC CGT ATT G-3
CPT-1	5-CCT CCG AAA AGC ACC AAA AC-3	5-GCT CCA GGG TTC AGA AAG TAC-3
PGC1-a	5-CAC CAA ACC CAC AGA AAA CAG-3	5-GGG TCA GAG GAA GAG ATA AAG TTG-3
Perilipin 5	5-CAT GAC TGA GGC TGA GCT AG-3	5-GAG TGT TCA TAG GCG AGA TGG-3

2.5. Haematoxylin and Eosin Staining. Adipose samples were fixed in formalin and paraffin embedded. Sections were prepared (5 μM) using Leica EG1150H Machine. Haematoxylin and Eosin (H&E) staining was conducted using Leica Autostainer XL and Leica CV5030. Sections were mounted using DPX media (Fisher Scientific, Ireland) and analyzed using Nikon 80i transmission light microscope.

2.6. Extraction of Fatty Acids from Gastrocnemius Muscle and Gas Chromatographic Analysis. Gastrocnemius muscle fragments (100 mg) were subjected to lipid extraction. For this, 0.5 mL chloroform/methanol (2 : 1; v/v) was added to 100 mg of gastrocnemius sample, well-vortexed and incubated at room temperature for 5 min. Additional volumes of 1.25 mL chloroform and 1.25 mL deionized H_2O were then added, and finally, following vigorous homogenization for 3 min, samples were centrifuged at 1200 g for 5 min, at room temperature to obtain two phases: aqueous phase in the top and organic phase in the bottom containing. The organic phase was collected, dried, and suspended in isopropanol. Triglyceride content was then determined in the homogenate. After that, for fatty acid composition determination, gastrocnemius lipid extracts were dried using atmospheric N_2 for evaporation of the solvent without fatty acid oxidation. The fractions of neutral and polar lipids were separated from these extracts by using a column chromatography. The polar (phospholipids) and neutral (triglycerides) fractions were methylated (for formation of methyl esters), using acetyl chloride and methanol. The methyl esters were analyzed in a gas chromatographer coupled to a flame ionizer detector (FID) (Varian GC 3900). Fatty acid composition was then determined by using standard mixtures of fatty acids with known retention times (Supelco, 37 Components).

For the analysis of fatty acids, a programmed chromatography was used with the characteristics described below. The reading was initiated at 170°C temperature for 1 minute and then a ramp of 2.5°C/min was employed to reach a final temperature of 220°C that was maintained for 5 min. The injector and detector were maintained at 250°C. We used the *CP wax 52 CB* column, with a 0.25 mm thickness, internal diameter of 0.25 mm, and 30 mm long, with hydrogen as the carrier gas.

2.7. Analysis of Inflammatory Parameters

2.7.1. Adipokines Content Measurements. Mice were euthanized on CO_2 chamber and retroperitoneal adipose tissue was rapidly collected. About 100 mg of retroperitoneal adipose tissue was used for the determination of TNF-α, IL-6, and IL-10 content. Adipose tissue was homogenized in RIPA buffer (0.625% Nonidet P-40, 0.625% sodium deoxycholate, 6.25 mM sodium phosphate, and 1 mM ethylenediaminetetraacetic acid at pH 7.4), containing 10 g/mL of a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Homogenates were centrifuged at 12.000 g for 10 min at 4°C, supernatant was collected, and protein concentration was determined using Bradford assay (Bio-Rad, Hercules, CA, USA). Bovine serum albumin was used as protein standard.

Ex Vivo Adipose Tissue Culture. Retroperitoneal adipose tissue explants (about 100 mg) were cultured in DMEM sterile medium (Gibco), containing 10% FBS, 2 mM glutamine, streptomycin, and penicillin for 24 h, at 37°C and 5% CO_2, humidified air environment. Thereafter, medium culture was collected and used for the determination of IL-1β and IL-10, using ELISA assays (DuoSet kits, R&D System).

2.7.2. Peritoneal Macrophage Isolation and Culture. Cytokine and nitric oxide (NO) production were evaluated in macrophages obtained by washing the peritoneal cavity with 6 mL RPMI culture medium (Gibco), containing 10% FBS and 4 mM glutamine. Macrophage-rich cultures (more than 90% of the cells were F4/80$^+$) were obtained by incubating peritoneal cells in 24-well polystyrene culture plates for 2 h at 37°C in a 5% CO_2, humidified air environment. Nonadherent cells were removed by washing with RPMI. Adherent cells were then incubated with 2.5 μg/mL of LPS (*E. coli*, serotype 0111:B4, Sigma Chemical Company, USA) for 24 h [26]. Medium was collected for determination of IL-6, IL-10, IL-1B and TNF-α by ELISA and nitrite content by Griess method [27].

2.8. Quantitative RT-PCR. Total RNA from the gastrocnemius muscle was extracted with Trizol reagent (Invitrogen Life Technologies, Grand Island, NY, USA), following the method described by Chomczynski and Sacchi [28]. Reverse transcription to cDNA was performed using the high-capacity cDNA kit (Applied Biosystems, Foster, CA, USA). Gene expression was evaluated by real-time PCR [29], using Rotor Gene (Qiagen) and SYBR Green (Invitrogen Life Technologies) as fluorescent dye. Primer sequences are shown in Table 2. Quantification of gene expression was carried out using the RPL-19 gene as internal control, as previously described [30].

Table 3: Effect of high fat diet, with or without supplementation of macadamia oil, on obesity characteristics.

	CD	CD + MO	HF	HF + MO
Initial body weight (g)	24.26 ± 5.02	24.77 ± 3.37	24.2 ± 3.42	24.4 ± 4.41
Final body weight (g)	26.2 ± 1.99	26.82 ± 3.32	34.49 ± 6.96$^{*\#}$	34.75 ± 4.96$^{*\#}$
Liver weight (g)	1.17 ± 0.14	1.10 ± 0.23	1.33 ± 0.52	1.24 ± 0.26
Mesenteric adipose tissue weight (g)	0.32 ± 0.14	0.28 ± 0.12	0.55 ± 0.28$^{*\#}$	0.52 ± 0.23$^{*\#}$
Epididymal adipose tissue weight (g)	0.63 ± 0.14	0.73 ± 0.34	1.45 ± 0.60$^{*\#}$	1.59 ± 0.70$^{*\#}$
Retroperitoneal adipose tissue weight (g)	0.21 ± 0.06	0.24 ± 0.12	0.53 ± 0.22$^{*\#}$	0.55 ± 0.17$^{*\#}$
Adiposity index (g)	1.16 ± 0.24	1.25 ± 0.53	2.53 ± 1.01$^{*\#}$	2.65 ± 1.01$^{*\#}$
Brown adipose tissue weight (g)	0.104 ± 0.06	0.116 ± 0.03	0155 ± 0.06	0.128 ± 0.02

Values represent the means ± S.D. of the data obtained from analysis of 15 animals per group. $^{*}P < 0.001$ versus CD; $^{\#}P < 0.001$ versus CD + MO.

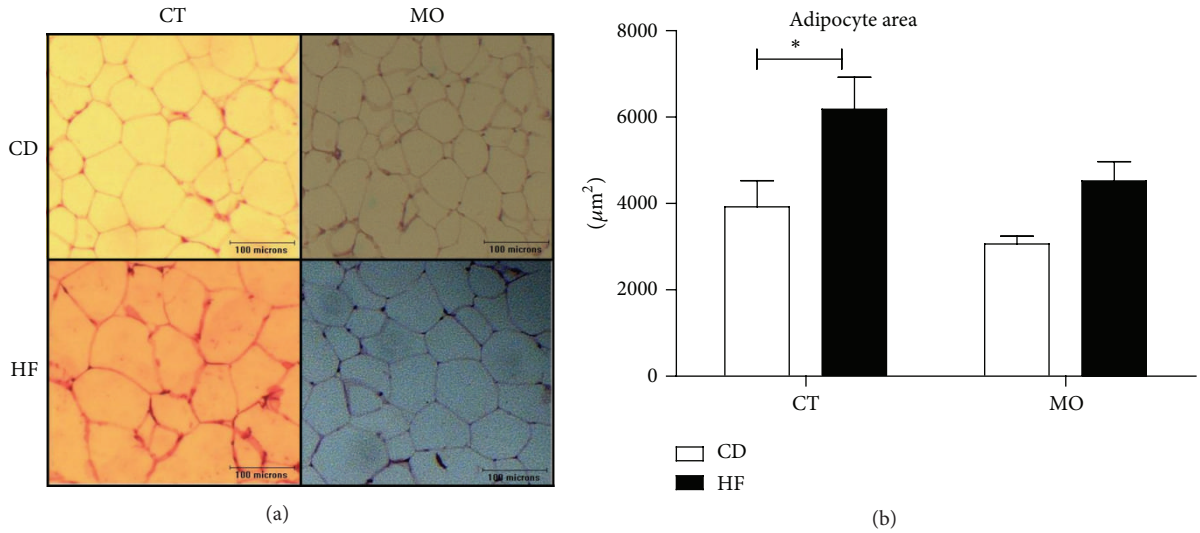

Figure 1: Effect of MO supplementation on adipose tissue histology. (a) Histological sections stained with H&E. (b) Area of adipocytes. CD = group of animals maintained on control diet; HF = group of animals fed high-fat diet; CD + MO = group of animals fed control diet supplemented with macadamia oil; HF + MO = group of animals fed high-fat diet supplemented with macadamia oil. The data are given as the means ± S.D. $^{*}P < 0.05$ ($n = 6$).

2.9. Statistical Analysis. Results are presented as mean ± S.D. All groups were compared by using two-way ANOVA followed by Bonferroni posttest. Significance level was set at $P < 0.05$.

3. Result

3.1. Characterization of the Experimental Model

3.1.1. Body Composition. Mice fed high-fat diet showed increased body weight gain, hypercholesterolemia, and insulin resistance. These modifications were similar to those observed in our previous studies [24, 25]. Animals fed the high-fat diet (HF and HF + MO) for eight weeks showed increased (by 2-fold) body weight gain and visceral adiposity index as compared with CD and CD + MO (Table 3). The weights of the liver and the brown adipose tissue depot were not altered with diet or supplementation (Table 3). Although the visceral adiposity index of mice fed high-fat diet (HF and HF + MO) was greater than in animals that received control diet (CD and CD + MO), the HF group had an increase (by 1,62-fold) of adipocytes size compared to the control diet (Figures 1(a) and 1(b)), with statistical difference not evidenced in HF + MO group. No difference was evidenced by diet or supplementation in LDL-c, NEFA, and glycerol (data not shown). Moreover, the basal glycemia and K_{itt} were increased in both groups treated with high-fat diet (data not shown). Homa-IR index was increased in the HF group (by 3-fold) as compared to the other groups including the HF + MO (Figure 2(a)). This result suggests a beneficial effect of macadamia oil supplementation on insulin responsiveness in the HF group.

The peripheral insulin resistance was confirmed by glucose uptake in incubated *soleus* muscle (data not shown), as also shown previously [24, 25]. In addition, both groups treated with high-fat diet showed decrease in GLUT-4 mRNA expression (Figure 2(b)). The PGC-1, IRS-1, CPT-1 and Perilipin 5 mRNA expression were not modulated in our treatment. The HF group showed an increase in triacylglycerol

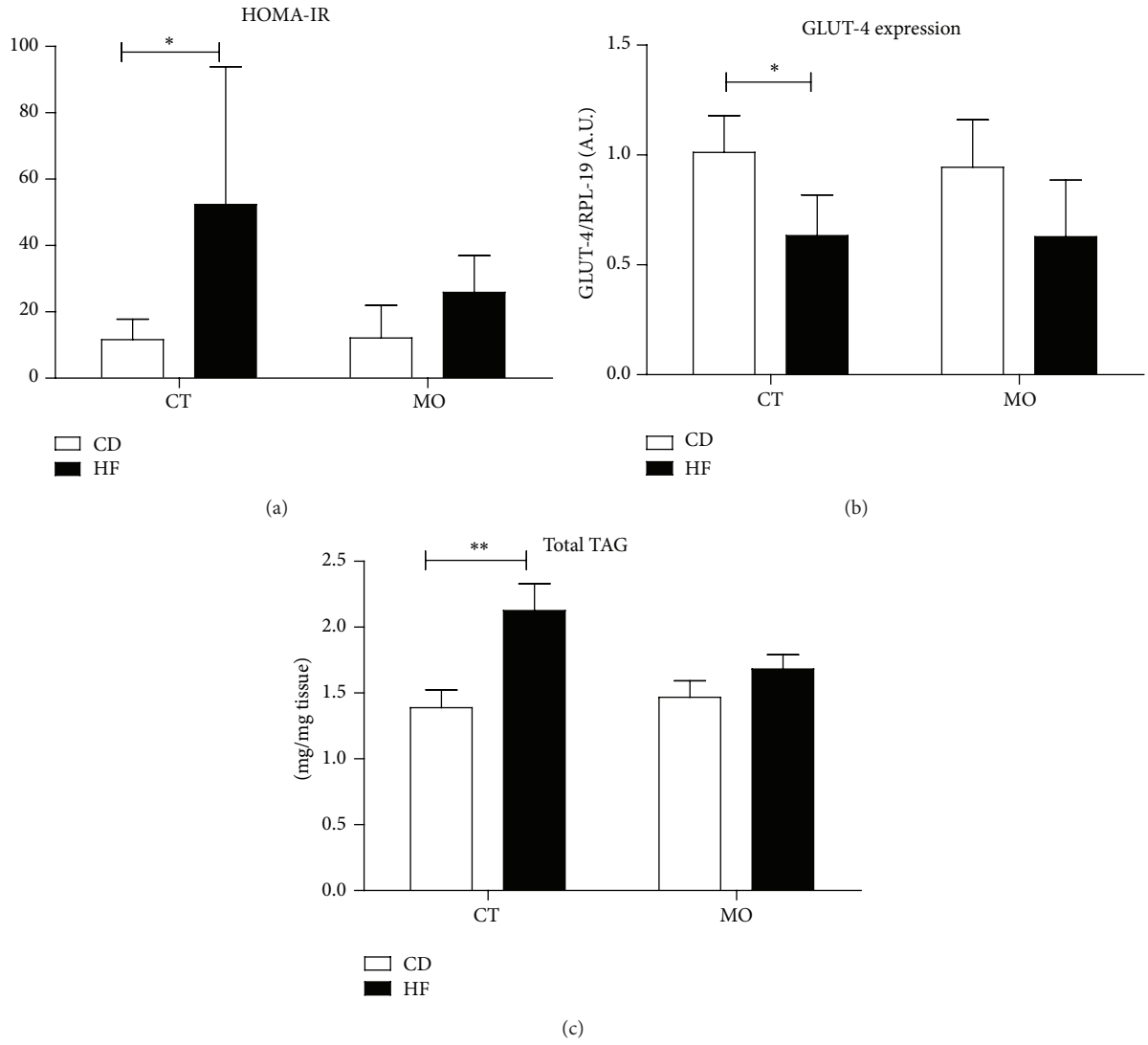

Figure 2: Insulin sensitivity and triacylglycerol content in skeletal muscle after 12 weeks. (a) HOMA-IR: homeostatic model assessment of insulin resistance; (b) GLUT-4 gene expression; (c) triacylglycerol content in gastrocnemius muscle. The data are given as the means ± S.D. In all experiments the animals were previously fasted for 6 hours. CD = group of animals maintained on control diet; HF = group of animals fed high-fat diet; CD + MO = group of animals fed control diet supplemented with macadamia oil; HF + MO = group of animals fed high-fat diet supplemented with macadamia oil. A.U. = arbitrary unit. $^{*}P < 0.05$; $^{**}P < 0.01$ ($n = 6$).

content in gastrocnemius muscle, but this effect was blunted in HF + MO (Figure 2(c)). The fatty acid composition in neutral or polar lipid fractions remains unchanged regardless of the diet given and MO supplementation (see Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/870634).

3.2. Macadamia Oil Supplementation Attenuates High-Fat Diet Induced Inflammation. The contents of the anti-inflammatory cytokine IL-10 were increased in the HF + MO group (approximately 4,09-fold) (Figure 3(a)), while IL-1b concentration in the medium of adipose tissue explants was increased in the HF group (Figure 3(b)).

When stimulated with LPS, macrophages from all groups showed increased IL-6 production (by 2.97-fold) (Figure 4(d)), whereas IL-10 and NO production were elevated in cells from the HF and CT + MO groups (2.39- and 4.08-fold compared to base line, resp.) (Figures 4(a) and 4(e)). No effect of LPS stimulation was observed on TNF-α production by macrophages from all groups (Figure 4(c)).

Moreover, macrophages from the HF group showed an increase (by 2,41-fold) of IL-1β production compared to unstimulated cells whereas the supplementation with MO abolished this elevation (Figure 4(d)). Similar results were found in NO production. MO attenuated nitrate production by LPS stimulation on macrophages from the HF group (Figure 4(a)). The production of IL-10 was decreased in the CT group compared to CT + MO and HF groups (by 1,88-fold) (Figure 4(e)). TNF-α and IL-6 production remained unchanged by diet or supplementation (Figures 4(c) and 4(d)).

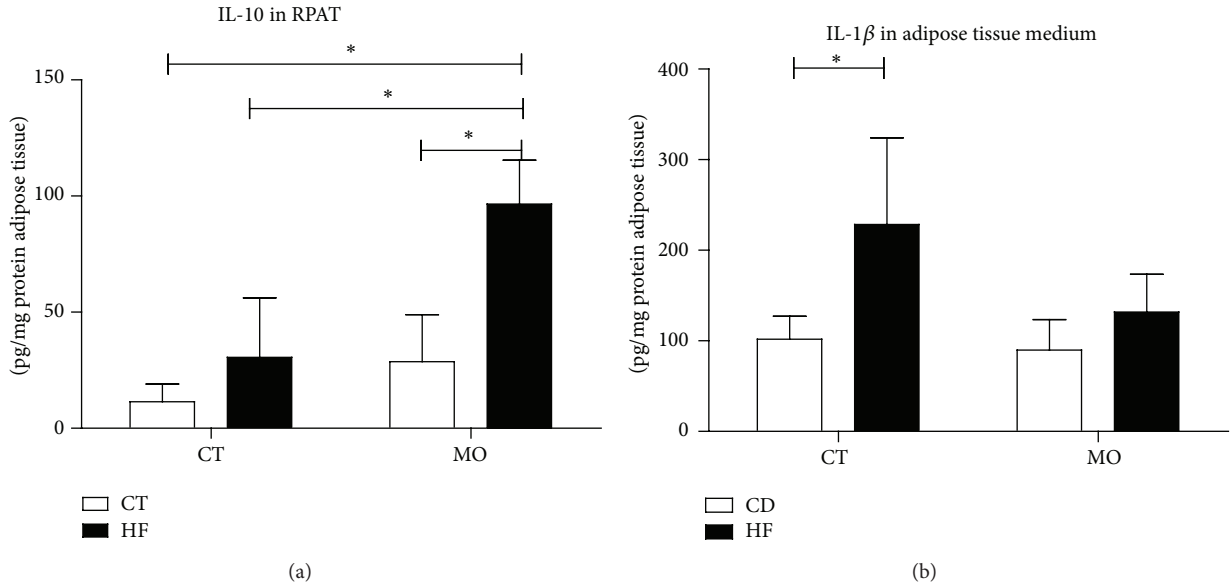

Figure 3: Inflammatory parameters in adipose tissue homogenate and adipose tissue explant incubation medium. IL10 content in adipose tissue homogenate (a) and IL1-β in the adipose tissue explant incubation medium, after 24 hours measured by ELISA (b). The animals received water or macadamia oil orally, 2 g/kg b.w., with or without association with a high-fat diet. CD = group of animals maintained on control diet; HF = group of animals fed high-fat diet; CD + MO = group of animals fed control diet supplemented with macadamia oil; HF + MO = group of animals fed high-fat diet supplemented with macadamia oil. The data are given as the means ± S.D. $^{*}P < 0.05$ (n = 5-6).

No significant difference was found in serum levels of adiponectin after 12 weeks of treatment (data not shown). As expected, leptin concentration was increased (by 5.69-fold) in the groups fed the high-fat diet (data not shown). The significant difference between the CD + MO and the CD + CT groups suggests that MO can enhance circulant leptin.

4. Discussion

We showed herein that twelve weeks of macadamia oil supplementation attenuate the increase in inflammation and adipocyte hypertrophy in mice fed a high-fat diet that exhibit signs of the metabolic syndrome.

High consumption of fat, sucrose, and industrialized foods in association with sedentary lifestyle is the main contributor to obesity and its related comorbidities, including dyslipidemias, insulin resistance, and cardiovascular diseases; evidence has been accumulated that low grade inflammation plays a key role in the obesity induced comorbidities [11, 31–37].

The increase in adipocytes size was attenuated by macadamia oil treatment. The increase in adipocyte diameter has been associated with disturbances in cellular homeostasis, such as insulin resistance, inflammation, and hypoxia [38]. The prevalence of large adipocytes increases leptin production and secretion, as observed in our study [39]. The increase in leptin is associated with an elevation in low grade inflammation. Leptin is known to stimulate proinflammatory cytokines production in lymphocytes [40–42], monocytes [43], and macrophages [44].

Mice fed the HFD for 8 weeks exhibited increased IL-10 content in retroperitoneal adipose tissue. This result may be associated with the increase in peroxisome proliferator activated receptor- (PPAR-) gamma activity. This nuclear receptor increased the number of small adipocytes and raised the IL-10 [45, 46]. The increase of IL-10 content in adipose tissue leads to macrophage polarization (type 2) that is important for remodeling and tissue repair [47, 48]. Moreover, the increase in IL-10 content in adipocytes is associated with increased insulin sensitivity in adipose tissue [49, 50].

IL-1β strongly induces the inflammatory response in innate immune cells [51], via JNK and NFκB pathway [52]. IL-1β is also a potent inductor of insulin resistance. This cytokine decreased insulin-stimulated glucose uptake via ERK activation [53]. Patients with high level of the circulating IL-1β are associated with greater risk on development of type 2 diabetes [54]. Adipose tissue and peritoneal macrophages are two sources of IL-1β, and macadamia oil supplementation was effective in decreasing the production of this cytokine in both. However, unexpectedly, the CDM showed an increased IL-1β production after LPS stimulation in peritoneal macrophages.

NO production is increased in LPS-stimulated macrophages being more pronounced in mice fed high-fat diet [24]. We demonstrated herein that the same pattern and the supplementation with macadamia oil prevented the production of NO by peritoneal macrophages from HF mice. Other bioactive compounds, such as epigallocatechin gallate and resveratrol [55], decrease NO production by macrophage inhibition through of MAP kinase, JNK, and NFκB signaling [56].

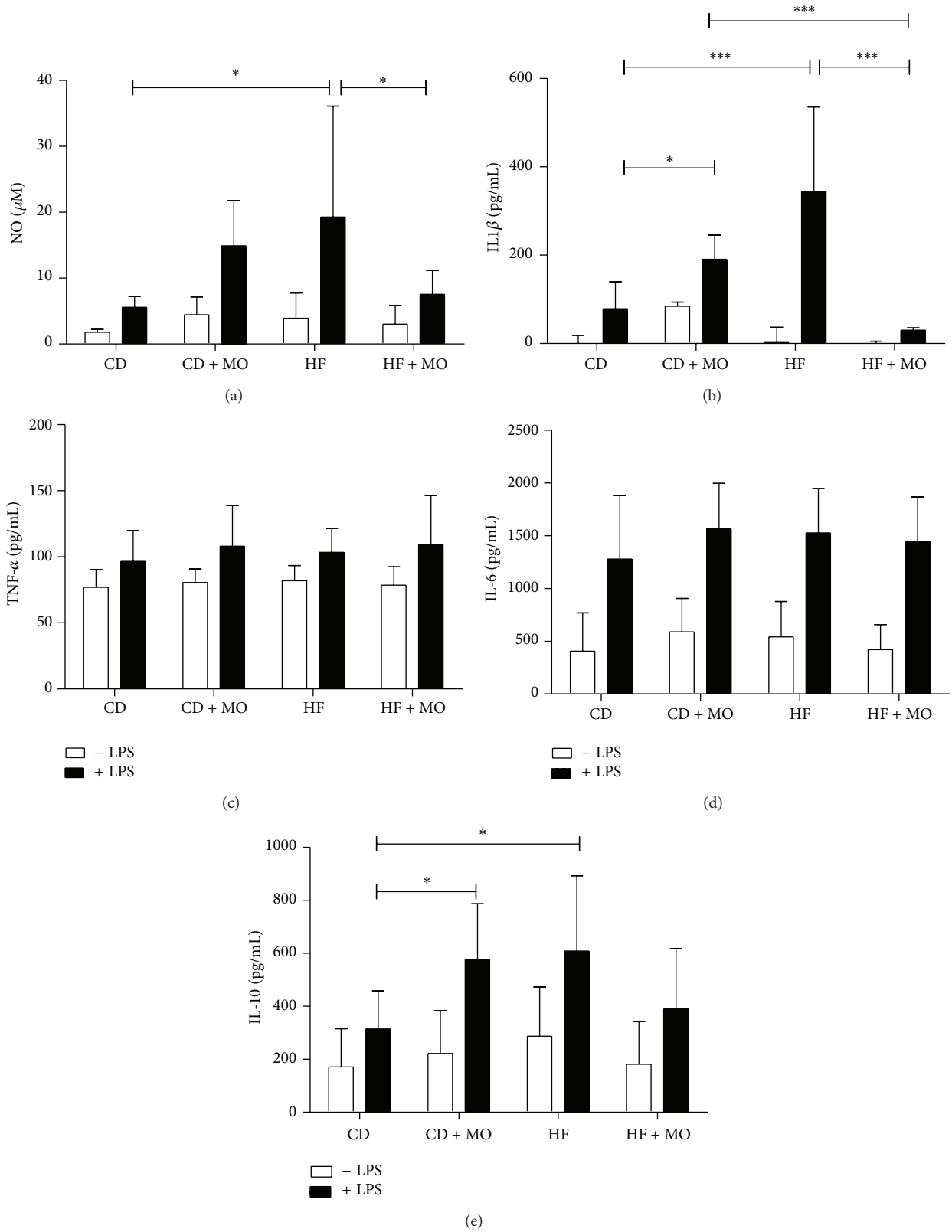

Figure 4: Nitric oxide and cytokine production by peritoneal macrophages. Peritoneal macrophages were collected and cultured for 24 h in the absence (white bars) or presence (black bars) of 2.5 μg/mL LPS. Nitric oxide (a), IL1-β (b), TNF-α (c), IL-6 (d), and IL-10 (e) were measured. CD = control diet; HF = high-fat diet; CD + MO = control diet + macadamia oil; HF + MO = high-fat diet + macadamia oil. The data are given as the means ± S.D. $^{*}P < 0.05$; $^{***}P < 0.001$ (n = 5-6).

In conclusion, macadamia oil supplementation attenuated inflammation and adipocyte hypertrophy in obese mice.

Conflict of Interests

The authors declare that they have no conflict of interests with the presented data.

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Research Article

Preventive Effects of Chitosan Coacervate Whey Protein on Body Composition and Immunometabolic Aspect in Obese Mice

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Functional foods containing bioactive compounds of whey may play an important role in prevention and treatment of obesity. The aim of this study was to investigate the prospects of the biotechnological process of coacervation of whey proteins (CWP) in chitosan and test its antiobesogenic potential. *Methods.* CWP (100 mg·kg·day) was administered in mice with diet-induced obesity for 8 weeks. The animals were divided into four groups: control normocaloric diet gavage with water (C) or coacervate (C-CWP), and high fat diet gavage with water (HF) or coacervate (HF-CWP). *Results.* HF-CWP reduced weight gain and serum lipid fractions and displayed reduced adiposity and insulin. Adiponectin was significantly higher in HF-CWP group when compared to the HF. The level of LPS in HF-W group was significantly higher when compared to HF-CWP. The IL-10 showed an inverse correlation between the levels of insulin and glucose in the mesenteric adipose tissue in the HF-CWP group. CWP promoted an increase in both phosphorylation AMPK and the amount of ATGL in the mesenteric adipose tissue in HF-CWP group. *Conclusion.* CWP was able to modulate effects, possibly due to its high biological value of proteins. We observed a protective effect against obesity and improved the inflammatory milieu of white adipose tissue.

1. Introduction

Over the past decades, the incidence of obesity in the population has increased severely, and it has become a public health challenge. Its etiology is multifactorial, encompassing environmental, dietary, physical inactivity, and genetic factors. Obesity is a complex disease associated with a high-calorie diet, which contributes to the development of several other chronic noncommunicable diseases [1]. Obesity is also associated with increased plasma endotoxin (lipopolysaccharide-LPS), saturated fatty acids [2, 3], and proinflammatory cytokines [3] all intricately involved in the development of comorbidities such as diabetes mellitus, hypertension, dyslipidemia, and metabolic syndrome.

The fat tissue is not merely an energy storage organ, as it plays crucial endocrine and immune roles. White adipose tissue (WAT) is an endocrine organ secreting pro- and anti-inflammatory adipokines such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), which are important inflammatory markers that stimulate the production of

several proteins and proinflammatory cytokines in different cell types, via nuclear factor κB activation (NF-κB) [4]. In addition, endotoxin and saturated fatty acids act in the same pathway of NF-κB activation. Many studies have shown that LPS (endotoxin) can activate these proteins in adipocytes, thereby increasing the gene expression of proinflammatory adipokines [5–7].

The main role of adipose lipolytic enzymes is to provide other tissues with FAs in case of energy demand. Triglyceride stored in the lipid droplet is first hydrolyzed by the adipose triglyceride lipase enzyme (ATGL), also known as desnutrin, releasing a diacylglycerol moiety and FA, which requires an abhydrolase domain containing 5 (ABHD-5) promoter to be activated. After hydrolysis by ATGL, diacylglycerols are then hydrolyzed sequentially by hormone-sensitive lipase (HSL) and monoglyceride lipase (MGL), producing nonesterified fatty acids (NEFAs) and glycerol [8]. Different lipases gain access to the lipid droplet when the proteins coating the vesicle (perilipins) are phosphorylated. Perilipin A normally prevents lipolysis of triglyceride by surrounding the lipid droplet, thus preventing the access of lipases.

Whey protein (WP) has been found to be an excellent prophylactic against obesity, because of the high biological value mediated by bioactive peptides. These act as antimicrobial agents, antihypertensive, and regulators of immune function, reducing body fat as well as a variety of related beneficial mechanisms for human health. They also have additional functions; for example, they have appetite suppressant effects [9], stimulate muscle protein synthesis, and regulate of body energy homeostasis [10]. There is plenty of evidence indicating the potential of the WP in anti-inflammatory and antioxidant effects of exercise [9, 11–13].

Chitosan complex coacervation with WP is composed of by-products from the processing of shrimp, crab (chitosan), and cheese, adding an environmental benefit to the product, as these by-products may be reused and not disposed of in landfill sites or released into rivers by producers [14]. In view of the above, together with the development of fractionation technique and whey protein preservation, employing the method may contribute to the recovery of this valuable nutrient and increase the expression of the functional properties [15]. Complex coacervation is defined as a colloidal separation forming two liquid phases. This process is essentially driven by attractive forces with opposite charges of a biopolymer. This phenomenon occurs by the formation of a system of balance between colloids and the diluted supernatant [16, 17]. Thus, the purpose of the present study was to investigate the prospect of a biotechnological process of complexation and separation of cheese whey proteins in chitosan and test their antiobesogenic potential through the modulation of inflammatory markers and lipolytic pathway present in obesity.

2. Methods

2.1. Coacervation and Characterization. In this study we used sweet cheese whey (SW 1108 bag 25 kg) with 1.5% fat marketed by Company Alibra-PR. Dissolving 10 g of the whey powder in 100 mL of distilled water. Chitosan was used for the coacervation medium molar mass with 75–85% degree of deacetylation and viscosity of 200–800 cps (Sigma-Aldrich 44887-7). A concentration of 0.75 mg/mL of Chitosan was used. Chitosan was dissolved in citric acid (208 mmol/L) and, after this step, added to the cheese whey in a proportion of 1 : 1 under stirring at room temperature for 1 h. The pH of the solution of chitosan and WP was adjusted to 6 with NaOH (250 mmol/L) and solubilized at room temperature (±25°C) with stirring. Solids coagulated with chitosan known as coacervate (CWP) were collected by centrifugation (1300 g).

In order to obtain on average 30% of the protein coacervate 3 L cheese whey was used. Thus, samples of CWP were obtained for their chemical analysis of total lipids, total protein, and lactose. Finally, for measurements of samples of mineral micronutrients, Ca, K, Mg, and P, of cheese whey, CWP, and Chitosan, 100 μg was subjected to an optical emission spectrometer for inductively coupled plasma (ICP OES, Perkin Elmer Optima 3000 DV, Norwalk, CT, USA). To determine the existing protein fractions in CWP the electrophoretic profile with reducing buffer containing 62.5 mM Tris-HCl, 20% glycerol, 2% sodium dodecyl sulfate(SDS) (10%), 5% β-mercaptoethanol, and bromophenol blue at pH 6.8 was performed.

2.2. Animals and Treatment. This study was approved by the Research Ethics Committee of the Universidade Federalde São Paulo, Escola Paulista de Medicina (UNIFESPEPM), as the search protocol number 0473/10. Experimental procedures are in accordance with Principles of Laboratory Animal Care formulated by the National Institutes of Health (National Institutes of Health Publication number 96-23, revised 1996).

Forty-nine male Swiss mice twelve-week-old from CEDEME (Centro de Desenvolvimento de Modelos Experimentais da Universidade Federal de São Paulo) were housed five in a cage in a standard experimental animal laboratory and kept under controlled conditions of light (12 h light-dark cycle with lights on at 6 am) and temperature (24 ± 1°C). All mice received water and food *ad libitum*. The animals were divided as follows: control diet plus tap water (C-W); control diet plus coacervate (C-CWP); high fat diet plus coacervate (HF-CWP); and high fat diet plus tap water (HF-W). All diets were prepared according to the recommendations of the American Institute of Nutrition [18] (Table 1). Coacervate (CWP) containing 100 mg·kg·day was given by gavage. The body weight gain was recorded twice a week.

2.3. Composition and Aspect of the Coacervate. Figure 1(a) shows the protein profile of the coacervate by the presence of the major proteins of larger fractions, namely, alpha-lactalbumin (α-La-14 kDa), beta-lactoglobulin (β-Lg—18 kDa monomer and 34 kDa dimer form), bovine serum albumin (BSA—66 kD), and lactoferrin (Lacf—86 kDa). The CWP has a flocculation aspect (Figure 1(b)) consisting of proteins and micronutrients such as calcium, potassium, magnesium, phosphorus, and sodium (Table 2).

Table 1: Composition of the control diet and diet enriched with saturated fatty acids according to AIN-93. Coacervate was resuspended in 300 μL of water.

Components (%)	Control diet (C)	High fat diet (HF)
Corn starch	72.07	40.87
Casein	14.0	14.0
Soybean oil	4.0	4.0
Lard	—	31.2
Cellulose	5.0	5.0
Vitamin mix	1.0	1.0
Mineral mix	3.5	3.5
L-cystine	0.18	0.18
Choline bitartrate	0.25	0.25
Butyl hydroquinone. g/kg	0.008	0.008
Energy. kcal/kg	3,802.8	5,362.8
Treatment by gavage		
Coacervate (CWP)	(C-CWP) 100 mg·kg·day	(HF-CWP) 100 mg·kg·day
Water (W)	(C-W) 300 μL·day	(HF-W) 300 μL·day
Fatty acids (%)		
Saturated (SFA)	17.12	34.13
Monounsaturated (MUFA)	25.63	39.14
Polyunsaturated (PUFA)	57.25	26.67
PUFA n3	4.32	6.37
PUFA n6	52.65	19.98

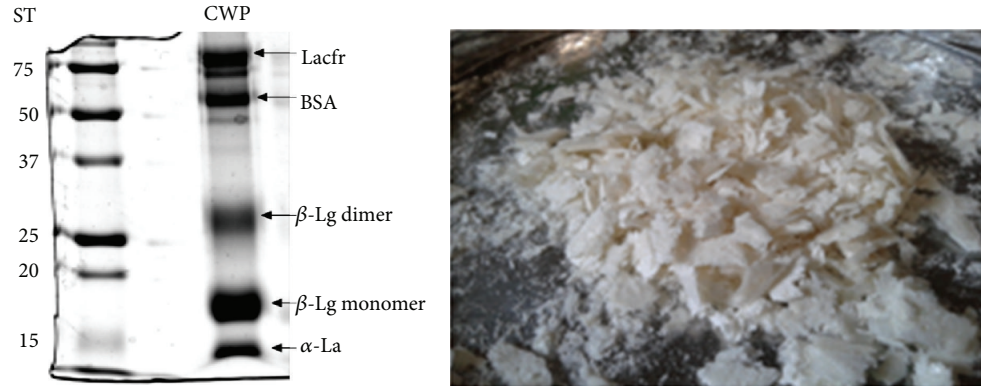


Figure 1: (a) Electrophoretic profile coacervate using 0.75 mg/ml of chitosan. ST: standard of different molecular weights, α-La: alpha-lactalbumin (14 kDa), β-Lg: beta-lactoglobulin monomer (18 kDa) and dimer (34 kDa), BSA: bovine albumin (66 kDa), and Lacfr: lactoferrin (86 kDa). (b) Aspect of CWP proteins in Chitosan, freeze-dried.

2.4. Fatty Acids Composition of Diets. For total lipid extraction, diet samples were homogenized in chloroform and methanol 2 : 1 (v/v), mixed, and incubated at room temperature for 5 min. Then, additional volumes of 1.25 mL chloroform and 1.25 mL deionized H_2O were added, and finally, following being vigorously homogenized for 3 min, samples were centrifuged at 1000 rpm for 5 min at room temperature. The chloroform layer was dried under N_2, and the total extract was converted into methyl esters and was analyzed in gas chromatography (GC), coupled with a flame ionizer detector (FID), (Varian GC 3900) and fatty acid profile was determined by calculating the retention time, using a pattern of fatty acids with known retention time (Supelco, 37 Components). The addition was initiated at a temperature of 170°C maintained for 1 minute and then a ramp of 2.5°C/min to a final temperature of 240°C, which was maintained for 5 minutes. The injector and detector were maintained at 250°C and 260°C, respectively. We used a column CP wax 52CB, with a thickness of 0.25 mm, internal diameter of 0.25 μm, and length of 30 m,with hydrogen as the carrier gas at a linear velocity of 22 cm s^{-1}.

2.5. Oral Glucose Tolerance Test (OGTT). After 12 hours of fasting, blood was collected from the tail vein to assess

Table 2: Partial composition of micronutrients by ICP and chemical macronutrients of CWP, WP, and Chitosan.

	Nutritional composition g/100 g		
	CWP	WP	Chitosan
Proteins	30	35	—
Lactose	1.7	40	—
Lipids	0.4	1.5	—
Ca	0.47	0.55	0.07
K	0.37	1.69	0.010
Mg	0.06	0.11	<0.001
P	0.46	0.53	0.003
Na	0.81	1.32	0.26

basal glucose concentration. Then, a glucose (Merck) solution (1.4 g/kg) was administrated by gavage. Blood samples were collected after 15, 30, 45, 60, and 120 minutes to measure glucose concentration using a glucose analyzer (AccuCheck Roche).

2.6. Experimental Procedures. At the end of the experimental period, animals were fasted for 12 h overnight prior to being sacrificed by decapitation. Trunk blood was collected and immediately centrifuged (1125 g/15 min at 4°C). Serum was separated and stored at −80°C for later biochemical and hormonal determination. The adipose tissue depots, retroperitoneal (RET), mesenteric (MES), and epididymal (EPI), were dissected, weighed, immediately frozen in liquid nitrogen, and stored at −80°C.

2.7. Biochemical and Hormonal Serum Analyses. Serum concentrations of glucose, total cholesterol, triglycerides, and HDL-c were measured by an enzymatic colorimetric method using commercial kits (Labtest, Brazil). Concentrations of insulin and adiponectin were measured using specific enzyme-linked immunosorbent assay (ELISA) kits (Milipore and R&D Systems). LPS was determined using a commercial kit (Lonza).

2.8. Mesenteric Adipose Tissue TNF-α, IL-6, and IL-10 Protein Level Determined by ELISA. Following euthanasia, mesenteric adipose tissue was removed, homogenized into a specific total protein extraction buffer [1% Triton X-100, 100 mm Tris-HCl (pH 7.4), 100 mm sodium pyrophosphate, 100 mm sodium fluoride, 10 mm EDTA, 10 mm sodium orthovanadate, 2.0 mm phenylmethylsulfonyl fluoride, and 0.1 mg aprotinin/mL], and centrifuged at 12,000 g for 30 min at 4°C. The supernatant was saved, and the protein concentration was determined using the BCA assay (Bio-Rad, Hercules, California) with bovine serum albumin (BSA) as a reference. Quantitative assessment of TNF-α, IL-6, and IL-10 proteins was carried out by ELISA (DuoSet ELISA, R&D Systems, Minneapolis, MN) following the recommendations of the manufacturer. All samples were run as duplicates and the mean value was reported.

2.9. Protein Analysis by Western Blotting. After euthanasia, the mesenteric adipose tissue was dissected and homogenized in 1.0 mL of solubilization buffer at 4°C [1% Triton X-100, 100 mm Tris-HCl (pH 7.4), 100 mm sodium pyrophosphate, 100 mm sodium fluoride, 10 mm EDTA, 10 mm sodium orthovanadate, 2.0 mm phenylmethylsulfonyl fluoride, and 0.1 mg aprotinin/mL]. Insoluble material was removed by centrifugation for 30 min at 9000 g in a 70 Ti rotor (Beckman, Fullerton, CA, USA) at 4°C. The protein concentration of the supernatants was determined using the BCA assay (Bio-Rad, Hercules, CA, USA). Proteins were denatured by boiling (5 min) in a Laemmli sample buffer containing 100 mM DTT and were run on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis in a Bio-Rad miniature slab gel apparatus.

The proteins were electrotransferred from gels to nitrocellulose membranes for ~1.30 h/4 gels at 15 V (constant) in a Bio-Rad semidry transfer apparatus. Nonspecific protein binding to the nitrocellulose was reduced by preincubation for 2 h at 22°C in blocking buffer (1% BSA, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20). The nitrocellulose membranes were incubated overnight at 4°C with antibodies against hormone-sensitive lipase (HSL), adipose triglyceride lipase (ATGL), abhydrolase domain containing protein 5 (ABHD-5), perilipin A, phospho 5′ AMP-activated protein kinase (p-AMPKα 1 e 2 - Thr 172), and alpha-tubulin obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) diluted 1 : 1000 with blocking buffer supplemented with 1% BSA and then washed for 30 min in blocking buffer without BSA. The blots were subsequently incubated with peroxidase-conjugated secondary antibody for 1 h at 22°C. To evaluate protein loading, the membranes were stripped and reblotted with an anti-alpha-tubulin antibody as appropriate. Specific bands were detected by chemiluminescence, and visualization/capture was performed by UVITEC gel-documentation system. Band intensities were quantified by optical densitometry of developed autoradiographs (Scion Image software, Scion Corporation, Frederick, MD, USA).

2.10. Statistical Analyses. All results are presented as mean ± standard error of the mean (SEM). Statistical significances were assessed using two-way analysis of variance (ANOVA) followed by Tukey's *post hoc* analysis to identify significant

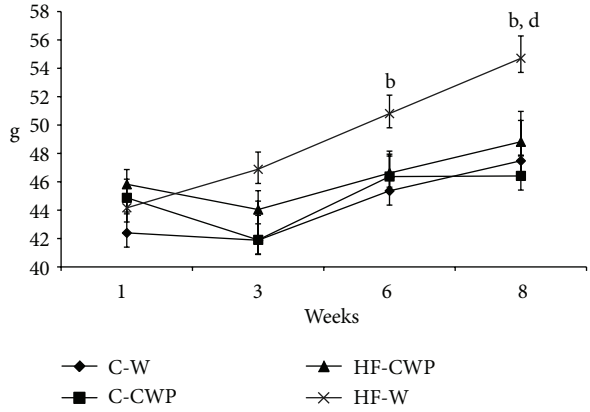


Figure 2: Evolution of the average gain in body mass (g) of mice for eight weeks of treatment with high fat diet (HF) or control normocaloric (C) associated with gavage of coacervate (CWP) or water (W). Data submitted with an average ± EPM. (b) C-W versus HF-W and (d) HF-CWP versus HF-W. ($P < 0.05$).

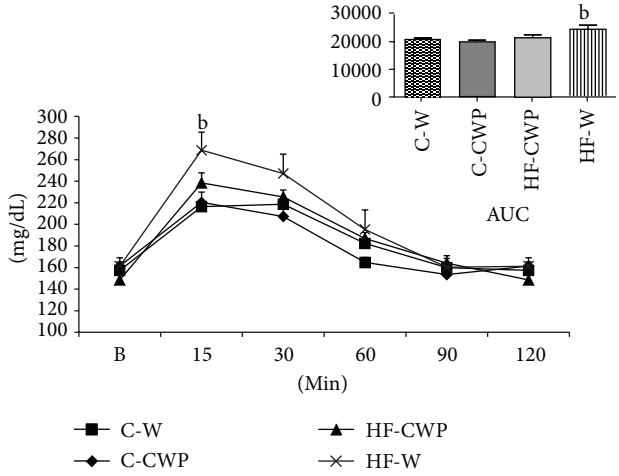


Figure 3: OGTT and AUC (area under the curve) after eight weeks of treatment with high fat diet (HF) or normocaloric control (C) associated with gavage of coacervate (CWP) or water (W). Glycemia in time zero (basal-B), 15, 30, 60, 90, and 120 minutes after gavage of 0.2 g/100 g body weight of glucose. Data submitted with an average ± EPM. (b) C-W versus HF-W. ($P < 0.05$).

differences among the groups. *Pearson's* correlation was used to assess the associations between the analyzed variables. Differences were considered significant for ($P \leq 0.05$) with the StatsDirect software.

3. Results

3.1. Body and Tissue Weights. After six weeks of treatment, the hyperlipidic diet promoted an increase in the body weight when compared to the control (C-W versus HF-W). On the other hand, HF-CWP showed a lower body weight when compared to HF-W (Figure 2). The hyperlipid diet increased the relative mass of epididymal and mesenteric depot and adiposity (Σ of epididymal, retroperitoneal, and mesenteric relative weight) when compared to control group (C-W versus HF-W), while the association with coacervate reduced these parameters (HF-W versus HF-CWP). The retroperitoneal depot was increased in the HF-W group when compared to C-W one (Table 3).

3.2. Levels of Serum Lipids, Insulin, Adiponectin, Lipopolysaccharides, and OGTT. The hyperlipid diet increased the triacylglycerol (TAG) and VLDL when compared to control group (C-W versus HF-W), while the association with coacervate reduced these parameters (HF-W versus HF-CWP). Insulin level and HOMA index were increased in the animals fed with hyperlipidic diet (C-W versus HF-W). When associated with coacervate, the hyperlipid diet promoted an increase in the adiponectin and a decrease in LPS concentrations (HF-W versus HF-CWP) (Table 4).

The oral glucose tolerance test showed that the hyperlipidic diet promoted an increase at 15 minutes when compared to control (HF-W versus C-W). The AUC (area under the curve) analysis increased HF-W compared with C-W (Figure 3).

3.3. Concentration of IL-6, IL-10, and TNF-Alpha in the Mesenteric Adipose Tissue. There was a significant decrease in IL-10 concentrations in the animal fed with high fat diet when compared to animals fed the control diet (HF-W versus C-W). The concentration of IL-6 in C-CWP group was lower when compared to C-W group. The IL-10/TNF-α ratio in mesenteric tissue showed no significant differences (Table 5).

3.4. Expression of Proteins Involved in Lipolysis Pathway. Figures 4(a), 4(b), 4(c), 4(d), and 4(e) show the data of protein expression of HSL, ATGL, Perilipin A, and ABHD-5 and AMPK activity, respectively, in mesenteric adipose tissue.

HSL protein expressions were reduced in C-CWP and HF-W when compared to the C-W group (Figure 4(a)). There was an increase in ATGL in HF-CWP group when compared to HF-W group (Figure 4(b)). Perilipin A was significantly higher in the HF-W group when compared to the C-W and HF-CWP groups (Figure 4(c)). There was a significant decrease in the protein expression of ABDH-5 in C-CWP when compared to C-W and HF-CWP groups (Figure 4(d)). The phosphorylation of AMPK (Figure 4(e)) was higher in HF-CWP compared to HF-W group.

3.5. Correlations. A positive correlation between AUC and TAG ($r = 0.86$ $P < 0.05$) was found in the HF-W group (Figure 5(a)). Figure 5(b) shows a positive correlation between insulin levels and STA in HF-W ($r = 0.96$ $P = 0.006$) group. Figures 5(c) and 5(d) show an inverse correlation between insulin ($r = -0.85$ $P = 0.02$) (Figure 5(c)) and glucose ($r = -0.88$ $P = 0.01$) levels with IL-10 in the mesenteric adipose tissue in HF-CWP group (Figure 5(d)).

4. Discussion

Numerous procedures for isolation and recovery of WP have been investigated and reported [19]. The functional,

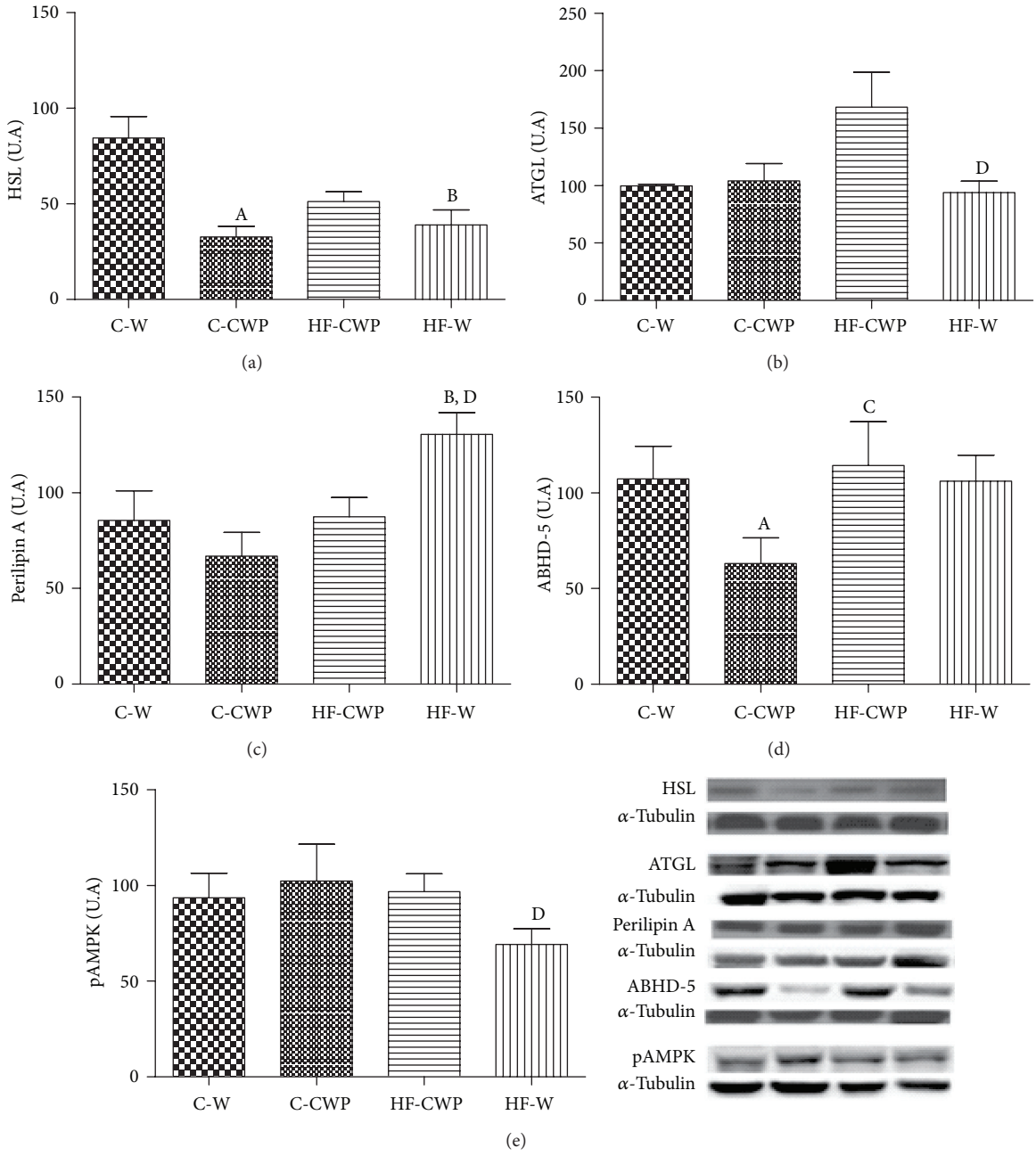
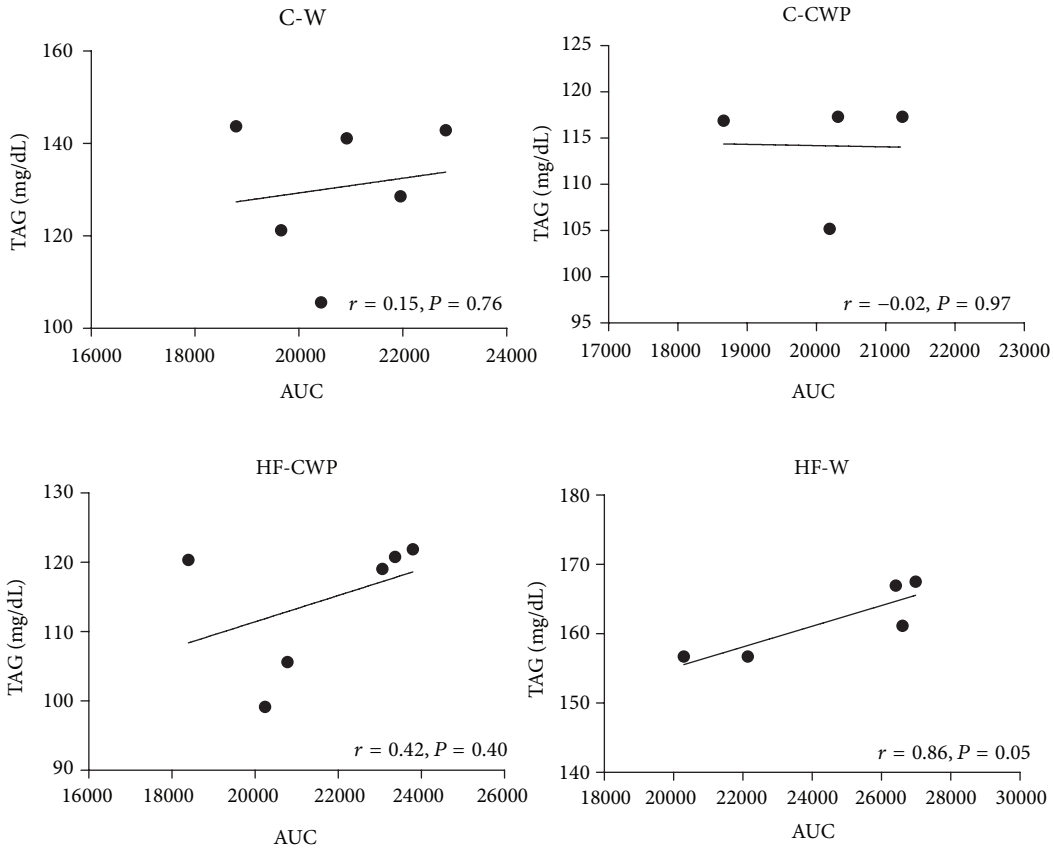


Figure 4: Protein expression of HSL (a); ATGL (b); perilipin A (c); ABHD-5 (d); and pAMPK (e) in the mesenteric adipose tissue. The data are expressed in arbitrary units (A.U). Data submitted with an average ± EPM. (A) C-W versus C-CWP; (B) C-W versus HF-W; (C) C-CWP versus HF-CWP; and (D) HF-CWP versus HF-W. ($P < 0.05$).

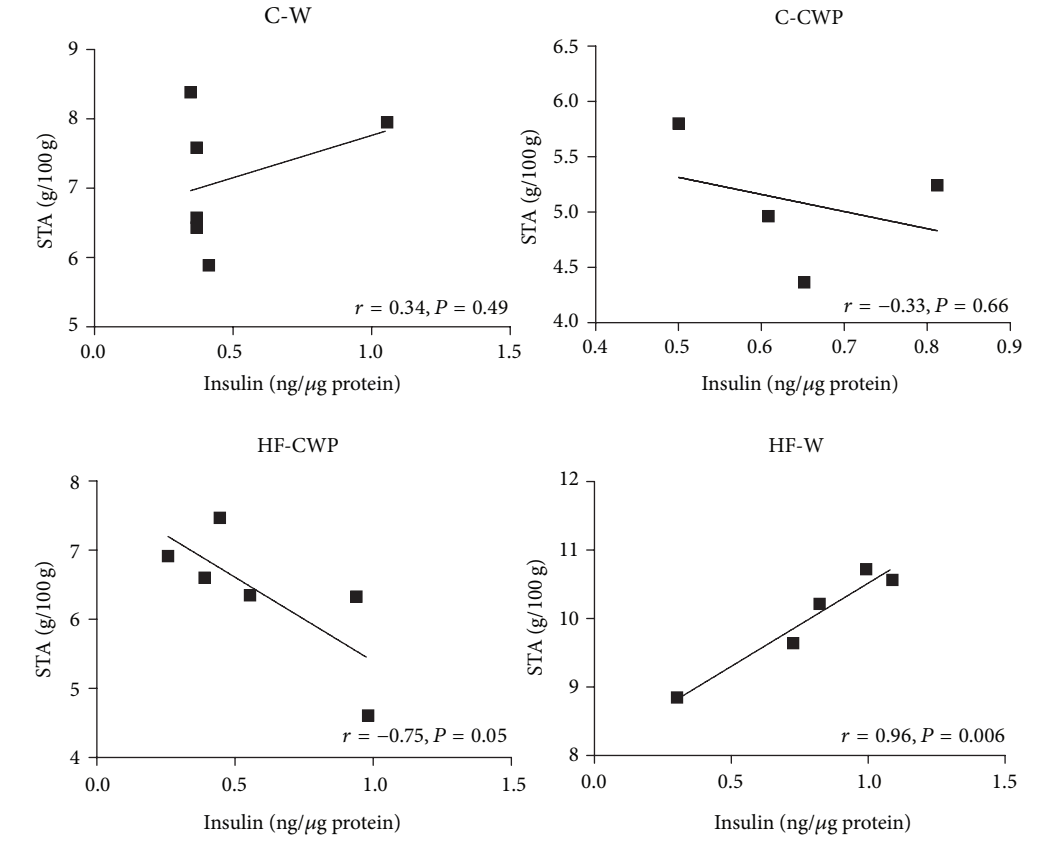
Table 3: Total mass (g), delta on the body mass gain (g/100 g body weight), and relative mass of tissue (RMT) (g/100 g body weight) of mice treated with high fat diet (HF) or control normocaloric (C) associated with gavage of coacervate (CWP) or water (W).

	C-W ($n = 10$)	C-CWP ($n = 10$)	HF-CWP ($n = 19$)	HF-W ($n = 19$)
RTM (g/100 g)				
EPI	4.04 ± 0.17	3.52 ± 0.21	4.06 ± 0.28	4.93 ± 0.44[b,d]
MES	1.80 ± 0.24	1.29 ± 0.21	1.72 ± 0.1	2.6 ± 0.36[b,d]
RET	1.12 ± 0.10	0.82 ± 0.04	1.35 ± 0.12	1.48 ± 0.18[b]
Σ adipose tissue	6.97 ± 0.38	5.64 ± 0.41	7.14 ± 0.46	9.02 ± 0.55[b,d]

EPI: epididymal; MES: mesenteric; RET: retroperitoneal. Data submitted with an average ± EPM. [b]C-W versus HF-W and [d]HF-CWP versus HF-W. ($P < 0.05$).



(a)



(b)

Figure 5: Continued.

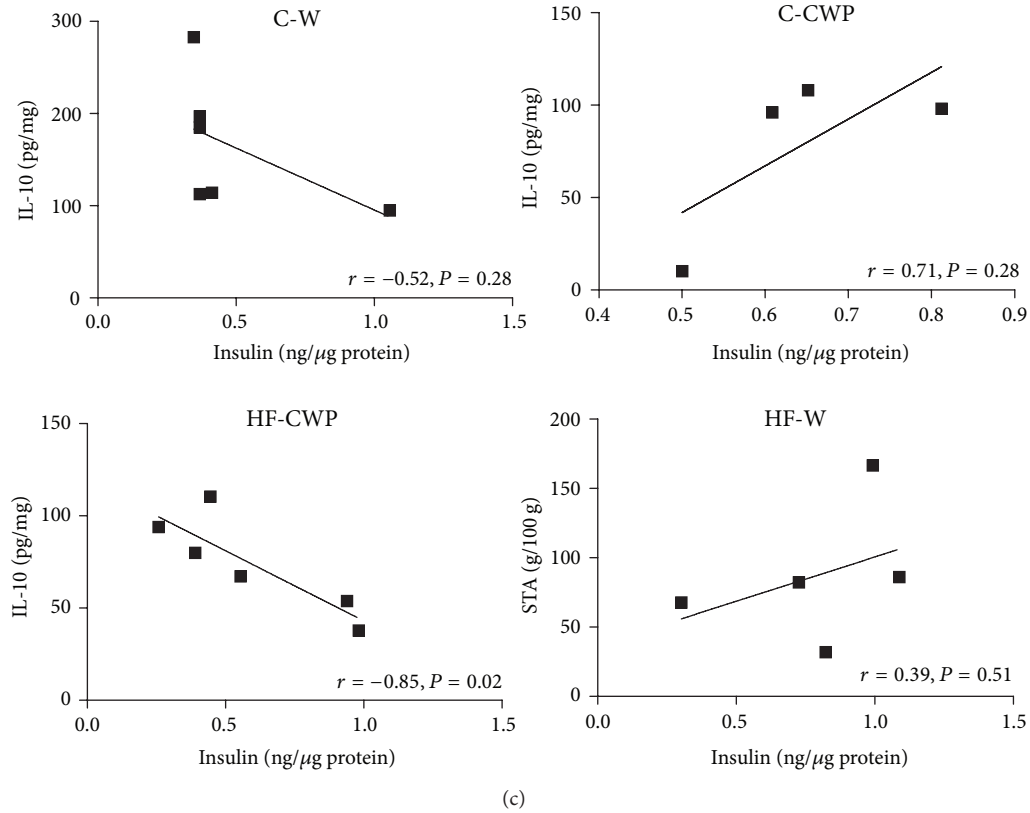


Figure 5: Correlation between different experimental groups: (a) TAG and AUC in different experimental groups. (b) Insulin and STA (sum of adipose tissues MES, RET, and EPI). (c) Insulin and IL-10 of serum and mesenteric adipose tissue, respectively, and (d) glucose and IL-10 of serum and mesenteric adipose tissue, respectively.

Table 4: Serum of triacylglycerol, total cholesterol, glucose, insulin, and adiponectin of mice treated with high fat diet (HF) or control normocaloric (C) associated with gavage of coacervate (CWP) or water (W).

Serum measurements	C-W ($n = 10$)	C-CWP ($n = 11$)	HF-CWP ($n = 17$)	HF-W ($n = 17$)
Triacylglycerol (mg/dL)	133.37 ± 4.28	127.09 ± 4.85	129.30 ± 4.31	141.55 ± 3.94[b,d]
Total cholesterol (mg/dL)	128.75 ± 4.22	129.46 ± 4.76	128.99 ± 3.35	150.03 ± 6.00
LDL (mg/dL)	68.81 ± 1.75	73.18 ± 3.07	75.66 ± 1.64	80.3 ± 3.28
VLDL (mg/dL)	26.32 ± 0.86	25.42 ± 0.97	25.86 ± 0.86	28.92 ± 0.76[b,d]
HDL (mg/dL)	33.62 ± 1.75	36.71 ± 3.07	35.13 ± 1.64	40.83 ± 3.28
Glucose (mg/dL)	100.27 ± 2.69	89.84 ± 4.45	100.80 ± 3.58	105.28 ± 3.47
Insulin (ng/mL)	0.51 ± 0.03	0.64 ± 0.01	0.65 ± 0.01	0.75 ± 0.02[b]
HOMA-IR	0.89 ± 0.26	0.97 ± 0.15	1.12 ± 0.21	1.25 ± 0.19[b]
Adiponectin (ng/mL)	3.55 ± 0.47	3.98 ± 0.26	4.04 ± 0.10	3.34 ± 0.29[d]
Lipopolysaccharides (EU/mL)	2.58 ± 0.98	1.61 ± 0.66	1.49 ± 0.50	3.07 ± 0.97[d]

Data submitted with an average ± EPM. [b]C-W versus HF-W and [d]HF-CWP versus HF-W. ($P < 0.05$).

Table 5: Concentrations of IL-6, TNF-α, IL-10, and IL-10/TNF-α (pg/mg of total protein content) in different experimental groups.

	C-W ($n = 6$)	C-CWP ($n = 7$)	HF-CWP ($n = 12$)	HF-W ($n = 12$)
IL-6	329.69 ± 43.64	132.08 ± 24.10[a]	215.80 ± 49.70	241.61 ± 29.27
TNF-α	111.93 ± 21.71	92.60 ± 22.86	54.48 ± 8.27	69.38 ± 17.35
IL-10	153.36 ± 27.01	119.30 ± 29.04	65.05 ± 9.96	100.74 ± 20.97[b]
IL-10/TNF-α	1.37 ± 0.027	1.28 ± 0.28	1.19 ± 0.10	1.45 ± 0.67

Data submitted with an average ± EPM. [a]C-W versus C-CWP; [b]C-W versus HF-W. ($P < 0.05$).

physical, and chemical characteristics vary according to the procedures used to obtain these proteins. In our study, it was possible to recover an average OF 30% in WP (Table 2). If we compare these numbers with published data (which often use conventional techniques such as ultrafiltration (UF)), our method seems to be a low efficiency process [19]. However, cost-benefit considerations should be taken into account. For example, the UF method is expensive not only in terms of deployment but also in terms of operation. In addition to being cost-effective, the coacervation process promotes the separation of WP and obtains a low-calorie product.

The emergence of food compounds with health benefits may eventually become a good strategy to improve public health. In recent years, functional food has attracted the attention from scientific community, consumers, and food manufacturers. The list of nutraceuticals compounds (vitamins, probiotics, bioactive peptides, and antioxidants among others) is extensive, and scientific evidence seems to increasingly support the concept of health promotion through food ingredients [20, 21].

Functional foods are usually marketed as food containing ingredients technologically manipulated to perform a benefit for health [22]. Our study lends support to previous studies showing the effectiveness of CWP as a nutraceutical able to stabilize fat mass gain in animals fed with high fat diet. Our findings agree with the WP intake benefits extensively reported in literature [23–27]. As demonstrated, there was a decrease in body weight in the HF-CWP group when compared to the HF-W group, accompanied by a reduction in the adiposity.

It is now well established that excessive consumption of saturated fat is related to the development of dyslipidemias [28, 29], and this study further corroborates it, as the animals fed with high fat diet increased TAG and VLDL. Studies have demonstrated the insulinotropic effect of WP [9, 11, 30]. In this study, we did not find any significant difference in blood glucose between the groups assessed. Regarding insulin and HOMA index, the hyperlipidic diet showed significantly higher values. Our results demonstrated the effectiveness of this experimental model of obesity. However, CWP treatment promoted improved glucose and insulin tolerance. A study undertaken by Huang et al. [31] utilizing different protein sources (cheese whey, soy, red meat, and milk) in obese mice (induced by high fat diet) has found increased adiponectin concentration and reduced insulin when the animals were fed with whey protein cheese. In the present study, we also detected the critical role of CWP in modulating adiponectin, as the values were higher for the HF-CWP group when compared to HF-W group. These findings indicate that CWP may have functional effects. Yamauchi et al. [32] have demonstrated the potential of adiponectin in reducing insulin resistance by enhancing fatty acid oxidation, leading to a reduction in TAG content in obese diabetic rats.

There is strong evidence of an immunomodulatory role of WP [33]. Products containing WP may be beneficial in the treatment of certain diseases. Studies have shown that WP

promotes improvement in the treatment of gastrointestinal symptoms of infant mice with rotavirus-induced diarrhea, a protective role in colorectal cancer in rats, reduced release of IL-6 in blood of rats undergoing transient ischemia/intestinal reperfusion and may provide protective effects against experimentally induced breast cancer in animals [25, 34, 35]. Most of the more recent studies in the literature, both *in vivo* and *in vitro*, have focused on the possible effects of these proteins in macrophages and lymphocytes. Although most proteins are degraded during the gastric digestion, certain cheese whey proteins, such as β-Lg, α-La, or GMP, are resistant to digestion and remain intact. Such proteins can directly stimulate leukocyte after their digestive absorption. Thus, it is important to understand the mechanisms underlying the impact of these proteins on immunity, stimulating anti-inflammatory processes in the body [33].

The IL-10 is a pleiotropic cytokine that controls inflammatory processes by eliminating the proinflammatory cytokines production such as IL-1, IL-6, and IL-8, and TNF-α is produced mainly by monocytes, macrophages, lymphocytes, mast cells, and mature adipocytes [14, 36]. The IL-10/TNF-α ratio has been considered an important indicator of inflammatory status as low values are often associated with increased morbidity and mortality risk. We did not observe an increase of the IL-10/TNF-α ratio in MES of group HF-CWP. We believe that because of the short period of the treatment the animals may not have developed their proinflammatory state. With increased length of treatment, we believe find results more expressive. Another possibility is that the coacervate may have protected the mice from a proinflammatory state triggered by the treatment diet, leading to the counterbalance of IL-10 unnecessary in HF-CWP group. However, a study involving a longer treatment period may be required to discern this possible effect.

In this sense, there is an immunomodulatory mechanism underlying CWP, most likely the IL-10 cytokine, which has a homeostatic metabolic effect in the mesenteric adipose tissue. Our result suggests that IL-10 may be a positive regulator of insulin sensitivity and increased glucose uptake. This mechanism can protect the adipose tissue against insulin resistance. Although the precise origin of the unchecked inflammatory response in obesity is still unclear, it is well known that in obesity the overproduction of proinflammatory cytokines affects metabolism. For example, TNF-α contributes to the inability of cells to respond to insulin and to increased levels of insulin [37], and IL-10 was associated with other variables closely linked to insulin sensitivity, such as fasting and postload insulin concentrations, HDL cholesterol, and triglyceride levels. Besides the tissue-specific effect of CWP, we showed a systemic effect in the decrease in the LPS serum level.

Regarding the composition of the CWP, we may highlight the presence of α-La and β-Lg protein, the major protein present in the coacervate, which has been proven effective in suppressing the release of proinflammatory cytokines [27, 38]. The presence of these proteins is a great indication that the coacervate components are able to modulate the proinflammatory milieu promoted by hyperlipidic diet. In another study by our group, mice, previously treated with high fat diet and fed with a supplementation of CWP (gavage, 36 mg protein/kg of body weight), showed a positive correlation between IL-10 and TNF-alpha in mesenteric adipose tissue, retroperitoneal adipose tissue, and liver tissue. We also observed a positive correlation between lipopolysaccharide and IL-10 in the liver tissue. Therefore, pretreatment with high fat diet promoted metabolic alterations and inflammation, and CWP modulated the inflammatory milieu [14].

Evidence suggests that eating WP causes the decrease in calorie intake, increased basal energy expenditure, and modulates insulin sensitivity and glucose homeostasis, leading to changes in lipid metabolism in adipose tissue, liver, and muscle [39–42]. The AMPK and adiponectin are key molecules to metabolic responses in different tissues [43, 44]; they are involved in the preventive response against negative physiological processes caused by the consumption of a diet high in saturated fatty acids. The activation of AMPK by bioactive components of foods or medicines has been regarded as goal, since it may reverse the metabolic changes associated with obesity and type 2 diabetes [45]. It is also known that adiponectin can activate AMPK in white adipose tissue [46]. The animals treated with coacervate showed an increase in AMPK activation associated with the decrease in HSL and increase in ATGL protein expression in mesenteric adipose tissue. Similar results were reported in the review undertaken by Bijland et al. [47].

A study conducted by Gaidhu et al. [43] showed that AMPK activation stimulated by AICAR (*5-aminoimidazol-4-carboxamida ribonucleotídeo)* initially promoted inhibition of lipolysis in adipocytes isolated as *in vivo*, reflecting a decrease in free fatty acid in serum. On the other hand, prolonged treatment with AICAR promoted an increase in lipolysis, which the authors attributed to an increase in the content of ATGL and reduced activity of HSL [48]. However, clear-cut conclusions are difficult to arrive at, due to a lack of tools for manipulating assays using specific AMPK. Furthermore, the overall effect of AMPK activation of lipolysis is still controversial. The duration and mode of activation of AMPK may be of particular importance when it comes to a process aimed at reducing the proinflammatory state caused by increased lipolysis [47]. In addition, adiponectin can suppress the activation of HSL, without changing ATGL and ABHD-5 in adipocytes in order to modulate a homeostatic control of lipolysis to avoid lipotoxicity [49].

Lipolysis does seem to play a crucial physiological role by recruiting a source of energy mobilized in times of stress and/or energy deprivation. Moreover, the very significant reduction in lipolysis is clearly harmful, as demonstrated in the clinical domain by the syndromes resulting from deficiencies in the lipolytic apparatus [50]. Given that, it is reasonable to question whether the inhibition of lipolysis, via HSL induced by CWP, is helpful or harmful, since enhanced lipolytic activity and concomitant increase of free fatty acid in the circulation is clearly deleterious and leads to several comorbidities. To clearly determine this, however, an analysis of free fatty acid and endogenous glycerol concentrations would need to be carried out.

Another interesting finding was the significantly higher protein expression of perilipin A (52%) in HF-W group, which also refers to larger deposits of triglycerides, since this protein is primarily anchored around the droplets of neutral lipids in adipocytes. This is in line with studies showing that increased protein expression perilipin A leads to increased storage of triglycerides by reducing its hydrolytic rate. [51].

Finally, there is plenty of evidence suggesting that the intake of WP may lower consumption of calories, increase baseline energy expenditure, and improve insulin sensitivity and glucose homeostasis, thus leading to changes in lipid metabolism in adipose tissue, liver, and muscle [39–42].

5. Conclusion

CWP were able to promote nutritional and physiological improvements in HF-CWP group, such as reduction in body mass and decreased serum lipid levels followed by decreased serum insulin and LPS. In addition, intervention with CWP resulted in higher adiponectin contents and attenuated processes that would lead to glucose intolerance. Therefore, CWP could play a beneficial role, in some way, in modulating lipolysis in animals treated with hyperlipidic diet.

Abbreviations

ABHD-5:	Abhydrolase domain containing 5
AUC:	Area under the curve
CWP:	Coacervate
C-CWP:	Control diet plus coacervate
C-W:	Control diet plus tap water
HF-CWP:	High fat diet plus coacervate
HF-W:	High fat diet plus tap water
HSL:	Hormone-sensitive lipase
LPS:	Lipopolysaccharide
MGL:	Monoglyceride lipase
NEFAs:	Nonesterified fatty acid
p-AMPKα 1 e 2 - Thr 172:	Phospho 5′ AMP-activated protein kinase
TAG:	Triacylglycerol
OGTT:	Oral glucose tolerance test
ATGL:	Triglyceride lipase enzyme
WP:	Whey protein.

Conflict of Interests

The authors declare that they have no competing interests.

Authors' Contribution

All authors read and approved the final paper.

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Research Article

The Influence of Autologous Bone Marrow Stem Cell Transplantation on Matrix Metalloproteinases in Patients Treated for Acute ST-Elevation Myocardial Infarction

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Background. Matrix metalloproteinase-9 (MMP-9), regulated by tissue inhibitor of metalloproteinase-9 (TIMP-1) and the extracellular matrix metalloproteinase inducer (EMMPRIN), contributes to plaque instability. Autologous stem cells from bone marrow (mBMC) treatment are suggested to reduce myocardial damage; however, limited data exists on the influence of mBMC on MMPs. *Aim*. We investigated the influence of mBMC on circulating levels of MMP-9, TIMP-1, and EMMPRIN at different time points in patients included in the randomized Autologous Stem-Cell Transplantation in Acute Myocardial Infarction (ASTAMI) trial ($n = 100$). Gene expression analyses were additionally performed. *Results*. After 2-3 weeks we observed a more pronounced increase in MMP-9 levels in the mBMC group, compared to controls ($P = 0.030$), whereas EMMPRIN levels were reduced from baseline to 2-3 weeks and 3 months in both groups ($P < 0.0001$). Gene expression of both MMP-9 and EMMPRIN was reduced from baseline to 3 months. MMP-9 and EMMPRIN were significantly correlated to myocardial injury (CK: $P = 0.005$ and $P < 0.001$, resp.) and infarct size (SPECT: $P = 0.018$ and $P = 0.008$, resp.). *Conclusion*. The results indicate that the regulation of metalloproteinases is important during AMI, however, limited influenced by mBMC.

1. Introduction

The early and late mortality after acute myocardial infarction (AMI) is declining, but cardiovascular disease (CVD) is still one of the leading causes of morbidity and death in the Western world. Ischemic heart disease is a feared, but often inevitable complication of atherosclerosis, the main underlying cause of myocardial infarction [1, 2]. Inflammation is considered to be a key process for development of atherosclerosis and this includes a number of cellular and molecular responses resulting in plaque formation [2, 3]. Despite well-documented treatment of AMI survivors, both medically and by percutaneous coronary intervention (PCI), some patients either do not receive this treatment, do not respond satisfactorily, or develop congestive heart failure despite treatment.

Several animal studies have shown that bone marrow stem cells differentiate to cardiomyocytes when infused into the affected myocardium [4]. Treatment with autologous stem cells from bone marrow has been suggested to reduce myocardial damage in patients with AMI. Results from clinical trials are, however, conflicting with regard to improvement of left ventricular ejection fraction [5–10]. Possible mechanisms by which autologous bone marrow stem cells

act are discussed to be cardiac transdifferentiation, paracrine effects, angiogenesis, and reduced apoptosis [11, 12].

Matrix metalloproteinases (MMPs), a class of 24 endopeptidases, participate in plaque instability by degrading the extracellular matrix. MMP-9, a zinc-dependent gelatinase, is found in the shoulder of the plaque, contributes to plack instability and rupture, and has been associated with acute coronary syndrome (ACS) [3, 13]. Circulating MMP-9 has been shown to be elevated in patients with AMI, stable, and unstable angina pectoris [13, 14], as well as in hypertensives [15] and smokers [16]. In addition, MMP-9 is discussed to be involved in adverse left ventricular remodelling and associated with higher cardiovascular risk score [13]. The MMPs are regulated by specific endogenous inhibitors, tissue inhibitor of metalloproteinases (TIMPs), and MMP-9 is specifically regulated when pro-MMP-9 binds to TIMP-1 [14, 17].

Lately, the extracellular matrix metalloproteinase inducer (EMMPRIN, CD147), a member of the immunoglobulin superfamily, has been discussed to be involved in both expression and release of MMP-9 [18], thus, having a potentially regulatory role in CVD. EMMPRIN has been shown to be expressed in atherosclerotic plaques [19] as well as in cell types like monocytes, macrophages, and platelets [20].

In humans, limited data exists on the influence of bone marrow stem cells on MMPs that may be of importance for the myocardium and the infarction process. In the "randomized Autologous Stem-Cell Transplantation in Acute Myocardial Infarction" (ASTAMI) trial [10], the main aim was to assess the effects of intracoronary injection of autologous mononuclear bone marrow-derived cells (mBMC) on left ventricular ejection fraction in patients with ST-elevation myocardial infarction (STEMI). The main hypothesis was that the treatment would reduce the infarction sequelae, which, however, could not be demonstrated [10].

The aim of this ASTAMI substudy was to investigate the influence of coronary injection of mBMC on MMP-9, TIMP-1, and EMMPRIN, circulating levels as well as on gene expression in leukocytes, in patients with STEMI undergoing successful PCI. Furthermore, to investigate any association between the measured biomarkers and infarct size and left ventricular function, our hypothesis was that MMP-9 levels would be reduced after mBMC treatment, in parallel with reduction of EMMPRIN.

2. Materials and Methods

2.1. Subjects and Study Design. The ASTAMI study design and treatment protocol have previously been described in details [21]. Briefly, it was a randomized 1 : 1 open-labelled study, where one arm was intracoronary treatment with mBMC, and the other controls without aspiration and injection of bone marrow. Patients, age between 40 and 75 years, both gender were included. They were all treated with PCI with stent implantation in the left anterior descending (LAD) coronary artery.

Exclusion criteria were previous Q-wave infarction, cardiogenic shock, or severe comorbidity interfering with compliance to the protocol.

Baseline recordings were performed during day 4-5 after AMI, before bone marrow aspiration (in the treatment group) [10].

The study protocol, including the biobank, was approved by the Regional Committee for Medical Research Ethics and all patients gave written, informed consent. The study is registered at ClinicalTrials.gov, NCT 00199823.

2.2. Laboratory Methods

2.2.1. Blood Sampling. A biobank, kept at −80°C consisting of plasma, serum, and PaxGene tubes (PreAnalytiX GmbH, Hombrechtikon, CH), the latter for gene expression measures in circulating leukocytes, was established. Blood samples were collected in fasting condition between 08.00 and 10.00 am the day before transplantation in the mBMC group (day-1) (baseline), the day after (day 1) and further day 3, after 2-3 weeks and after 3 months. The same time interval was used for the control group, except baseline sampling (day-1) which was drawn median 4 days after PCI compared to 5 days in the mBMC group.

2.2.2. Enzyme Immunoassays. For analyses of MMP-9, TIMP-1, and EMMPRIN commercial enzyme linked immunosorbent assays (ELISA) (R&D Systems Europe, Abingdon, Oxford, UK) were used on serum samples, which were performed within 1 hour by centrifugation at room temperature 2500 ×g for 10 min. The interassay coefficients of variation (CV) were 7.3% for MMP-9, 4.4% for TIMP-1, and 5.4% for EMMPRIN.

2.2.3. Gene Expression Analysis. Isolation of RNA from PaxGene tubes was performed according to the manufacturers instruction (PreAnalytix, Qiagen GmbH, Germany) in a subset of randomly selected samples ($n = 47$), with an additional cleaning step (Rneasy MinElute Cleanup Kit, Qiagen). A complementary DNA (cDNA) of the messenger RNA (mRNA) content was achieved by inversely transcribing total RNA in the samples. The genetic expression of mRNA of MMP-9 and EMMPRIN was performed by use of real-time PCR on the ViiA 7 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) and the ΔΔCt method was applied [22]. This relative or comparative Ct method determines the relative target quantity (RQ-values) in the samples, by measuring the amplification (crossing threshold Ct) of the target samples and in a reference sample and normalized to an endogenous control (house-keeping gene). Assays for the target genes were Hs00234579_m1 for MMP-9, Hs00936295_m1 for EMMPRIN, and β-2 macroglobulin (Hs99999907_m1) was chosen as house-keeping gene.

2.2.4. Measures of Myocardial Function. Left ventricular ejection fraction (LVEF) and infarct size were obtained by electrocardiogram-gated single photon emission computed tomography (SPECT) (GE Medical Systems with 4D-MSPECT software) at baseline (4.0 ± 1.4 days after the AMI). Infarct size was expressed as percentage of the LAD-area.

Table 1: Characteristics of the study population according to the randomized groups. Values are presented as proportion, means ± SD, or medians[1] with 25th and 75th percentiles.

	mBMC group ($n = 50$)	Control group ($n = 50$)
Age (years)	58.1 (8.6)	56.7 (9.6)
Gender (% female)	16	16
Hypertension (%)	35	34
Diabetes (%)	10	8
Smokers (%)	39	48
BMI (kg/m^2)	26.3 (3.3)	27.1 (4.1)
SBP/DBP (mmHg)	131/82 ± 21/14	132/83 ± 23/17
Tot. chol (mmol/L)	4.4 ± 0.9	4.5 ± 0.9
LDL chol (mmol/L)	2.9 ± 0.8	2.9 ± 0.7
HDL chol (mmol/L)	1.0 ± 0.3	1.1 ± 0.3
Triglycerides (mmol/L)[1]	1.3 (1.0, 1.7)	1.3 ± (1.1, 1.5)
Symptom start to PCI (min)[1]	210 (180, 330)	230 (180, 330)
LVEF (%)	41.3 (10.6)	42.6 (11.7)
Infarct size (%)	43.7 ± 17.6	40.7 ± 19.3
Peak CK (U/L)	3338 ± 2398	3532 ± 2650
Thrombolysis before PCI (%)	30	28
Medication at discharge		
Aspirin (%)	100	100
Clopidogrel (%)	100	100
ACE-I/ATII antagonist (%)	100	100
β-blocker (%)	100	100
Diuretics (%)	43	32
Statin (%)	100	100

ATII antagonist: angiotensin II receptor antagonist; ACE-I: angiotensin-converting enzyme inhibitor; BMI: body mass index; DBP: diastolic blood pressure; LVEF: left ventricular ejection fraction; PCI: percutaneous coronary intervention; and SBP: systolic blood pressure.

2.3. Statistical Analysis. MMP-9, TIMP-1, and EMMPRIN levels were all skewly distributed and nonparametric statistics were used throughout. Median values and 25 and 75 percentiles are given unless otherwise stated. For group comparisons the Mann-Whitney test was used for continuous data and Chi square test for categorical data. Friedman test was performed to analyze for differences between any time points within the groups. For changes from baseline (day-1) to the subsequent time points, Wilcoxon test was used only when Friedman test was significant. For differences in changes between the randomized groups, Mann-Whitney test was used. Spearman's rho was used for correlation analysis. The level of significance was set to $P < 0.05$. The SPSS software package version 18.0 was used throughout.

3. Results

Baseline characteristics of the study population according to the randomized groups are shown in Table 1.

One hundred patients included in the ASTAMI study, 50 randomized to mBMC treatment and 50 controls, were followed. From one patient we did not obtain blood samples. The groups did not differ regarding baseline characteristics. They were all medically treated according to current guidelines, thus, all patients were on statins, beta-blockers, ACE-inhibitors/ATII antagonists, and antithrombotic agents.

3.1. Circulating Levels of MMPs (Table 2). Although blood samples at baseline were obtained one day later (median) from symptom start of AMI in the mBMC group compared to controls, no significant differences between the groups were recorded at baseline (day-1). No differences in MMP-9, TIMP-1, or EMMPRIN between the mBMC group and controls were seen at any further time points.

In the mBMC group there was a significant increase in MMP-9 levels from baseline to 2-3 weeks ($P = 0.009$), which could not be demonstrated in the control group. We observed no within group changes in TIMP-1 levels in either groups. EMMPRIN levels were significantly reduced from baseline to 2-3 weeks and 3 months in both groups ($P < 0.0001$, all).

When analyzing for differences between the groups in changes from baseline to further time points, we could demonstrate a significant difference in change in MMP-9 levels between the two groups at 2-3 weeks and after 3 months, showing a more pronounced increase in the mBMC group ($P = 0.030$ and $P = 0.05$, resp.). No differences in changes between the groups were observed for TIMP-1 and EMMPRIN.

3.2. Gene Expression Levels. We observed no differences in MMP-9 gene expression between the two groups at any time points, while the EMMPRIN gene expression was significantly lower in the mBMC group ($n = 23$) versus the controls

Table 2: Circulating levels of MMP-9, TIMP-1, and EMMPRIN at baseline and follow-up in the randomized groups. Median values (25th and 75th percentiles) are given.

	Baseline (Day-1)		Day 1		Day 3		2-3 weeks		3 months	
	mBMC	Control	mBMC	Control	mBMC	Control	mBMC	Control	mBMC	Control
MMP-9	205	235	286	276	287	273	**281***†	302	**270**†	210
(ng/mL)	(79, 419)	(83, 502)	(98, 432)	(103, 518)	(115, 426)	(116, 499)	(141, 574)	(106, 419)	(153, 410)	(110, 398)
TIMP-1	211	228	234	253	222	253	216	213	204	220
(ng/mL)	(158, 326)	(151, 327)	(157, 326)	(158, 354)	(173, 322)	(182, 333)	(171, 336)	(172, 306)	(164, 282)	(182, 273)
EMMPRIN	4324	4329	4317	4357	4339	4367	**3754***	**3857***	**3654***	**3775***
(pg/mL)	(3663, 4878)	(3752, 4994)	(3739, 4966)	(3976, 4952)	(3667, 4837)	(3768, 4853)	(3249, 4436)	(3440, 4476)	(3312, 4099)	(3364, 4245)

No differences between the groups were observed at any time points.

*Refers to P value < 0.05 for intragroup changes from baseline to later time points.

†Refers to P value ≤ 0.05 for differences in changes from baseline between the groups.

($n = 24$) after 3 months ($P = 0.03$) (Table 3). The levels of both MMP-9 and EMMPRIN gene expressions were significantly reduced from baseline to 3 months in the mBMC group ($P < 0.0001$ and $P = 0.002$, resp.). This could not be demonstrated in the control group. There were, however, no differences between the groups in changes from baseline to any later time points. When defining baseline mRNA level (RQ-values) in the total population to 1, there was a 20% reduction in MMP-9 gene expression from baseline to 2-3 weeks, and a 50% reduction after 3 months in the mBMC group. A similar pattern was seen in gene expression of EMMPRIN, with a 20% reduction after 2-3 weeks and 60% reduction after 3 months in the mBMC group. The results of the gene expression presented as fold changes are illustrated in Figure 1.

3.3. Correlations. In the total population at baseline we observed a significant correlation between MMP-9 and EMMPRIN ($r = 0.25$, $P = 0.011$). A strong correlation was also shown between MMP-9 and TIMP-1 ($r = 0.66$, $P < 0.0001$) and also between TIMP-1 and EMMPRIN ($r = 0.36$, $P = 0.01$). Significant correlations between CK and baseline levels of both MMP-9 and EMMPRIN were found ($r = 0.29$ $P = 0.005$ and $r = 0.43$ $P < 0.001$, resp.). MMP-9 and EMMPRIN, but not TIMP-1, showed also significant correlations to infarct size measured by SPECT ($r = 0.24$, $P = 0.018$ and $r = 0.27$ $P = 0.008$, resp.). EMMPRIN levels were also found to be inversely correlated to LVEF at baseline ($r = -0.31$ $P = 0.002$).

We observed no significant correlations between circulating MMP-9 and gene expression of MMP-9 at any time points in the total population, or in the single groups. Likewise there were no correlations between circulating EMMPRIN and gene expression of EMMPRIN, or between gene expression of EMMPRIN and MMP-9 levels in either groups or in the total population.

4. Discussion

The main finding in the present study was that there was limited influence of intracoronary injection of mBMC transplantation after AMI on circulating levels of MMP-9, TIMP-1, and EMMPRIN, other than a more pronounced increase in MMP-9 after 2-3 weeks in the mBMC group. EMMPRIN levels were reduced after 2-3 weeks and 3 months in both groups. At baseline both MMP-9 and EMMPRIN were significantly correlated to myocardial injury assessed by biomarkers and infarct size and might therefore support their predictory ability for later outcome.

All patients were medically treated according to current guidelines; thus, any influence by medication on the measured variables would be equally affected in the randomized groups.

In both *in vitro* and *in vivo* studies, stem cell transplantation has been shown to reduce MMPs after AMI and improve ventricular remodeling [23]. Our hypothesis was therefore that treatment with mBMC would reduce the circulating levels of the selected biomarkers. In a study using modified mesenchymal stem cell transplantation into AMI rat hearts [24], a reduction in MMP-9 levels was shown. Mesenchymal stem cells are multipotent stromal cells that can differentiate into a variety of cell types [25], and the results are thus not quite comparable to ours. In addition, stem cell injection was performed one hour after AMI. In contrast, we found a significantly more pronounced increase in MMP-9 levels from baseline to 2-3 weeks in the mBMC group compared to controls. In accordance with our findings Roderfelt et al. demonstrated a transient inflammatory response and upregulation of MMP-9 activity after bone marrow transplantation in *Abcb4*$^{-/-}$ (hepatic fibrosis) mice [26]. We have previously shown that MMP-9 levels are reduced 1 day after AMI [27]. Therefore we assume that the levels were normalized when baseline sampling in the present study was performed and limited influenced by the acute phase reaction. In the control group in our study, bone marrow aspiration was not performed. This procedure which itself is a trauma could influence the release of inflammatory markers and contribute to the elevated levels in the mBMC group.

In the study by Shu et al. using mesenchymal stem cell transplantation, TIMP-1 levels did not vary significantly [24], which is in accordance with our findings of no changes in this variable during the observation period in any of the groups.

The significant reduction in genetic expression of MMP-9 seen at 3 months might be discussed as compensatory to the increase observed in the circulating levels. MMP-9 expression is a crucial pathogenic feature in a range of conditions and disease states, also other than CVD [28–30], in which treatment with stem cells has been shown to suppress or downregulate the MMP-9 expression [30] and thereby improving the current condition.

The underlying mechanisms for the influence of stem cells on MMPs are not clarified. In cell culture of cardiac fibroblasts Wang et al. [31] could demonstrate that the protein expression and activity of MMP-2, but not MMP-9, were increased in response to hypoxia and decreased when cocultured with mesenchymal stem cells. It has also been demonstrated that early endothelial progenitor cells increased MMP-9 expression *in vitro*, whereas MMP-2 was increased in outgrowth endothelial cells [32]. The type of stem cells seems to be of importance regarding the degree of influence on MMPs [33].

The importance of EMMPRIN as an inducer of MMP-9 has been explored to a limited extent in humans. In our study circulating levels of MMP-9 and EMMPRIN were significantly correlated, indicating a common regulatory pathway [18]. Circulating levels as well as genetic expression of EMMPRIN were significantly reduced along with the increase in MMP-9. This might be discussed as a negative feedback mechanism. There was, however, no influence of mBMC on circulating levels or gene expression of EMMPRIN, shown by the significant reduction in both groups during the observation period. Expression of the EMMPRIN-gene in circulating leukocytes, also reported by Xu et al. assessed by flow cytometry [34], may indicate that the leukocytes contribute to the circulating levels, although no correlation between circulating levels and gene expression was observed. The reduction over time seen in EMMPRIN expression, with

Table 3: The levels of gene expression, RQ values of MMP-9, and EMMPRIN at baseline and follow-up.

	Baseline (Day-1)		Day 1		Day 3		2-3 weeks		3 months	
	mBMC	Control	mBMC	Control	mBMC	Control	mBMC	Control	mBMC	Control
MMP-9	0.483 (0.309, 0.749)	0.441 (0.289, 0.826)	0.460 (0.255, 0.772)	0.466 (0.322, 0.655)	0.384 (0.229, 0.652)	0.379 (0.265, 0.543)	0.444 (0.328, 0.760)	0.395 (0.275, 0.640)	**0.247**[*] (0.159, 0.367)	0.357 (0.223, 0.521)
EMMPRIN	0.567 (0.248, 0.961)	0.566 (0.305, 1.016)	0.358 (0.251, 0.797)	0.467 (0.226, 0.953)	0.490 (0.196, 0.758)	0.273 (0.174, 1.005)	0.512 (0.231, 0.810)	0.567 (0.182, 0.927)	**0.295**[*#] (0.177, 0.533)	0.649 (0.223, 1.166)

[*]Refers to P value < 0.05 for intragroup changes from baseline to later time points.
[#]Refers to P value < 0.05 for differences between the groups.

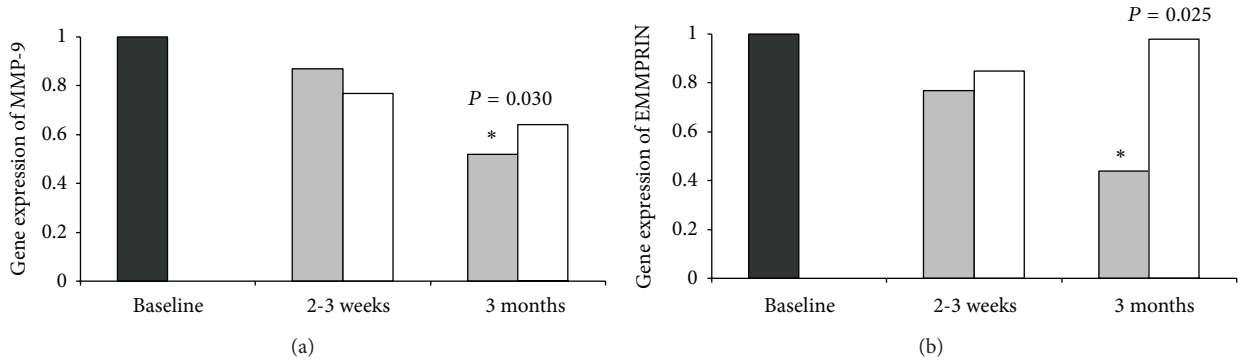

Figure 1: Gene expression of MMP-9 (a) and EMMPRIN (b) after 2-3 weeks and 3 months in the mBMC group and controls relative to baseline expression in the total population. Black bars: total group at baseline; grey bars: mBMC group; white bars: controls. * indicates reduction from baseline to 3 months in the mBMC group *P* values refer to differences in expression relative to baseline between groups.

subsequent reduction in MMP-9 gene expression, contributes to the assumption that EMMPRIN is an inducer of MMP-9.

The significant correlations found between both MMP-9 and EMMPRIN, and myocardial injury assessed by biomarkers as well as infarct size measured by SPECT, have been sparsely explored in humans. In experimental AMI, MMP-9 has been shown to increase infarct size and left ventricular fibrosis [13], in accordance with our findings.

An association between EMMPRIN and the degree of myocardial injury and LVEF has previously been reported in the work by Nie et al. [35], but this was a post mortem immunohistochemistry study which showed a strong increase in EMMPRIN around the zone of necrosis in the AMI group. This can to some degree be compared to our findings of the correlations between MMPs and biomarkers of AMI and also to our results of an inverse correlation between EMMPRIN and LVEF. An additional contribution to this understanding has been demonstrated in $CD147^{+/-}$ mice, where the disruption of the EMMPRIN-Cyclophilin A interaction reduced infarct size [36]. Our findings contribute to the suggestion that the expression of EMMPRIN is a decisive factor in regulating MMP-9 activity and thereby involved in myocardial remodeling.

The strength of the present study is the randomized design, the rather frequent sample collection for determination of the profiles, and gene expression analysis in a relatively large subpopulation. All patients were medically treated according to current guidelines; thus any influence by medication on the measured variables would therefore be equal in the randomized groups.

A limitation is that there was no bone marrow aspiration in the control group. There was, however, no significant difference between the two groups at baseline, that is, after the bone marrow aspiration in the mBMC group. It should be noted that the measures of circulating MMP-9 and TIMP-1 were performed in serum, as also used in many other studies. The proteins are released upon platelet activation during clotting, thus, the levels measured do not reflect the "true" circulating levels. However, the same standardized procedure for serum preparation was applied throughout the study, and therefore we assume that the comparisons between the randomized groups have been limited influenced. Confirmation of our results based on plasma samples is warranted. From the literature there are both coincide results and not when using plasma or serum samples, thus, it is important to take this into account when comparing results between studies. The gene expression analyses were performed in whole blood with circulating leukocytes as the RNA source, which might not be the most important source of either MMP-9 or EMMPRIN.

When performing stem cell transplantation as treatment regimen, several studies have discussed the timing, type of stem cells, and the procedure of the transplantation for optimization of the results [37, 38], but a conclusion has not yet been made.

5. Conclusions

Limited effects of intra coronary injection of mBMC transplantation on circulating levels as well as gene expression of MMP-9 and EMMPRIN in patients with STEMI treated with PCI could be demonstrated. EMMPRIN levels were reduced in both groups, whereas MMP-9 showed increased levels in the mBMC group. Both MMP-9 and EMMPRIN were significantly correlated to myocardial injury and infarct size, indicating that the regulation of metalloproteinases is important in the process of an AMI. The results contribute to the understanding of the pathophysiology of metalloproteinases in AMI, but further investigations are needed regarding timing and type of stem cells.

Conflict of Interests

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

Authors' Contribution

Eline Bredal Furenes contributed in development of the study protocol, laboratory analyses, statistics, preparation, and discussion of paper. Trine Baur Opstad contributed to laboratory analyses, statistics, evaluation of results, and discussion of paper. Svein Solheim contributed in developing the study protocol, inclusion of patients, and discussion of paper. Ketil

Lunde contributed in developing the study protocol, inclusion of patients, and discussion of paper. Harald Arnesen contributed in developing the study protocol, evaluation of study results, and discussion of paper. Ingebjørg Seljeflot contributed in developing the study protocol, evaluation of study results, and discussion of paper.

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Research Article

Resolution of Sterile Inflammation: Role for Vitamin C

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Introduction. Macrophage reprogramming is vital for resolution of acute inflammation. Parenteral vitamin C (VitC) attenuates proinflammatory states in murine and human sepsis. However information about the mechanism by which VitC regulates resolution of inflammation is limited. *Methods*. To examine whether physiological levels of VitC modulate resolution of inflammation, we used transgenic mice lacking L-gulono-γ-lactone oxidase. VitC sufficient/deficient mice were subjected to a thioglycollate-elicited peritonitis model of sterile inflammation. Some VitC deficient mice received daily parenteral VitC (200 mg/kg) for 3 or 5 days following thioglycollate infusion. Peritoneal macrophages harvested on day 3 or day 5 were examined for intracellular VitC levels, pro- and anti-inflammatory protein and lipid mediators, mitochondrial function, and response to lipopolysaccharide (LPS). The THP-1 cell line was used to determine the modulatory activities of VitC in activated human macrophages. *Results*. VitC deficiency significantly delayed resolution of inflammation and generated an exaggerated proinflammatory response to *in vitro* LPS stimulation. VitC sufficiency and *in vivo* VitC supplementation restored macrophage phenotype and function in VitC deficient mice. VitC loading of THP-1 macrophages attenuated LPS-induced proinflammatory responses. *Conclusion*. VitC sufficiency favorably modulates macrophage function. *In vivo* or *in vitro* VitC supplementation restores macrophage phenotype and function leading to timely resolution of inflammation.

1. Introduction

Resolution of inflammation typically follows an ordered series of events orchestrated by different cell types [1]. During the early stages of inflammation, leukocytes such as polymorphonuclear neutrophils (PMN) are the first immune cells to arrive at the site of inflammation. PMN are recruited by gradients of proinflammatory signals and usually reach peak numbers within 24–48 hrs. PMN have short half-lives and are normally cleared from sites of inflammation by undergoing apoptosis [2]. Mobilized monocyte-derived macrophages extravasate to inflammatory tissue sites and clear apoptotic PMN in a nonphlogistic fashion by the process of efferocytosis. Apoptotic PMN release "find-me" signals that are sensed by extravasated macrophages [3]. Following phagocytosis, apoptotic PMN provides resolution cues to macrophages by evoking distinct signaling events that block release of proinflammatory mediators thus allowing further engulfment of apoptotic cells. Mantovani et al. and Fleming and Mosser note that mobilized macrophages are divided into three groups based upon their activation states [4, 5]. These include the M1, M2, and the recently described regulatory macrophages (M_{res}). M1 macrophages, classically referred to as activated macrophages, secrete proinflammatory factors that mediate

host defense against invading pathogens. M2 macrophages, termed alternatively activated macrophages, are considered to be anti-inflammatory [6, 7]. Finally, M_{res} macrophages secrete considerable amounts of anti-inflammatory cytokines that prevent inflammatory and autoimmune pathology [8, 9]. M_{res} macrophages also secrete various lipid mediators that play critical roles in resolution of inflammation (see below). Extensive new research has identified expression markers or phenotypic signatures for the various macrophage activation states in mice. They include gene expression changes in IL-1β, TNFα, and iNOS for classical activation and arginase-1 (Arg1), chitinase 3-like 3 (YM-1), and TGFβ for the alternatively activated macrophages [7].

Resolution of inflammation and restoration of normal tissue function prevent the development of "complications" of excessive inflammation, a process referred to as catabasis [10]. Catabasis is driven by synthesis and release of proresolution lipid mediators such as resolvins, protectins, and lipoxins [11]. Lipoxins [12] and protectins [13] are synthesized by lipoxygenase enzymes (such as 15-lipoxygenase (15-Lox)). Resolvins are derived from omega-3 polyunsaturated fatty acids such as docosahexaenoic acid and eicosapentaenoic acid [14]. They are products of metabolism involving 15-Lox and cyclooxygenase. Russell and Schwarze have reviewed the proresolution effects of proresolution mediators in a variety of inflammatory states [15]. However their regulation by vitamin C (VitC, ascorbic acid, AscA) has yet to be examined.

VitC readily functions as one or two electron-reducing agents for many oxidants and serves as a primary chemical antioxidant in most cell types. It modulates complex biochemical pathways that form an essential part of normal metabolism of immune cells [16]. Intracellular levels of VitC in cells differ significantly from circulating plasma levels. In particular, VitC accumulates in millimolar quantities in immune cells such as PMN and macrophages in which intracellular VitC concentrations are typically 40–60 fold higher than that present in circulation [17, 18]. In PMN, Vissers and Wilkie showed that intracellular VitC levels regulate neutrophil apoptosis [19]. Further, VitC contributes to the antioxidant defenses as well as normal PMN and macrophage function. Oberritter and colleagues showed that intracellular concentrations of VitC in macrophages are in the low millimolar range in freshly prepared peritoneal macrophages and *in vivo* or *in vitro* activation of peritoneal macrophages results in a significant decline in their VitC content [20]. Li et al. found that VitC deficiency worsens the inflammatory response following infection with the influenza virus [21]. Moreover, mice deficient in VitC generate excessive proinflammatory responses upon infection with the virulent bacterium *Klebsiella pneumonia* [22]. In humans, VitC levels are significantly reduced in critically ill patients and specifically in patients with poorly resolving proinflammatory states (e.g., sepsis, systemic inflammatory response syndrome) [23, 24]. Several studies performed in septic patients have found that plasma VitC levels correlate inversely with the incidence of organ failure and directly with survival [25, 26]. We recently showed that VitC attenuates inflammation and normalizes PMN function in septic mice [27, 28]. We further showed that parenteral VitC attenuates proinflammatory biomarkers and reduces mortality in human sepsis [29]. However information is limited regarding the mechanism by which VitC regulates the progression and eventual resolution of inflammatory states.

In the current study we examined the progression and resolution of inflammation using a murine thioglycollate (TG-)elicited peritonitis model in VitC sufficient and deficient mice. While humans lack L-gulono-γ-lactone oxidase (Gulo), the final enzyme in the biosynthesis pathway of VitC [30], mice express functional Gulo, resulting in cells and tissues generally maintaining high levels of VitC thereby complicating the translatability of VitC studies in mice, to humans. In order to establish the studies more relevant to humans, TG-induced peritonitis was performed in transgenic mice lacking Gulo ($Gulo^{-/-}$). Our studies reveal that progression and resolution of TG-induced inflammation is significantly delayed in VitC *deficient* mice. In particular, the spatiotemporal profile of pro- and anti-inflammatory mediator production by TG-elicited macrophages was significantly different between the VitC *sufficient* and *deficient* mice. Further, macrophage function and phenotype, as well as the antioxidant capacity of VitC *deficient* macrophages, was significantly impaired by the decline in intracellular VitC levels. Infusion of parenteral VitC as ascorbic acid (AscA) partly restored macrophage phenotype and function in VitC deficient mice.

2. Materials and Methods

2.1. Animals. $Gulo^{-/-}$ mice were bred in-house from an established homozygous colony as previously described [27]. In order to maintain their plasma VitC levels similar to that observed in humans, VitC sufficient mice were fed ad libitum with regular chow and water supplemented with vitamin C (0.33 g/L) renewed twice per week. $Gulo^{-/-}$ mice were made VitC deficient by reducing VitC supplementation (0.033 g/L) for 1 week, followed by complete removal of dietary VitC for additional 2 weeks. We and others have shown that this reduced supplementation significantly decreases the concentration of VitC in immune cells, plasma, and organs [27, 31, 32].

2.2. Thioglycollate Induced Peritonitis and Isolation of Mouse Peritoneal Macrophages. Thioglycollate-mediated peritonitis was established by intraperitoneal (i.p.) injection of 1 mL aged, sterile 3% TG solution to 9–11-week old $Gulo^{-/-}$ mice. Thirty minutes following i.p. challenge, some VitC deficient mice were randomized to receive daily i.p. injection of VitC as AscA (200 mg/kg in saline) for a further 3 or 5 days. Untreated mice received i.p. saline instead of VitC. Mice were euthanized on day 3 or day 5, and the peritoneal cavity lavaged with 7 mL of Hanks' balanced salt solution (HBSS) containing 1% bovine serum albumin (BSA). The lavage was centrifuged and the resulting leukocyte pellet was washed with HBSS and resuspended in RPMI-1640 medium. Cell counts of the peritoneal lavage were performed using a Hemocytometer. Cytochemical staining of peritoneal cells was performed using HARLECO Hemacolor solution (EMD Millipore) [28].

PMNs were then separated from macrophages by adherence to a plastic dish as described previously [28, 33]. Peritoneal macrophages were plated at a density of 2×10^6 cells in 35 mm dishes in growth media (DMEM, 10% FBS). Media were changed after 2 h to remove floating cells prior to experimentation.

2.3. Cell Culture. Human acute monocytic leukemia suspension cell line (THP-1) was obtained from ATCC (Manassas, VA). THP-1 cells were maintained in RPMI-1640 medium containing 10% FBS according to the instructions supplied. For induction of macrophages, PMA (100 nM) was added to the medium and cells were seeded at a density of 0.1×10^6 cells/cm^2 into tissue culture dishes and maintained in a humidified atmosphere of 95% air and 5% CO_2. Media containing PMA were replaced every 2 days, and experiments started after 5 days in culture, when the cells were phenotypically macrophage [34].

2.4. Vitamin C Analysis. Plasma and intracellular VitC levels of peritoneal macrophages seeded onto 35 mm dishes were measured using a fluorescence end-point assay adopted from Vislisel et al. [35]. Plasma was deproteinized as described previously [29]. Briefly, 0.2 mL of cold 20% trichloroacetic acid (TCA) and 0.2 mL of cold 0.2% dithiothreitol (DTT) were added to 0.1 mL of plasma, vortexed for 2 min, and centrifuged (10,000 g, 10 min, 4°C). Supernatants were aliquoted and frozen at −70°C for batch analysis. Peritoneal macrophages were similarly extracted with TCA and DTT and frozen at −70°C for batch analysis. Supernatant or AscA standards were transferred in triplicate to a 96-well plate. Assay buffer containing 1 M sodium acetate, pH 5.5, and 1 mM TEMPOL was added to each well and the plate was incubated for 10 minutes at room temperature. Freshly prepared o-phenylenediamine (OPDA) solution (5.5 mM OPDA in acetate buffer of pH 5.5) was then added. After a further 30 min incubation in the dark, fluorescence was measured at an emission wavelength of 425 nm following excitation at 345 nm and values determined after comparison to a standard curve. Intracellular AscA levels were estimated spectrophotometrically from the standard curve and the intracellular concentrations derived from the measured amount of AscA and the known macrophage cell volume [36].

2.5. RNA Isolation and Real-Time Quantitative PCR (QPCR) Analysis. Isolation of total RNA and real-time QPCR analyses were performed as described previously [37]. Briefly total RNA was extracted and purified using QIAshredders and RNeasy columns according to the manufacturer's specifications (Qiagen). Total RNA (1 μg) was reverse-transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit. Complimentary DNA (cDNA) was diluted (1 : 500) and real time QPCR performed using POWER SYBR Green QPCR Master Mix. Primers were designed to anneal to sequences on separate exons or to span two exons. Primers used for QPCR are listed in Table 1. Cycling parameters were 95°C, 10 min, 40 cycles of 95°C, 15 sec, and 60°C, 1 min. A dissociation profile was generated after each run to verify specificity of amplification. All PCR assays were performed in triplicate. No template controls and no reverse transcriptase controls were included. The mRNA expression in macrophages from a "sufficient" mouse or a media well was set to "1." The mRNA expression of all other samples was compared relative to this sample which was used as the baseline. 18S rRNA was used as housekeeping gene against which all the samples were normalized for differences in the amount of total RNA added to each cDNA reaction and for variation in the reverse transcriptase efficiency among the different cDNA reactions. Automated gene expression analysis was performed using the Comparative Quantitation module of MxPro QPCR Software (Agilent).

2.6. Western Blot Analysis. Mouse macrophage and THP-I whole-cell extracts were isolated for western blot analysis as described previously [37]. Proteins were resolved by SDS polyacrylamide gel electrophoresis (4–20%) and electrophoretically transferred to polyvinylidene fluoride membranes (0.2 μm pore size). Immunodetection was performed using chemiluminescent detection with the Renaissance Western Blot Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences Inc., Boston, MA). Blots were stripped using the Restore Western Blot Stripping Buffer (Pierce Biotechnology Inc., Rockford, IL) as described by the manufacturer. Purified rabbit polyclonal antibodies to phospho-NFκB p65 (Ser276, Cell Signaling), NFκB p65 (sc-109, Santa Cruz Biotechnology), iNOS (sc-650, Santa Cruz Biotechnology), and actin (sc-1616, Santa Cruz Biotechnology) were used. Optical densities of antibody-specific bands were determined using Quantity One acquisition and analysis software (Bio-Rad, Hercules, CA).

2.7. Flow Cytometry. Mouse peritoneal lavage obtained on day 3 and day 5 from VitC sufficient or deficient mice following induction of TG-induced peritonitis was pelleted by centrifugation at 4°C. Cells were resuspended in FACS buffer containing Fc receptor block (CD16/CD32 eBioscience) for 10 min at 4°C. Aliquots of the suspension were incubated at 4°C for 30 min (in the dark) with fluorescein isothiocyanate (FITC-)conjugated anti-mouse CD45 (eBioscience) and allophycocyanin (APC-)conjugated anti-mouse CD11b (eBioscience). Unstained and single color controls were employed for each experiment. Samples were then fixed with 1% formaldehyde for 20 min at room temperature. All runs were performed on a BD Accuri C6 Flow Cytometer (BD Accuri Cytometers, MI, USA) and analyzed using FlowJo software (Tree Star, Ashland, OR).

2.8. Fluorescence Microscopy. Fluorescence microscopy for evaluation of mitochondrial reactive oxygen species (ROS) in macrophages was performed using the cell-permeant probe MitoTracker Red CMXRos as described by the manufacturer. Briefly, macrophages from VitC sufficient or deficient mice were grown on Ibidi 6-channel IbiTreat μ-slide VI. Following treatments (H_2O_2, 18 hours) culture media were aspirated and cells were fixed in 3.7% paraformaldehyde in PBS for 10 minutes at 4°C. Fluorescence imaging was performed

Table 1: Primer sequences for real time quantitative PCR.

Name	Sequences 5′ to 3′
Murine IL-1β forward	CTGAACTCAACTGTGAAATGCC
Murine IL-1β reverse	CAGGTCAAAGGTTTGGAAGC
Murine TNFα forward	GATGAGAAGTTCCCAAATGGC
Murine TNFα reverse	TTGGTGGTTTGCTACGACG
Murine MCP-1 forward	TTCTGGGCCTGCTGTTCACAG
Murine MCP-1 reverse	CCAGCCTACTCATTGGGATCATCTTGC
Murine YM1 forward	CAAGACTTGCGTGACTATGAAGC
Murine YM1 reverse	AGGTCCAAACTTCCATCCTCC
Murine Arg1 forward	AGGAAAGCTGGTCTGCTGG
Murine Arg1 reverse	TTGAAAGGAGCTGTCATTAGGG
Murine IL-10 forward	CAAGGAGCATTTGAATTCCC
Murine IL-10 reverse	ATTCATGGCCTTGTAGACACC
Murine Gal1 forward	CAGCAACCTGAATCTCAAACC
Murine Gal1 reverse	AGTGTAGGCACAGGTTGTTGC
Murine 15-Lox forward	TGGTGGCTGAGGTCTTTGC
Murine 15-Lox reverse	TCTCTGAGATCAGGTCGCTCC
Human IL-6 forward	GGATTCAATGAGGAGACTTGCC
Human IL-6 reverse	TCTGCAGGAACTGGATCAGG
Human IL-8 forward	GTGTGAAGGTGCAGTTTTGC
Human IL-8 reverse	GAGCTCTCTTCCATCAGAAAGC
Human TNFα forward	CCTCTTCTCCTTCCTGATCG
Human TNFα reverse	CGAGAAGATGATCTGACTGCC

using an Olympus model IX70 inverted phase microscope (Olympus America, Melville, NY) outfitted with an IX-FLA fluorescence observation system equipped with a TRITC filter cube (530–560 nm excitation and 590–650 nm emission, Chroma Technology Corp. Brattleboro, VT) through an Uplan FI objective (40x). Fluorescence images were digitized and captured by a MagnaFire digital camera (Optronics, Goleta, CA).

2.9. Lipid Extraction and Analysis. Quantitative analysis of eicosanoids was performed as previously described by us with minor modifications [38–43]. Briefly, peritoneal lavage was clarified by centrifugation and 0.05% BHT and 10 ng of each internal standard added. The internal standards used were (d_4) 8-iso $PGF_{2\alpha}$, (d_{11}) 5-iso $PGF_{2\alpha}$-VI, (d_4) 6k $PGF_{1\alpha}$, (d_4) $PGF_{2\alpha}$, (d_4) PGE_2, (d_4) PGD_2, (d_4) LTB_4, (d_5) Lipoxin A4, (d_5) Resolvin D2, (d_4) TXB_2, (d_4) LTC_4, (d_5) LTD_4, (d_5) LTE_4, (d_8) 5-hydroxyeicosatetranoic acid (5HETE), (d_8) 15-hydroxyeicosatetranoic acid (15HETE), (d_8) 14,15 epoxyeicosatrienoic acid, (d_8) arachidonic acid, and (d_5) eicosapentaenoic acid. The samples were mixed by vortexing and subjected to purification via solid phase extraction (SPE) using a 24 port vacuum manifold (Sigma-Aldrich). Strata-X SPE columns (Phenomenex) were washed with methanol (2 mL) and then dH_2O (2 mL). The samples were applied to the column. Thereafter the sample vials were rinsed with 5% MeOH (2 mL), which was then used to wash the columns. Finally, the eicosanoids were eluted with isopropanol (2 mL). The eluents were then dried under vacuum and reconstituted in LCMS grade 50 : 50 EtOH : dH_2O (100 μL) for eicosanoid quantitation via UPLC ESI-MS/MS analysis. A 14-minute reversed-phase LC method utilizing a Kinetex C18 column (100 × 2.1 mm, 1.7 μm) and a Shimadzu UPLC was used to separate the eicosanoids at a flow rate of 500 μL/min at 50°C. The column was first equilibrated with 100% Solvent A (acetonitrile : water : formic acid (20 : 80 : 0.02, v/v/v)) for two minutes and then 10 μL of sample was injected. 100% Solvent A was used for the first two minutes of elution. Solvent B (acetonitrile : isopropanol (20 : 80, v/v)) was increased in a linear gradient to 25% Solvent B to 3 minutes, to 30% by 6 minutes, to 55% by 6.1 minutes, to 70% by 10 minutes, and to 100% by 10.1 minutes. 100% Solvent B was held until 13 minutes and then was decreased to 0% by 13.1 minutes and held at 0% until 14 minutes. The eluting eicosanoids were analyzed using a hybrid triple quadrapole linear ion trap mass analyzer (ABSciex 6500 QTRAP) via multiple-reaction monitoring in negative-ion mode. Eicosanoids were monitored using species specific precursor → product MRM pairs. The mass spectrometer parameters used were curtain gas: 30; CAD: High; ion spray voltage: −3500 V; temperature: 300°C; Gas 1: 40; and Gas 2: 60; declustering potential, collision energy, and cell exit potential were optimized per transition.

2.10. Statistical Analysis. Statistical analysis was performed using SAS 9.3 and GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). Data are expressed as mean ± SE. Results were compared using one-way ANOVA and the post

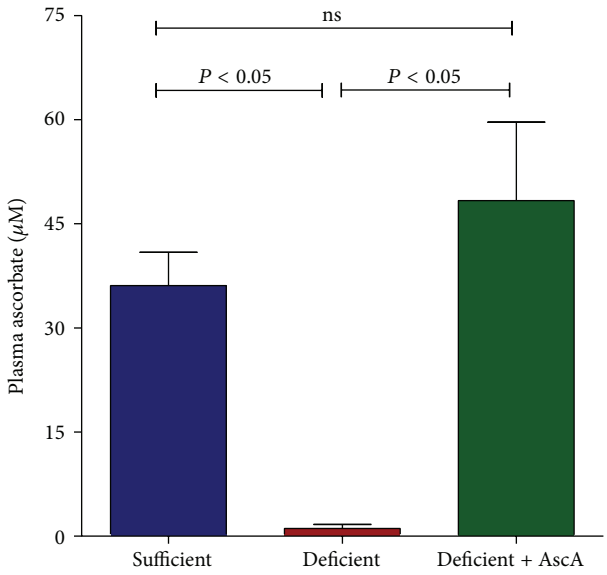

Figure 1: Vitamin C deficiency alters the progression of TG-induced peritoneal inflammation. Plasma VitC levels were measured in VitC *sufficient* and *deficient* Gulo$^{-/-}$ mice as well as in *deficient* mice treated daily with i.p. AscA for 3 days (N = 3–6 mice/group, ns = not significant).

Table 2: Differential cell counts from peritoneal exudates following thioglycollate-induced peritonitis (N = 6–8 mice/group, n.d. = not determined).

		Sufficient	Deficient	Deficient + AscA
Day 1	PMN (×10^6)	20.02 ± 3.4	23.2 ± 2.6	n.d.
	M0 (×10^6)	5.0 ± 0.8	3.9 ± 0.6	n.d.
Day 3	PMN (×10^6)	1.8 ± 0.4	8.1 ± 1.9[a]	3.8 ± 0.4
	M0 (×10^6)	18.4 ± 3.2	13.1 ± 3.1	14.5 ± 3.2
Day 5	PMN (×10^6)	1.0 ± 0.6	4.4 ± 1.1[b]	0.4 ± 0.2[c]
	M0 (×10^6)	18.8 ± 5.6	22.1 ± 4.2	14.3 ± 4.1

[a]Sufficient versus deficient, $P = 0.006$.
[b]Sufficient versus deficient, $P = 0.02$.
[c]Deficient versus deficient + AscA, $P = 0.02$.

hoc Tukey test. Statistical significance was confirmed at a P value of <0.05.

3. Results

3.1. VitC Deficiency Alters the Progression of TG-Induced Peritoneal Inflammation. In order to make the Gulo$^{-/-}$ mice VitC deficient, supplementation of water with AscA was withdrawn as described in the Methods section. Within 3 weeks of removal of VitC supplementation, plasma VitC levels of Gulo$^{-/-}$ mice declined significantly (Figure 1). This decline was not associated with deleterious changes in weight or health status in the VitC deficient mice (data not shown). To determine whether VitC deficiency impacts the progression of peritoneal inflammation, VitC sufficient or deficient mice were injected with TG and the progression of inflammation was monitored on days 3 and 5 (as described in Section 2). Some VitC deficient mice were injected i.p. with AscA (200 mg/kg) prior to harvest of peritoneal lavage (see Section 2). Daily i.p. administration of ascorbate for 3 days restored circulating plasma VitC concentrations in these mice to levels observed in the VitC sufficient mice (Figure 1). In all 3 groups, the infiltration of inflammatory cells on day 1 was similar to that observed in wild type mice [44] and was in agreement with our previous observations (Table 2) [28]. As seen in Table 2, there was also no difference in the total number of cells elicited from the peritoneal exudation of day 3 and day 5. However, significant differences in the cellular composition of the lavage were evident on day 3 and day 5 between the 3 groups. In the VitC sufficient mice group, mononuclear cells were the predominant cell type on days 3 and 5 (Table 2). PMN numbers, which peaked on day 1 [28], returned to baseline by days 3 and 5. In contrast, significantly elevated numbers of PMNs persisted in the peritoneal exudates of VitC deficient mice on days 3 and 5 (Table 2). Infusion of AscA reduced PMN numbers by day 3 with a significant decline in PMN numbers to baseline similar to the VitC sufficient mice by day 5 Table 2.

3.2. Spatiotemporal Profiling of Inflammatory Mediators following TG-Induced Peritoneal Inflammation. We previously observed that TG-elicited PMN from VitC deficient mice (on day 1) demonstrated increased expression of the proinflammatory genes TNFα and IL-1β [28]. Here we examined the expression of multiple pro- and anti-inflammatory mediators originating from macrophages, the predominant cell type recruited to the inflamed peritoneum on days 3 and 5. As seen in Figure 2, significant differences were evident in the magnitude of pro- and anti-inflammatory mediator expression on days 3 and 5. On day 3, increased expression of the proinflammatory mediators (IL-1β, TNFα, and MCP-1) was observed in macrophages from VitC deficient mice when compared to macrophages from VitC sufficient mice (Figure 2(a), (A), (C), and (E)). Proinflammatory gene expression was significantly attenuated by i.p. infusion of AscA in the VitC deficient mice (Figure 2(a), (A), (C), and (E)). In contrast, anti-inflammatory gene expression (YM1 and Arg1, but not IL-10) was elevated in macrophages from VitC sufficient mice (Figure 2(a), (B), (D), and (F)). Daily AscA infusion induced YM1 expression in VitC deficient macrophages but failed to restore Arg1 expression. IL-10 expression on the other hand was significantly lowered by AscA infusion on day 3 (Figure 2(a), (B), (D), and (F)).

On day 5 (Figure 2(b)), proinflammatory gene expression remained persistently elevated in macrophages from VitC deficient mice (IL-1β and MCP-1) but was attenuated by AscA infusion. In contrast, anti-inflammatory gene expression in VitC deficient macrophages was significantly higher when compared to macrophages from VitC sufficient mice (Arg1, IL-10). AscA infusion did not alter anti-inflammatory gene expression on day 5 although Arg1 levels were now similar to that observed in VitC sufficient mice (Figure 2(b), (B), (D), and (F)).

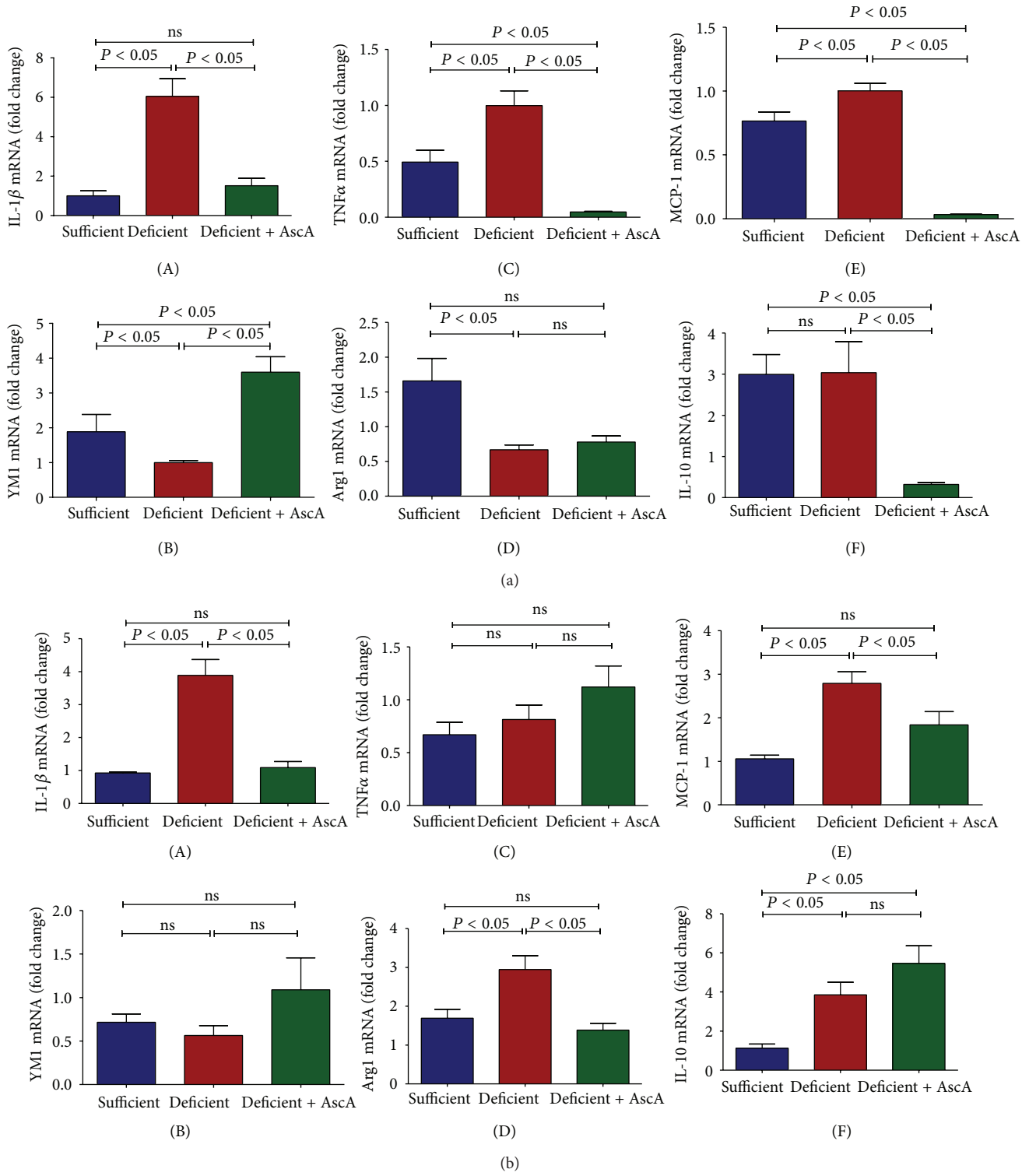

Figure 2: Spatiotemporal profiling of inflammatory mediators following TG-induced peritoneal inflammation. Real time QPCR for IL-1β, TNFα, MCP-1, YM1, Arg1, and IL-10 mRNA from peritoneal macrophages elicited on day 3 (a) and day 5 (b) following TG-induced peritonitis from VitC sufficient and deficient Gulo$^{-/-}$ mice. Following TG challenge, some VitC deficient mice were randomized to receive daily i.p. injection of VitC as AscA (200 mg/kg in saline) for a further 3 days (day 3, deficient + AscA) or 5 days (day 5, deficient + AscA) ($N = 6$ mice/group, ns = not significant).

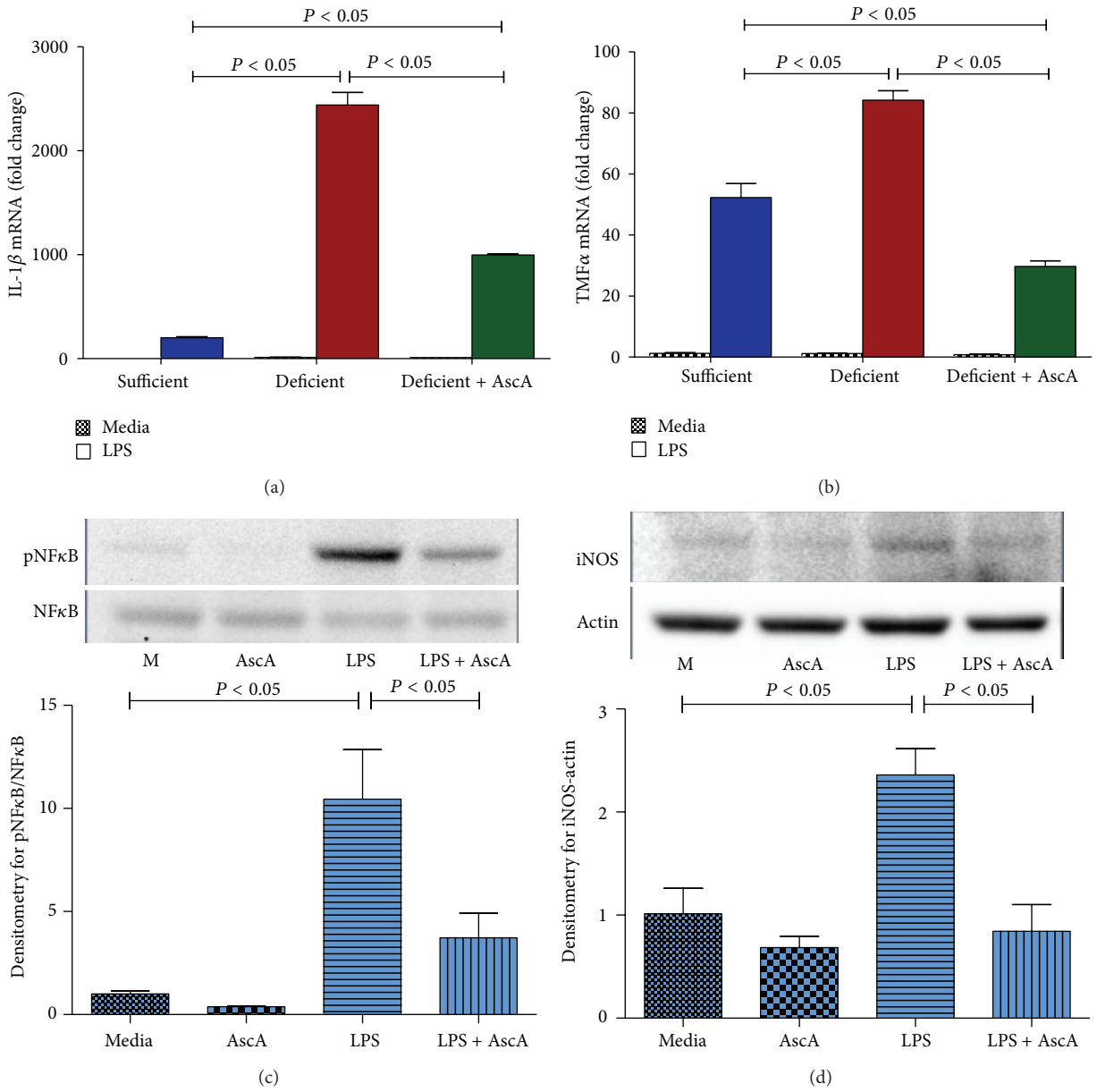

Figure 3: LPS differentially activates proinflammatory gene expression in macrophages from vitamin C sufficient and deficient mice. Peritoneal macrophages elicited on day 3 following TG-induced peritonitis from VitC sufficient and deficient Gulo$^{-/-}$ mice were exposed to LPS (50 ng/mL) for 4 hours. Macrophages from some VitC deficient mice were incubated with AscA (3 mM, 16 hours) prior to LPS exposure (deficient + AscA). Real time QPCR was performed for IL-1β (a) and TNFα (b) (N = 6/group). (c) Upper panel: representative western blot for expression of phospho-NFκB and NFκB from VitC deficient macrophages exposed to media alone (M), AscA (3 mM, 16 hours (AscA)), LPS (50 ng/mL) for 1 hour (LPS), or AscA for 16 hours followed by LPS for 1 hour (LPS + AscA). Lower panel: densitometry for normalized expression of phospho-NFκB from macrophages (N = 3/group). (d) Upper panel: representative western blot for expression of iNOS and actin from macrophages groups described in (c) and exposure to LPS (50 ng/mL) for 4 hour. Lower panel: densitometry for normalized expression of iNOS from macrophages (N = 3/group).

3.3. Ex Vivo Bacterial Lipopolysaccharide Differentially Activate Proinflammatory Gene Expression in Macrophages from VitC Sufficient and Deficient Mice. Canali et al. recently showed that in contrast with baseline physiological activation, exposure to a second "hit" such as an inflammatory stimulus results in a markedly different modulation of gene expression in human peripheral blood mononuclear cells in the presence or absence of VitC supplementation [45]. To examine whether peritoneal macrophages would exhibit an altered modulation of gene expression, we exposed day 3 peritoneal macrophages from VitC sufficient and deficient mice to bacterial lipopolysaccharide (LPS, 50 ng/mL). Some macrophages were incubated with AscA (3 mM, 16 hours) prior to LPS exposure. As seen in Figures 3(a) and 3(b), LPS

exposure resulted in a robust increase in expression of proinflammatory markers (IL-1β, TNFα) in macrophages from VitC sufficient mice. Proinflammatory gene expression was also induced in macrophages from VitC deficient mice, but the magnitude of induction was significantly greater than that observed in the VitC sufficient macrophages (Figures 3(a) and 3(b)). Importantly, exposure of VitC deficient macrophages to AscA prior to LPS significantly attenuated IL-1β and TNFα expression. Increased NFκB activation (Figure 3(c)) and iNOS protein expression (Figure 3(d)) was observed upon exposure of VitC deficient macrophages to LPS ($P < 0.05$). AscA pretreatment attenuated NFκB activation and iNOS expression in VitC deficient macrophages.

3.4. VitC Regulates Macrophage Function during the Resolution of Inflammation. Macrophages undergo reprogramming to adopt a variety of functional phenotypes upon receiving differentiation cues from their surrounding environment [46]. It was recently shown that macrophage reprogramming is vital for resolution of acute inflammation [47]. We examined whether macrophage VitC sufficiency or deficiency could influence macrophage function during resolution of acute inflammation. Macrophages were isolated on day 3 following TG-mediated peritonitis from VitC sufficient or deficient mice and intracellular concentrations of VitC measured (as described in Section 2). Some VitC deficient mice were injected daily with i.p. AscA (200 mg/kg) prior to harvest of peritoneal lavage (see Section 2). As seen in Figure 4(a), macrophages from VitC sufficient mice have high intracellular VitC concentrations. In contrast, intracellular ascorbate levels were significantly depleted in macrophages from VitC deficient mice. Daily i.p. administration of AscA for 3 days also restored macrophage intracellular concentrations to levels observed in the VitC sufficient mice (Figure 4(a)).

Gal-1 and 15-Lox expression is induced in macrophages during peritonitis. Their expression is associated with generation of proresolving lipid mediators [11] and successful resolution of inflammation [48]. Therefore we examined Gal-1 and 15-Lox expression in day 3 and day 5 macrophages from VitC sufficient or deficient mice. As seen in Figure 4(b), Gal-1 and 15-Lox expression was significantly induced in macrophages from VitC sufficient mice on day 3 when compared to macrophages from VitC deficient mice. AscA infusion restored Gal-1 expression in VitC deficient macrophages but did not affect 15-Lox expression on day 3 (Figure 4(b)). In contrast, Gal-1 expression in VitC deficient macrophages was delayed and observed to be higher on day 5 following TG-induced peritonitis (Figure 4(c)). 15-Lox expression was induced by AscA infusion on day 5 and was higher than that observed in macrophages from VitC sufficient or deficient mice. In agreement with the expression data seen above, resolvin (Figure 4(d)) production was higher on day 5 in VitC deficient mice indicating delayed resolution of inflammation.

3.5. VitC Influences Macrophage Phenotype during Resolution of Inflammation. Rostoker et al. recently showed that Gal-1 was selectively expressed in CD11b^{high} macrophages, and its expression declined significantly once these cells converted toward a CD11b^{low} phenotype [49]. Moreover, CD11b^{low} macrophages are the predominant subtype to depart the peritoneum [49]. To determine whether VitC regulated reprogramming of peritoneal macrophages to proresolution CD11b^{low} phenotype we used flow cytometry to examine the distribution of CD11b^{high} and CD11b^{low} population on macrophages isolated on day 3 and day 5 following TG-induced peritonitis in VitC sufficient or deficient mice. As seen in Figure 5, there was a significant transition from CD11b^{high} to a CD11b^{low} phenotype observed from day 3 to day 5 in the VitC sufficient macrophages. This was not evident in the macrophages from VitC deficient mice indicative of a delay in the resolution of TG-induced peritonitis in these mice.

3.6. Macrophages Deficient in VitC Have Reduced Antioxidant Capacity. Activated macrophages potentially generate mitochondria-damaging deleterious reactive oxygen species (ROS). Release of large amounts of ROS during activation exposes macrophages themselves to oxidant stresses not encountered by most other cell types [50]. To test whether VitC deficiency affected mitochondrial function in macrophages, we exposed peritoneal macrophages (day 3) from VitC sufficient or deficient mice to varying concentrations of H_2O_2 for 18 hours and stained the cells with Mito-Tracker Red CMXRos as described in Section 2. This probe is selectively retained by mitochondria, where it is oxidized to its fluorescent form. As seen in Figures 6(a) and 6(e), control macrophages from VitC sufficient or deficient mice were stained brightly with the probe. Oxidative stress from exposure to H_2O_2 decreased fluorescent staining in macrophages from both VitC sufficient and VitC deficient mice. However, the magnitude of decrease was significantly greater in macrophages from VitC deficient mice (Figures 6(f)–6(h)). This decrease was partially reversed by pretreatment of VitC deficient macrophages with AscA (Figures 6(j) and 6(k)). These studies indicate that VitC deficient macrophages sustain greater mitochondrial dysfunction when challenged with ROS.

3.7. VitC Attenuates Proinflammatory Gene Expression in Human Monocyte/Macrophages. To address whether the modulatory activities of VitC are effective in human monocyte/macrophages, we exposed THP-1 cells to bacterial LPS and examined the mRNA expression of the proinflammatory genes IL-6, IL-8, and TNFα. Since the culture medium in which THP-1 cells are grown contains no VitC, we increased intracellular concentrations of VitC by loading cells with AscA prior exposure to LPS. As seen in Figure 7(a), exposure of THP-1 cells to LPS resulted in a robust activation of mRNA for IL-6, IL-8, and TNFα. Loading cells with AscA did not affect baseline proinflammatory gene expression. However LPS exposure of AscA loaded cells resulted in significant attenuation of mRNA expression of these proinflammatory genes. Attenuation of mRNA expression was likely achieved by reduction in activation of the transcription factor NFκB following LPS exposure (Figure 7(b)).

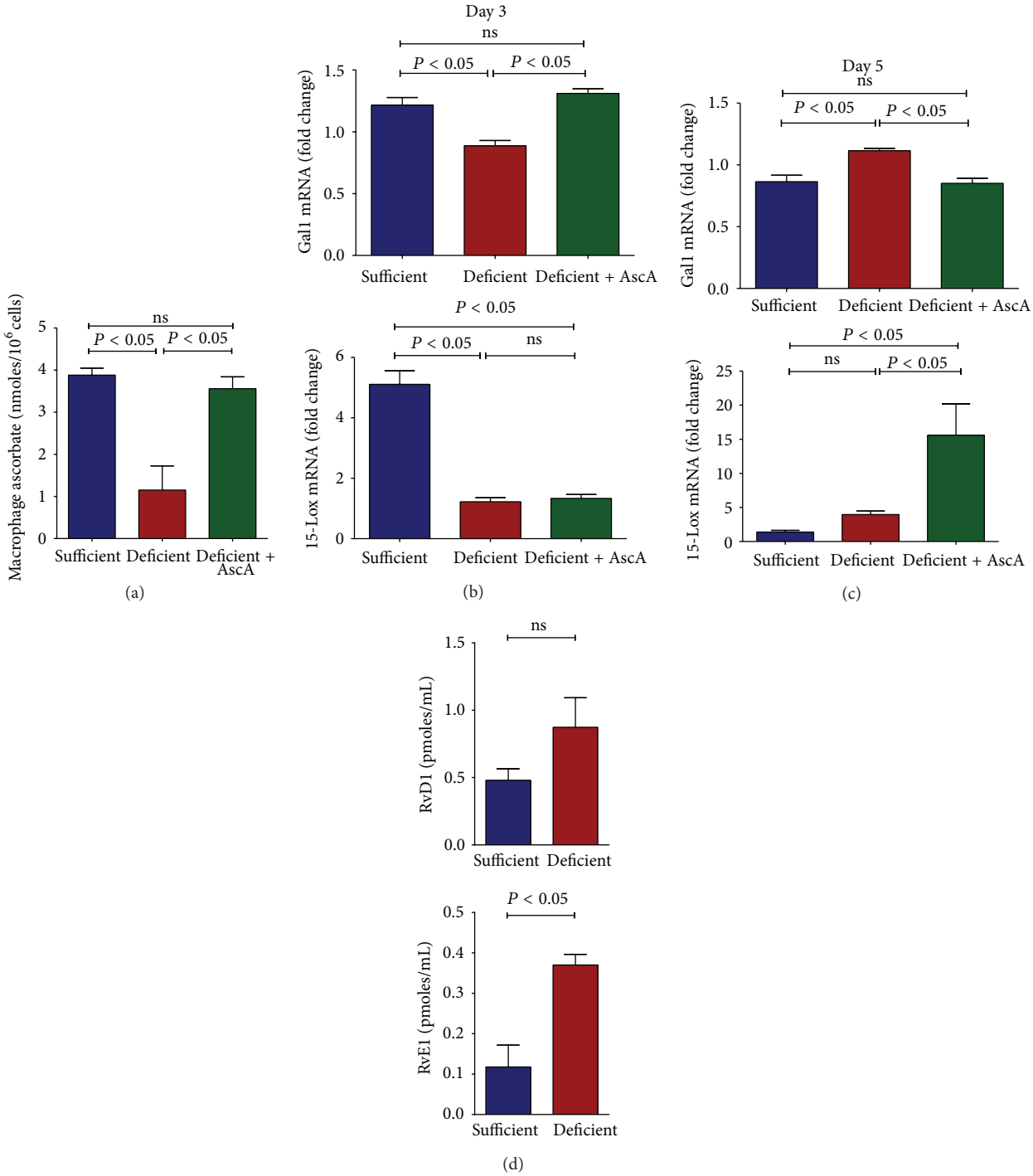

FIGURE 4: Vitamin C regulates macrophage function during the resolution of inflammation. (a) Macrophages were isolated on day 3 following TG-mediated peritonitis from VitC sufficient or deficient mice as well as in deficient mice treated daily with i.p. AscA for 3 days and intracellular concentrations of VitC measured (N = 3–10 mice/group, ns = not significant). (b) Real time QPCR for Gal1 and 15-Lox from peritoneal macrophages elicited on day 3 following TG-induced peritonitis from VitC sufficient and deficient Gulo$^{-/-}$ mice. Thirty minutes following TG challenge, some VitC deficient mice were randomized to receive i.p. injection of VitC as AscA (200 mg/kg in saline) for a further 3 days (deficient + AscA). (N = 6 mice/group, ns = not significant). (c) Real time QPCR for Gal1 and 15-Lox from peritoneal macrophages elicited on day 5 following TG-induced peritonitis from VitC sufficient and deficient Gulo$^{-/-}$ mice. Thirty minutes following TG challenge, some VitC deficient mice were randomized to receive i.p. injection of VitC as AscA (200 mg/kg in saline) for a further 5 days (Deficient + AscA) (N = 6 mice/group, ns = not significant). (d) UPLC ESI-MS/MS quantification of resolvin D1 (RvD1) and E1 (RvE1) in peritoneal lavage on day 5 following TG-induced peritonitis from VitC sufficient and deficient Gulo$^{-/-}$ mice (N = 3-4 mice/group, ns = not significant).

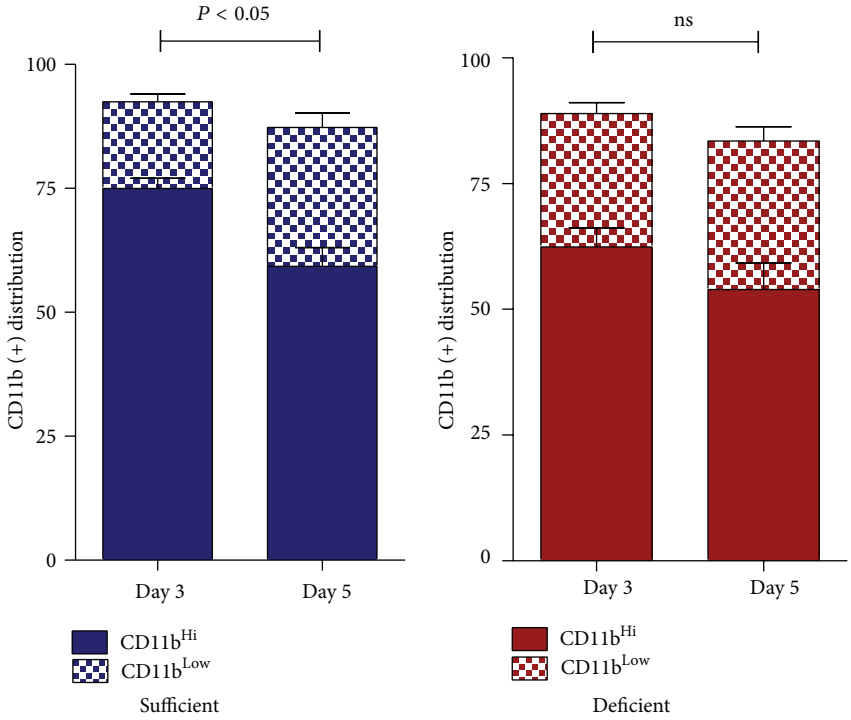

Figure 5: Vitamin C influences macrophage phenotype during resolution of inflammation. Flow cytometry for distribution of $CD11b^{high}$ and $CD11b^{low}$ population from macrophages isolated on day 3 and day 5 following TG-induced peritonitis in VitC sufficient or deficient mice ($N = 5$ mice/group, $P < 0.05$, $CD11b^{low}$ day 3 versus day 5, ns = not significant).

4. Discussion

In this study, we examined the mechanism by which VitC regulates the resolution of sterile inflammation. Using mice lacking the ability to synthesize VitC, we show that subnormal cellular VitC levels negatively impact the progression and resolution of sterile inflammation. In particular, our results demonstrate that low circulating VitC levels are associated with significant delays in the timing of resolution of inflammation. This apparent VitC-dependent process primarily occurs due to failure of macrophages to transition from a proinflammatory to a proresolving phenotype.

The initial response to sterile inflammation was identical in VitC sufficientanddeficient mice. During the early proinflammatory phase no differences in the cell numbers or cell types were observed. However, by days 3 and 5, VitC deficient mice exhibited significant numbers of PMN in peritoneal exudates (Table 2). Spatiotemporal mRNA profiling of macrophage-derived inflammatory mediators revealed dramatic differences in the magnitude of pro- and anti-inflammatory mediator gene expression (Figure 2). Macrophages from VitC sufficient mice displayed prominent anti-inflammatory phenotypes, while VitC deficient macrophages persistently expressed mRNA for IL-1β, TNFα, and MCP-1, findings characteristic of a proinflammatory phenotype. LPS activation of day 3 macrophages from VitC deficient mice led to proinflammatory gene expression that was significantly greater in magnitude than that observed in VitC loaded macrophages (Figure 3). LPS stimulation was characterized by enhanced NFκB activation and iNOS induction in VitC deficient macrophages (Figure 3). Importantly, on day 3, VitC sufficient macrophages demonstrated cues for reprogramming into resolution type macrophages, a vital step required for resolution of inflammation. In day 3 macrophages from VitC sufficient mice, expression of Gal-1 and 15-Lox mRNA was robust (Figure 4). In contradistinction, enhanced Gal-1 and 15-Lox mRNA expression was delayed to day 5 in VitC deficient macrophages. The delays in resolution we observed in VitC deficient mice were confirmed by quantification of resolvins in peritoneal exudates; increases of which were present only on day 5 (Figure 4). Further confirmation of altered spatiotemporal relationships was achieved by studying macrophage phenotypic changes by examining the distribution of CD11b on macrophages from VitC sufficient or deficient mice on day 3 and day 5 following TG-induced peritonitis (Figure 5). Phenotypic changes in macrophages were accompanied by alterations in macrophage function as demonstrated by the increased susceptibility of VitC deficient macrophages to mitochondrial dysfunction when exposed to reactive oxygen species (Figure 6). In final studies, we employed the human monocyte/macrophage cell line THP-1, which lacks VitC in culture medium when cultured under standard conditions. We demonstrated increased proinflammatory gene expression in THP-1 when exposed to LPS under VitC-deprived conditions. Loading THP-1 cells with AscA significantly

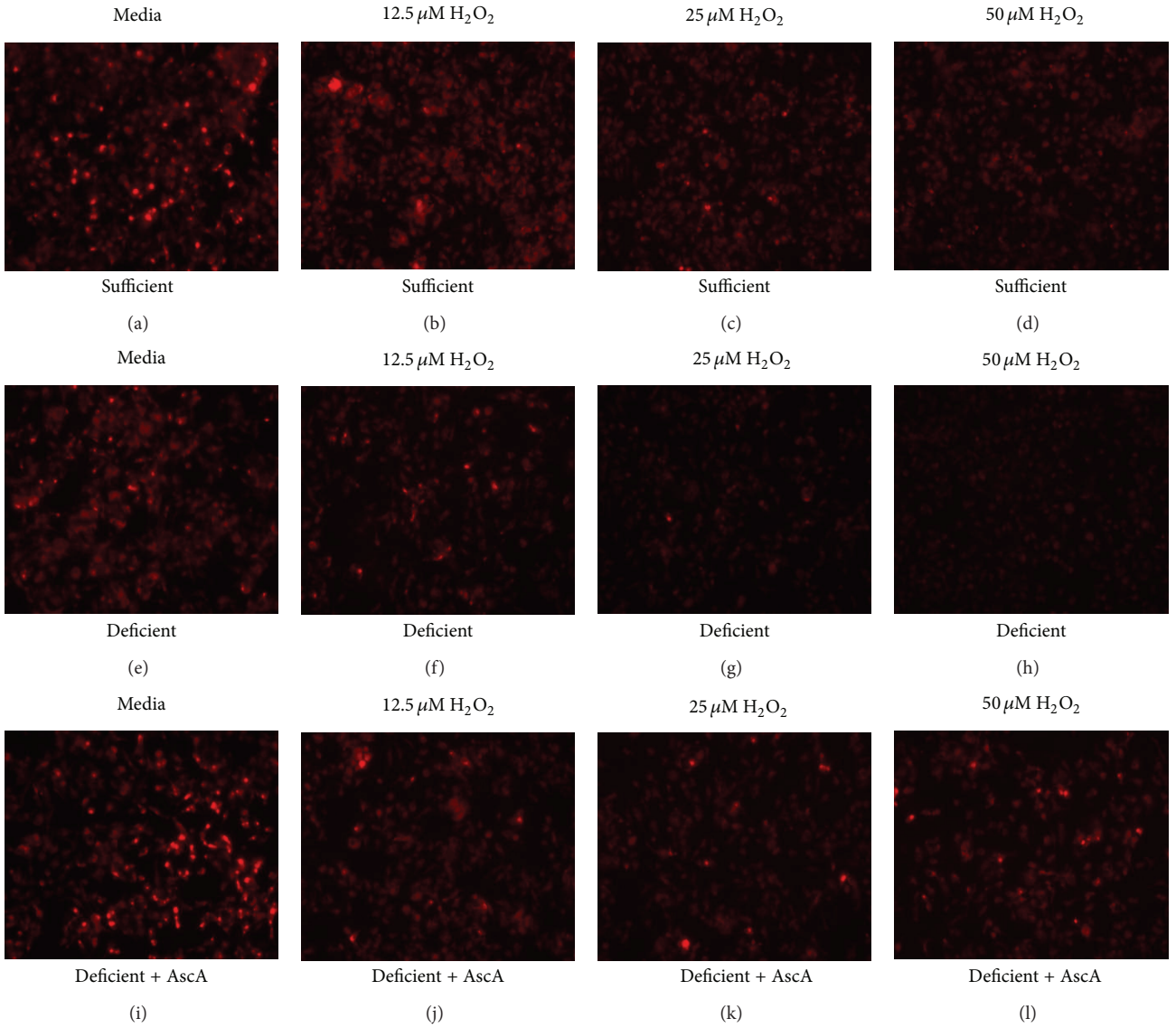

Figure 6: Macrophages deficient in Vitamin C have reduced antioxidant capacity. Peritoneal macrophages elicited on day 3 following TG-induced peritonitis from VitC sufficient ((a)–(d)) and deficient Gulo$^{-/-}$ ((e)–(h)) mice were exposed to 12.5, 25, and 50 μM H_2O_2 for 18 hours and probed with MitoTracker Red CMXRos. Macrophages from some VitC deficient mice were incubated with AscA (3 mM, 16 hours) prior to exposure to H_2O_2 followed by staining with MitoTracker Red CMXRos (Deficient + AscA, ((i)–(l))).

attenuated mRNA expression of proinflammatory genes via a mechanism likely involving reduced activation of the transcription factor NFκB. VitC loading was effective both *in vitro* and *in vivo* since daily AscA infusion following induction of peritonitis significantly restored macrophage phenotype and function in the VitC deficient mice.

Few studies have examined the role of VitC in resolution of sterile inflammation. Ganguly et al. initially reported that VitC deficiency affected migration of guinea pig macrophages under *in vitro* conditions [51]. They further showed that addition of exogenous VitC partially restored the migratory response. May et al. showed that activated macrophages use ascorbate to lessen self-generated oxidant stress [18]. They later showed that ascorbate deficient peritoneal macrophages were more susceptible to H_2O_2-induced mitochondrial dysfunction and apoptosis [52]. However no studies to date have examined macrophage function during resolution of inflammation in mice lacking the ability to synthesize their own VitC. Our observation of persistence of PMN at the site of inflammation in VitC deficient mice is in agreement with our previous results [28] and those of Vissers and Wilkie who used a similar TG model of peritonitis to show impairment in PMN apoptosis and clearance [19]. It has been suggested that the engulfment of apoptotic cells is generally anti-inflammatory or immunologically silent [53] due to the fact that it sequesters dying cells thus preventing release of potentially toxic cell contents into the local environment. Based on the observations that PMN persists for up to 5

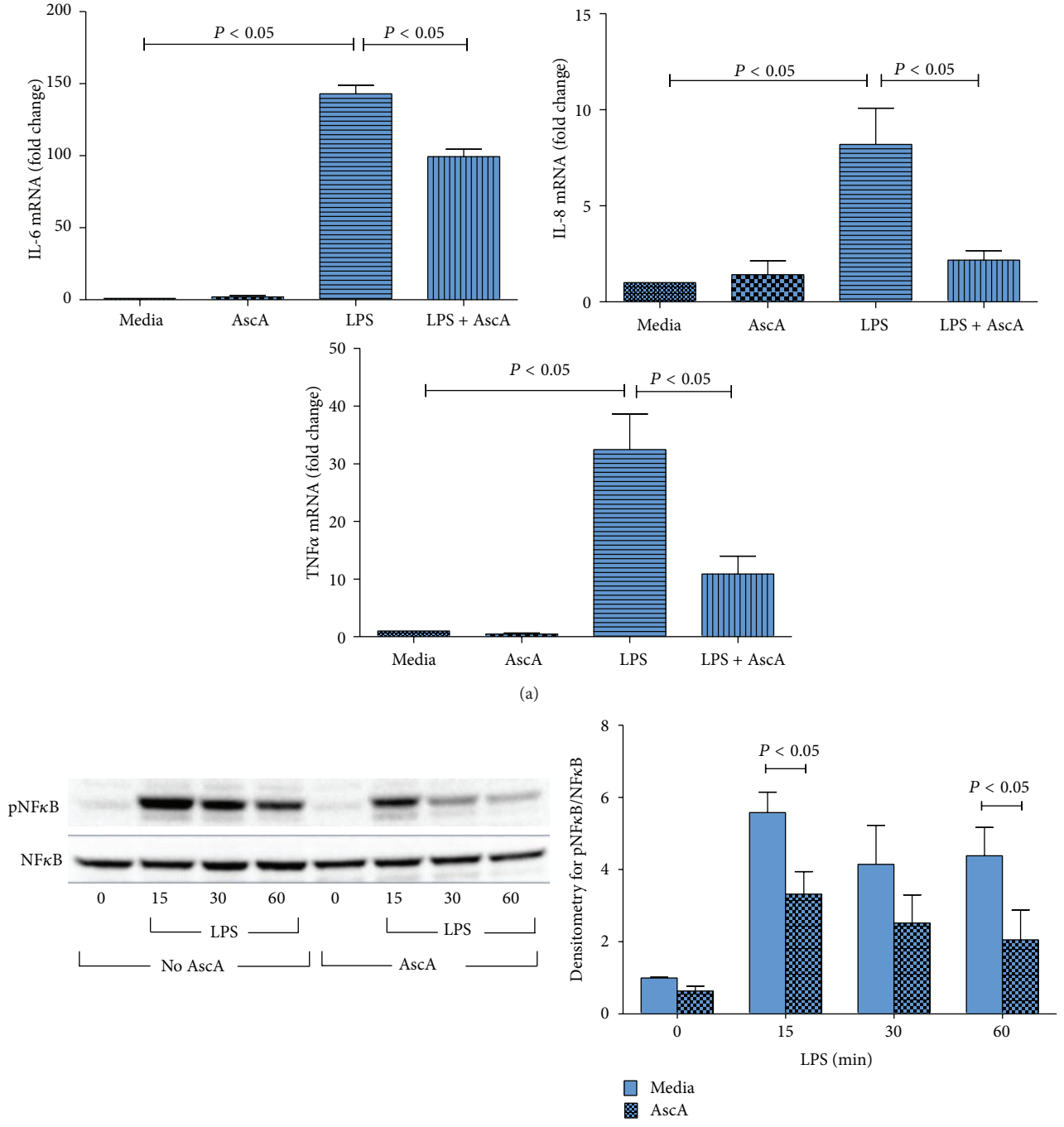

Figure 7: Vitamin C attenuates proinflammatory gene expression in human THP-1 monocyte/macrophages. THP-1 macrophages were exposed to media alone (Media), AscA (3 mM, 16 hours (AscA)), and LPS (50 ng/mL) for 4 hour (LPS) or AscA for 16 hours followed by LPS for 4 hour (LPS + AscA). (a) Real time QPCR for IL-6, IL-8, and TNFα was performed as described in Section 2 ($N = 4$/group; $P < 0.05$, Media versus LPS and LPS versus LPS + AscA). (b) Left panel: representative western blot for expression of phospho-NFκB and NFκB from THP-1 groups described above following exposure to LPS (50 ng/mL) for 0, 15, 30, and 60 minutes. Right panel: densitometry for normalized expression of phospho-NFκB from THP-1 ($N = 4$/group, $P < 0.05$, LPS versus LPS + AscA).

days in the peritoneum of VitC deficient mice (Table 2), it is therefore possible that the apoptosis-resistant PMN can cause strong proinflammatory responses from the macrophages that extravasate to sites of inflammation. Indeed strong and persistent proinflammatory responses were evident in VitC deficient macrophages elicited on day 3 and even day 5 (Figure 2).

Efferocytosis, a process by which dead and/or dying cells are being engulfed and removed by other cells, has been reported to induce production of anti-inflammatory mediators from macrophages that suppress inflammation thereby silently clearing apoptotic cells and thus dampening proinflammatory responses [54]. VitC sufficient mice exhibited anti-inflammatory mediator expression in macrophages early (day 3) in the post TG-induced inflammatory process, a phenomenon indicative of functional efferocytosis. In contrast, VitC deficient macrophages failed to upregulate anti-inflammatory mediator production until day 5 (Figure 2).

Gal-1 and 12/15-Lox play vital roles in resolution of inflammation. Rostoker et al. have shown that Gal-1 promotes the generation of M2-like macrophages, which then favors tissue repair during early resolution of inflammation [49]. Ariel and Timor demonstrated that Gal-1 promotes generation of M_{res} from M2 macrophages, which generates proresolving lipid mediators. This phenotype change promotes macrophage departure from peritoneal cavities with resolving inflammation, thus allowing return of tissue to homeostasis [55]. Moreover, Gal-1 expression, which is enhanced in $CD11b^{high}$ macrophages, declines sharply as cells revert to the $CD11b^{low}$ phenotype. $CD11b^{low}$ macrophage phenotypes, as noted previously, promote departure from peritoneal cavities with resolving inflammation [49]. Our findings (Figures 4 and 5) which agree with the above studies implicate VitC as a critical regulator of macrophage transition during resolution of inflammation. Expression and function of 12/15-Lox produce key mediators (e.g., lipoxins, resolvins, protectins, and maresins) that promote resolution of proinflammatory pathologies [56]. In particular, human and murine monocytes/macrophages expression of 15-Lox is upregulated by efferocytosis with production of mediators such as RvD1, a mediator shown to promote the resolution of murine peritonitis [47, 57]. Further, Gal-1 directly promotes 15-lipoxygenase expression and activity in macrophages during the inflammatory and resolving phases of peritonitis [49]. The earlier increases in Gal-1 and 15-Lox mRNA expression in VitC sufficient macrophages (Figures 4(b) and 4(c)) and the delayed resolvin production in the VitC deficient macrophages (Figure 4(d)) indicate for the first time that VitC influences multiple processes leading to the resolution of inflammation.

5. Conclusions

The findings in this mouse model have significant human relevance since VitC levels are subnormal in multiple human inflammatory disease states including sepsis, systemic inflammatory response syndrome (SIRS), trauma, and cancer, among others. In a recently completed Phase I trial (ClinicalTrials.gov identifier NCT01434121) of intravenous AscA in critically ill patients with severe sepsis, we showed that septic patients exhibited abnormally low VitC plasma levels and that intravenous AscA infusion could significantly increase circulating VitC levels [29]. Further, AscA infusion significantly reduced the proinflammatory biomarkers C-reactive protein and procalcitonin as well as multiple organ dysfunction [29]. Our findings here add a previously unrecognized element to our understanding of the machinery that governs the resolution of inflammation.

Conflict of Interests

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

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Research Article

Jussara (*Euterpe edulis* Mart.) Supplementation during Pregnancy and Lactation Modulates the Gene and Protein Expression of Inflammation Biomarkers Induced by *trans*-Fatty Acids in the Colon of Offspring

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Maternal intake of *trans*-fatty acids (TFAs) in the perinatal period triggers a proinflammatory state in offspring. Anthocyanins contained in fruit are promising modulators of inflammation. This study investigated the effect of Jussara supplementation in the maternal diet on the proinflammatory state of the colon in offspring exposed to perinatal TFAs. On the first day of pregnancy rats were divided into four groups: control diet (C), control diet with 0.5% Jussara supplementation (CJ), diet enriched with hydrogenated vegetable fat, rich in TFAs (T), or T diet supplemented with 0.5% Jussara (TJ) during pregnancy and lactation. We showed that Jussara supplementation in maternal diet (CJ and TJ groups) reduced carcass lipid/protein ratios, serum lipids, glucose, IL-6, TNF-α, gene expression of IL-6R, TNF-αR ($P < 0.05$), TLR-4 ($P < 0.01$), and increase *Lactobacillus* spp. ($P < 0.05$) in the colon of offspring compared to the T group. The IL-10 ($P = 0.035$) and IL-10/TNF-α ratio ($P < 0.01$) was higher in the CJ group than in the T group. The 0.5% Jussara supplementation reverses the adverse effects of perinatal TFAs, improving lipid profiles, glucose levels, body composition, and gut microbiota and reducing low-grade inflammation in the colon of 21-day-old offspring, and could contribute to reducing chronic disease development.

1. Background

Variations in maternal nutrition during pregnancy and lactation may alter the physiological and morphological development of the fetus and the newborn by epigenetic modification. This process, known as metabolic programming or metabolic imprinting, can alter gene expression and permanently affect the structure and function of organs and tissues, increasing an individual's susceptibility to the development of chronic diseases [1–3].

The composition of fatty acids in the maternal diet during pregnancy and/or lactation is thus a key factor in determining whether fetal and postnatal development proceeds normally. We previously demonstrated that maternal intake of *trans*-fatty acids (TFAs), obtained industrially by partial hydrogenation of vegetable oils [4], can promote adverse effects in offspring as well as increasing the tumor necrosis factor-α (TNF-α) mRNA expression, plasminogen activator inhibitor-1 (PAI-1) mRNA expression, and TNF receptor-associated

factor-6 (TRAF-6) protein in the adipose tissue of 21-day-old offspring [5, 6]. Furthermore, in adult offspring of dams fed TFAs, increased PAI-1 mRNA expression in the adipose tissue [6], increased serum endotoxin levels, NF-κBp65, TLR-4, and MyD88 protein expression, and induced hypothalamic increases in IL-6, TNF-α, and IL-1β [7].

Other studies have demonstrated that nutritional fatty acid exposure at perinatal stages modulates the functionality and inflammatory status of a variety of tissues and organs, including white and brown adipose tissue, skeletal muscle, and liver [5–9]. Additionally, differences in maternal dietary fat can change gut phospholipids, microbiota, intestinal permeability, and the colonic inflammatory response in offspring, particularly in animal disease models [10, 11].

Furthermore, it has been established that dietary fats modulate the gut microflora, increase colonic permeability, and trigger low-grade colon inflammation in healthy adult animals [12, 13].

The gastrointestinal tract is the first organ exposed to dietary components, and its functionality and integrity have systemic implications. In this sense, modification of microbiota, inflammation of the gut, and increases in intestinal permeability could mediate or contribute to disease development and metabolic disorders as has been proposed recently [14–16]. This process evolves with damage to the integrity of the intestinal barrier, causing an increase in bacterial translocation, and therefore an increase in the serum concentration of the external cellular membranes of gram-negative intestinal bacteria, which include lipopolysaccharide (LPS) and result in Toll-like receptor-4- (TLR-4-) mediated inflammatory responses [16–18].

LPS-induced TLR-4 provokes an inflammatory response through activation of the NF-κB signaling pathway and subsequent expression of proinflammatory cytokines such as TNF-α and IL-6 [19, 20]. Likewise, elevated serum levels of free fatty acids or saturated fatty acids (SFAs) can stimulate the TLR-4 and NF-κB signaling pathways and the inflammatory response [20–22].

During the perinatal period, diets rich in lipids, particularly TFAs, increase TFA-free, long-chain SFAs and decrease polyunsaturated fatty acids (PUFAs) in breast milk [23, 24]. In addition, intake of TFAs changes the lipid profile and elevates LPS serum concentration, increasing proinflammatory cytokines in offspring [5–7].

In contrast, *cis*-unsaturated fatty acids in the diet reduce the production of inflammatory cytokines and downregulate inflammation by inhibiting the NF-κB signaling pathway [25, 26]. Dietary fibers, especially prebiotics, also have favorable effects on the expression of inflammatory cytokines [27, 28] by decreasing colonic pH, stimulating the gut probiotic bacterial colonization and reducing intestinal permeability and consequently the migration of LPS to circulation [18, 28–30].

Evidence has highlighted the contribution of the maternal flora to gut growth and function in the newborn. Some strains of the mother's bacterial flora are transferred through the maternal skin, fecal and vaginal contact, or breast milk [31, 32]. The transmission of maternal flora is an important variable in offspring development and health because the mother's microbiota and milk content can be affected by dietary factors [10, 32].

Foods rich in flavonoids have been identified as promising modulators of inflammation and oxidative stress [33, 34]. The fruit of the Jussara palm (*Euterpe edulis* Mart.) is a species native to the Atlantic Forest/Brazil. The fruits are rich in *cis*-unsaturated fatty acids, PUFAs, and dietary fiber and are a source of anthocyanins, flavonoids that have been shown to have high antioxidant activity, inhibit cell proliferation, and play an important role in inflammation modulation in adult animals [35–38].

Studies investigating the effect of supplementation of the maternal diet with fruits phenolics content during gestation and lactation on the inflammatory process of the offspring and the influence of early-life nutritional factors on the gut intestinal tract of healthy offspring are rare. Thus, the aim of this study was to investigate the effect of Jussara supplementation on the TFA-induced proinflammatory state in the intestinal tract of 21-day-old offspring.

2. Materials and Methods

2.1. Animals and Treatments. All experimental procedures were approved by the Experimental Research Committee of the Federal University of Sao Paulo (Protocol number 859814).

Rats were kept under controlled conditions of light (12 : 12 h light-dark cycle with lights on at 07:00) and temperature (24 ± 1°C), with *ad libitum* water and food.

Twelve-week-old female Wistar rats of first-order parity were left overnight to mate. Copulation was verified the following morning by the presence of sperm in vaginal smears. On the first day of gestation, rats were isolated in individual cages and randomly assigned to one of four groups receiving a control diet (C diet, C group), a control diet supplemented with Jussara 0.5% freeze-dried powder (CJ diet, CJ group), a diet enriched with hydrogenated vegetable fat (T diet, T group), or a T diet supplemented with 0.5% Jussara freeze-dried powder (TJ diet, TJ group).

The diets were prepared according to the recommendations of the American Institute of Nutrition (AIN-93G) [39, 40] and had similar caloric and lipid content. The source of lipids for the C and CJ diets was soybean oil; the principal source for the T and TJ diets was partially hydrogenated vegetable fat rich in TFAs. The CJ and TJ diets were prepared by adding 5 g/kg of Jussara freeze-dried powder to each diet. Jussara pulp (*Euterpe edulis* Mart.) was obtained from the agroecological Project Juçara/IPEMA—Institute of Permaculture and Ecovillages of the Atlantic (Ubatuba, SP, Brazil) and then freeze-dried to powder using a lyophilizer. Diets were then stored at −20°C. The phenolic compounds and anthocyanin contents of the Jussara pulp were previously analyzed in our laboratory [36]. The centesimal composition of the diets is presented in Table 1. The fatty acid profile of C and T diets was previously described by Pisani et al. [6].

Dams' diets were maintained during pregnancy and lactation. After birth, litter sizes were adjusted to eight pups that remained with the mother. The pups were weighed and

Table 1: Composition of the control diet (C), control diet supplemented with 0.5% freeze-dried Jussara powder (CJ), diet enriched with hydrogenated vegetable fat, TFAs (T), and diet enriched with TFAs supplemented with 0.5% freeze-dried Jussara powder (TJ) according to AIN-93.

Ingredient	Diet (g/100 g)			
	C	CF	T	TF
Casein*	20.0	20.0	20.0	20.0
L-cystine†	0.3	0.3	0.3	0.3
Cornstarch†	62.0	62.0	62.0	62.0
Soybean oil‡	8.0	8.0	1.0	1.0
Hydrogenated vegetable fat$	—	—	7.0	7.0
Butylhydroquinone†	0.0014	0.0014	0.0014	0.0014
Mineral mixture§	3.5	3.5	3.5	3.5
Vitamin mixture#	1.0	1.0	1.0	1.0
Cellulose†	5.0	5.0	5.0	5.0
Choline bitartrate†	0.25	0.25	0.25	0.25
Freeze-dried Juçara powder£	—	0.5	—	0.5
Energy (kcal/g)	4.00	4.02	4.00	4.02

*Casein was obtained from Labsynth, São Paulo, Brazil.

†L-cystine, cornstarch, butylhydroquinone, cellulose and choline bitartrate were obtained from Viafarma, São Paulo, Brazil.

‡Oil was supplied from soybean (Lisa/Ind. Brazil).

$Hydrogenated vegetable fat was supplied from Unilever, São Paulo, Brazil.

§Mineral mix (9 mg/kg diet): calcium, 5000; phosphorus, 1561; potassium, 3600; sodium, 1019; chloride, 1571; sulfur, 300; magnesium, 507; iron, 35; copper, 6.0; manganese, 10.0; zinc, 30.0; chromium, 1.0; iodine 0.2; selenium, 0.15; fluoride, 1.00; boron, 0.50; molybdenum, 0.15; silicon, 5.0; nickel, 0.5; lithium, 0.1; vanadium, 0.1 (AIN-93G, mineral mix, Rhoster, Brazil).

#Vitamin mix (mg/kg diet): thiamin HCL, 6.0, riboflavin, 6.0; pyridoxine HCL, 7.0; niacin, 30.0; calcium pantothenate, 16.0; folic acid, 2.0; biotin, 0.2; vitamin B12, 25.0; vitamin A palmitate 4000 IU; vitamin E acetate, 75; vitamin D3, 1000 IU; vitamin KI, 0.75 (AIN-93G, vitamin mix, Rhoster, Brazil).

£Freeze-dried Juçara powder: Juçara pulp (*Euterpe edulis* Mart.) was obtained from agroecological Project Juçara/IPEMA—Institute of Permaculture and Ecovillages of the Atlantic (Ubatuba, SP, Brazil)—and by freeze-drying to powder using a lyophilizer.

measured (nasoanal length) at birth and on postnatal days 7, 14, and 21. After 21 days the offspring were decapitated. Trunk blood was collected and centrifuged. Serum was separated and stored at −80°C for later determination of triacylglycerol (TAG), total cholesterol, HDL-cholesterol, and glucose levels. The colon was removed and the fecal content was isolated; both were stored at −80°C.

2.2. Biochemical Serum Analyses. Glucose, triacylglycerol, total cholesterol, and HDL-cholesterol serum concentrations were measured with an enzymatic colorimetric method using commercial kits (Labtest Brazil).

2.3. Carcass Lipid and Protein Content. The carcasses were eviscerated and the remnants were weighed and stored at −20°C. The lipid content was measured as described by Stansbie et al. [41] and standardized using the method described by Oller Do Nascimento and Williamson [42]. The carcass was autoclaved at 120°C for 90 min and homogenized with water at a volume twice the carcass mass. Triplicate aliquots of approximately 3 g were digested in 3 mL of 30% KOH and 3 mL of ethanol for ≥2 h at 70°C in capped tubes. After cooling, 2 mL of 12 N H_2SO_4 was added and the samples were washed three times with petroleum ether to extract the lipids. The results are expressed as grams of lipid per 100 g of carcass. To measure the protein content, aliquots of the same homogenate, approximately 1 g, were heated to 37°C for 1 h in 0.6 N KOH with constant shaking. After clarification by centrifugation, protein content was measured using the Bradford assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a reference.

2.4. RNA Extraction and Real-Time Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from tissues with Tri-reagent (Sigma, St. Louis, MO, USA) and its concentration was determined from 260/280 nm absorbance ratios taken with a NanoDrop 2000/2000c (NanoDrop Technologies Inc., Wilmington, DE, USA). The TLR-4, TNF-αR, and IL-6R mRNA expression from colons were quantified by real-time polymerase chain reaction using a SYBR Green primer in StepOne Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA). Relative levels of the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT) were measured. The PCR primers used are listed in Table 2. Results were obtained using StepOne Software 2.1 (Applied Biosystems) and are expressed as a relative increase, using the method of $2^{-\Delta\Delta Ct}$ described by Livak and Schmittgen [43].

2.5. Genomic DNA Extraction from Fecal Samples and RT-PCR. Genomic DNA was extracted from colon fecal samples with the Qiagen QIAamp DNA Stool Minikit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations. The DNA concentration per microliter was measured using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA), and the readings were acquired at wavelengths of 260, 280, and

Table 2: Nucleotide sequence of the forward and reverse primers for the RT-PCR.

Target mRNA	Forward primer	Reverse primer
HPRT	5′-CTCATGGACTGATTATGGACAGGA-3′	5′-GCAGGTCAGCAAAGAACTTATAGC-3′
TLR-4	5′-GCATCATCTTCATTGTCCTTGAGA-3′	5′-CTACCTTTTCGGAACTTAGGTCTACT-3′
TNF-αR	5′-GAA CAC CGT GTG TAA CTG CC-3′	5′-ATT CCT TCA CCC TCC ACC TC-3′
IL-6R	5′ AAGCAGGTCCAGCCACAATGTAG 3′	5′ CCAACTGACTTTGAGCCAACGAG 3′
All bacteria	5′-TCC TAC GGG AGG CAG CAG T-3′	5′-GAC TAC CAG GGT ATC TAA TCC TGT T-3′
Lactobacillus spp.	5′-AGC AGT AGG GAA TCT TCC A-3′	5′-CAC CGC TAC ACA TGG AG-3′

Table 3: Serum glucose, total cholesterol, HDL-cholesterol, and triacylglycerols in 21-day-old offspring.

	C (15)	CJ (20)	T (19)	TJ (19)
Glucose	110.19 ± 3.44	103.99 ± 1.73$^{\#}$	118.51 ± 2.96	98.83 ± 1.14$^{*\#}$
Total cholesterol	120.05 ± 4.25	114.43 ± 3.57$^{\#}$	136.01 ± 2.85*	108.79 ± 2.23$^{\#}$
HDL-cholesterol	26.56 ± 1.48	28.92 ± 1.29	25.84 ± 0.71	28.34 ± 1.03
Triacylglycerols	180.20 ± 11.37	131.11 ± 4.06$^{*\#}$	209.19 ± 16.33	135.15 ± 3.72$^{*\#}$

C: offspring of dams fed control diet; CJ: offspring of dams fed control diet supplemented with 0.5% freeze-dried Jussara powder; T: offspring of dams fed diet enriched with hydrogenated vegetable fat, TFAs; TJ: offspring of dams fed diet enriched with TFAs supplemented with 0.5% freeze-dried Jussara powder. Data are presented as mean ± SEM. The number in parentheses refers to the sample value.
$^{*}P < 0.05$ versus C. $^{\$}P < 0.05$ versus CJ. $^{\#}P < 0.05$ versus T. $^{\&}P < 0.05$ versus TJ.

230 nm. The purity was estimated by the 260/280 nm ratio, which must range between 1.8 and 2.0 for nucleic acids. All samples were maintained at −80°C.

2.6. Lactobacillus spp. Quantified by RT-PCR. Relative levels of *Lactobacillus* spp. DNA were quantified in real time, using a SYBR Green primer in an ABI Prism 7500 Sequence Detector (both from Applied Biosystems, Foster City, CA, USA). Relative levels of the housekeeping gene of all bacteria were measured. The PCR primers used are listed in Table 2. The results were obtained using Sequence Detector software (Applied Biosystems) and are expressed as a relative increase, using the method of $2^{-\Delta\Delta Ct}$, described by Livak and Schmittgen [43].

2.7. Colon TNF-α, IL-6, and IL-10 Protein Levels by ELISA. The colon was homogenized and centrifuged at 12,000 rpm for 40 min at 4°C; the supernatant was saved and the protein concentration determined using the BCA assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA) as a reference. Quantitative assessment of TNF-α, IL-6, and IL-10 proteins was carried out by ELISA (DuoSet ELISA, R&D Systems, Minneapolis, MN, USA) following the recommendations of the manufacturer. All samples were run as duplicates and the mean value was reported.

2.8. Statistical Analysis. Statistical analyses were performed using the Sigma Stat 3.5. The data were analyzed by ANOVA followed by a Bonferroni posthoc or Kruskal-Wallis test. All results are presented as the mean ± SEM and $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Body Weight, Body Weight Gain, Length of the Animal, and Carcass Lipid and Protein Content. At birth, offspring of the CJ group were longer than those of the T group ($P < 0.05$) (Figure 1(c)). The body weight (BW) and length were reduced in the pups of the T group compared to those of the C group ($P = 0.02$ and $P < 0.05$, resp.) (Figures 1(a) and 1(c)). At postnatal day 21, the length of pups did not differ between the groups and the BW of the CJ group was lower than the C ($P = 0.042$), T ($P = 0.006$), and TJ ($P = 0.006$) groups (Figure 1(a)). Furthermore, the CJ and TJ groups displayed longer body length than the C group ($P < 0.001$ and $P = 0.017$) at postnatal day 14 (Figure 1(c)). The CJ group also exhibited decreased BW gain compared with the T and C groups ($P < 0.001$) three weeks after birth (Figure 1(b)).

The relative carcass lipid levels in the CJ and TJ groups were significantly lower than in the C group ($P < 0.05$). The TJ group exhibited higher relative carcass protein levels than the T group ($P = 0.003$) and the T group contained less relative carcass protein than the C group ($P = 0.006$). The lipid to protein carcass ratio was lower in the CJ and TJ groups than in the T group ($P < 0.05$) (Figure 1(d)).

3.2. Biochemical Serum. The T group had increased serum concentrations of total cholesterol compared to the C group at postnatal day 21 ($P = 0.008$) while the CJ and TJ groups exhibited reduced serum levels of total cholesterol ($P < 0.001$) and glucose ($P < 0.05$) compared to the T group. Furthermore, in the TJ group the glucose concentration was lower than in the C group ($P < 0.05$). Triacylglycerol levels were also reduced in the CJ and TJ groups compared to the C and T groups ($P < 0.05$). HDL-cholesterol levels in the serum were similar in all groups (Table 3).

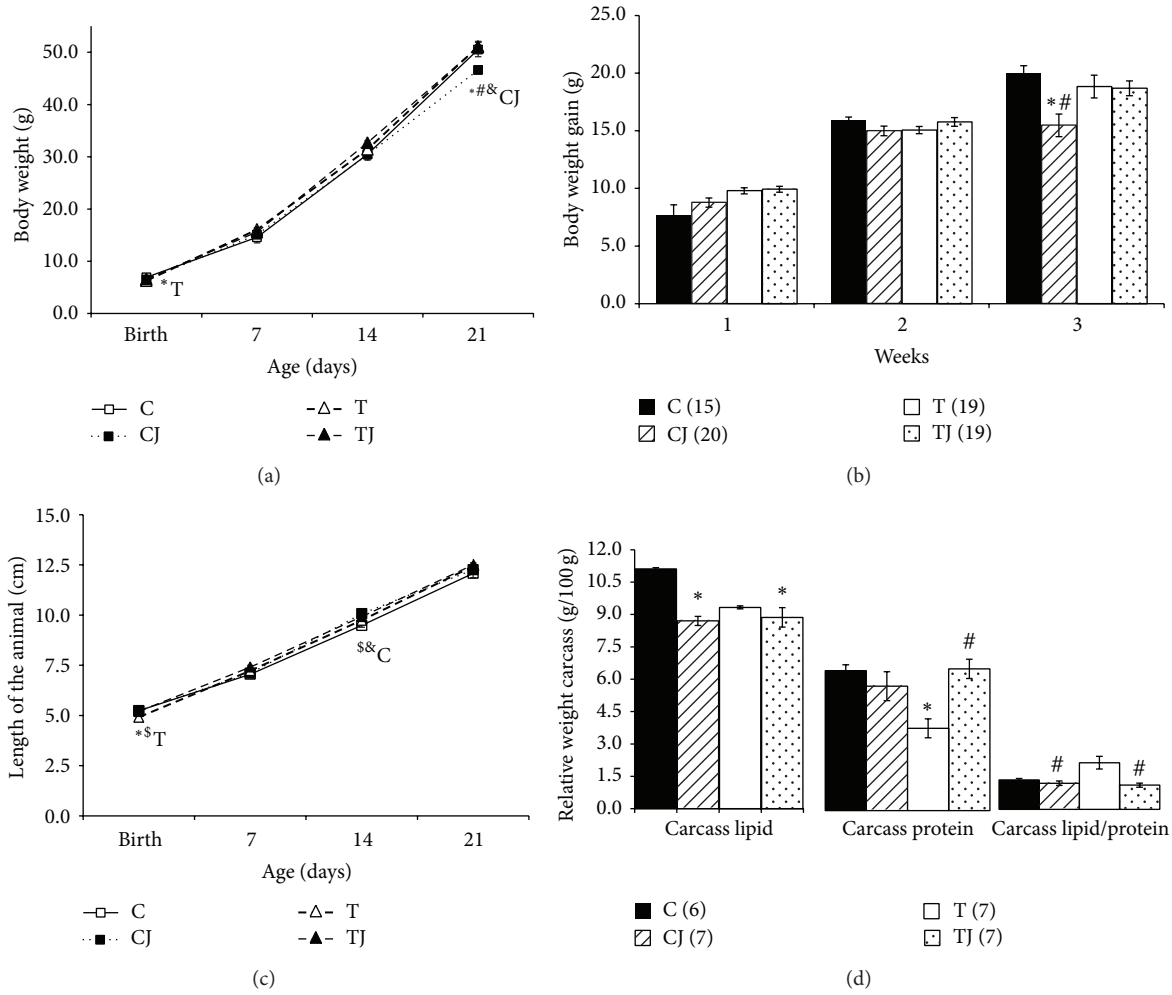

Figure 1: Body weight (a), body weight evolution (b), length (c), and carcass lipid, protein content, and lipid/protein ratio (d). C: offspring of dams fed control diet; CJ: offspring of dams fed control diet supplemented with 0.5% freeze-dried Jussara powder; T: offspring of dams fed diet enriched with hydrogenated vegetable fat, TFAs; TJ: offspring of dams fed diet enriched with TFAs supplemented with 0.5% freeze-dried Jussara powder. Data are means ± SEMs. The number in parentheses refers to the sample value. $^{*}P < 0.05$ versus C. $^{\$}P < 0.05$ versus CJ. $^{\#}P < 0.05$ versus T. $^{\&}P < 0.05$ versus TJ.

3.3. IL-6R, TNF-αR, and TLR-4 Gene Expression. The TLR-4 gene expression in the colon of 21-day-old offspring was higher in the T group (83.1%) than in the C group ($P = 0.047$). Levels of TNF-αR mRNA expression also increased in the T group (49.8%), but this difference was not significant. However, the CJ and TJ groups showed lower levels of TNF-αR mRNA expression (CJ group 52.1%, $P = 0.013$ versus T group; TJ group 48.9%, $P = 0.027$ versus T group) and TLR-4 mRNA expression (CJ group 63.1%, $P = 0.002$ versus T group; TJ group 66.5%, $P = 0.002$ versus T group) in offspring at postnatal day 21 (Figures 2(b) and 2(c)). In addition, the IL-6R gene expression decreased in the CJ and TJ groups compared to the T group (50.9%, $P < 0.001$; 30.2%, $P = 0.02$, resp.) and also in the CJ group compared to C group (40.7%, $P = 0.005$) (Figure 2(a)).

3.4. Levels of Lactobacillus spp. in Colon. The levels of *Lactobacillus* spp. genomic DNA in colon fecal content in the CJ and TJ groups were 4.2-fold higher and 2.6-fold higher, respectively, than in the T group ($P < 0.05$). The T group level of *Lactobacillus* spp. genomic DNA was 2.1-fold lower than the C group level; however, this difference was not significant (Figure 2(d)).

3.5. Cytokine Profile of the Colon. The protein levels of IL-6 (36.5%) and TNF-α (36.2%) were significantly higher ($P = 0.048$ and $P = 0.013$, resp.) in the T group than in the C group in offspring at postnatal day 21. However, the Jussara supplementation in the CJ and TJ groups reduced the levels of IL-6 (CJ group 31.3%, $P = 0.011$; TJ group 40.2%, $P < 0.001$) and TNF-α (CJ group 28.1% $P = 0.013$ and TJ group 35.8%, $P < 0.001$) compared to the T group (Figures 3(a) and 3(c)). Furthermore, in the CJ group, the expression of IL-10 protein was higher than in both the T group (63.4%, $P = 0.035$) and TJ group (80.2%, $P = 0.011$) (Figure 3(b)). Thus, the IL-10/TNF-α ratio in the CJ group increased compared to the T

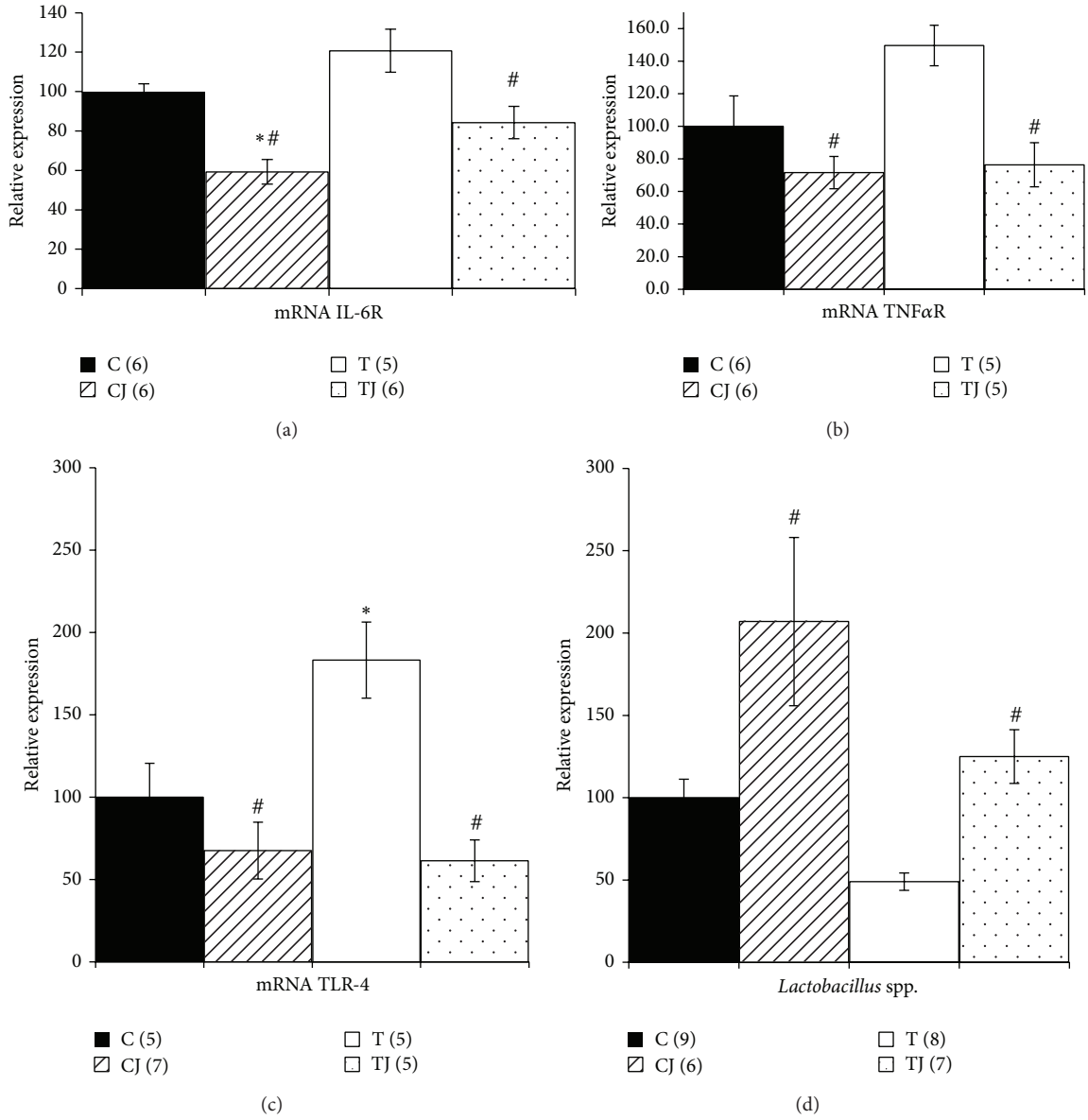

Figure 2: Gene expression of IL-6 receptor (IL-6R) (a), tumor necrosis factor-α receptor (TNF-αR) (b), Toll-like receptor 4 (TLR4) (c), and DNA levels of *Lactobacillus* spp. in 21-day-old offspring colon (d). C: offspring of dams fed control diet; CJ: offspring of dams fed control diet supplemented with 0.5% freeze-dried Jussara powder; T: offspring of dams fed diet enriched with hydrogenated vegetable fat, TFAs; TJ: offspring of dams fed diet enriched with TFAs supplemented with 0.5% freeze-dried Jussara powder. Data are means ± SEMs. The number in parentheses refers to the sample value. Results are expressed in arbitrary units, stipulating 100 as the control value. $^{*}P < 0.05$ versus C. $^{\#}P < 0.05$ versus T.

group (70.2%, $P = 0.003$) and was reduced in the T group compared to the C group (35.1%, $P = 0.026$) (Figure 3(d)).

4. Discussion

In this study, supplementing the maternal diet with 0.5% Jussara attenuated the adverse effects of perinatal TFAs. We showed that the maternal intake of Jussara in perinatal period modulates the inflammatory state and improves the lipid profile, glucose levels, body composition, and intestinal microbiota of 21-day-old offspring.

In our study, Jussara supplementation of the maternal diet did not affect the growth of pups at birth but in 21 day of life offspring from Jussara-supplemented dams had lower BW gain and better body composition with lower lipid content and higher carcass protein (Figure 1).

Corroborating our data, a study with açaí (*Euterpe Oleracea* Mart.) a fruit similar to Jussara, reported that supplementation with hydroalcoholic extract (200 mg/kg/day) during the pregnancy does not change BW in offspring at birth [44]. However, Rahal et al. [45] found that supplementation with 3% blueberry, which is rich in anthocyanin, to

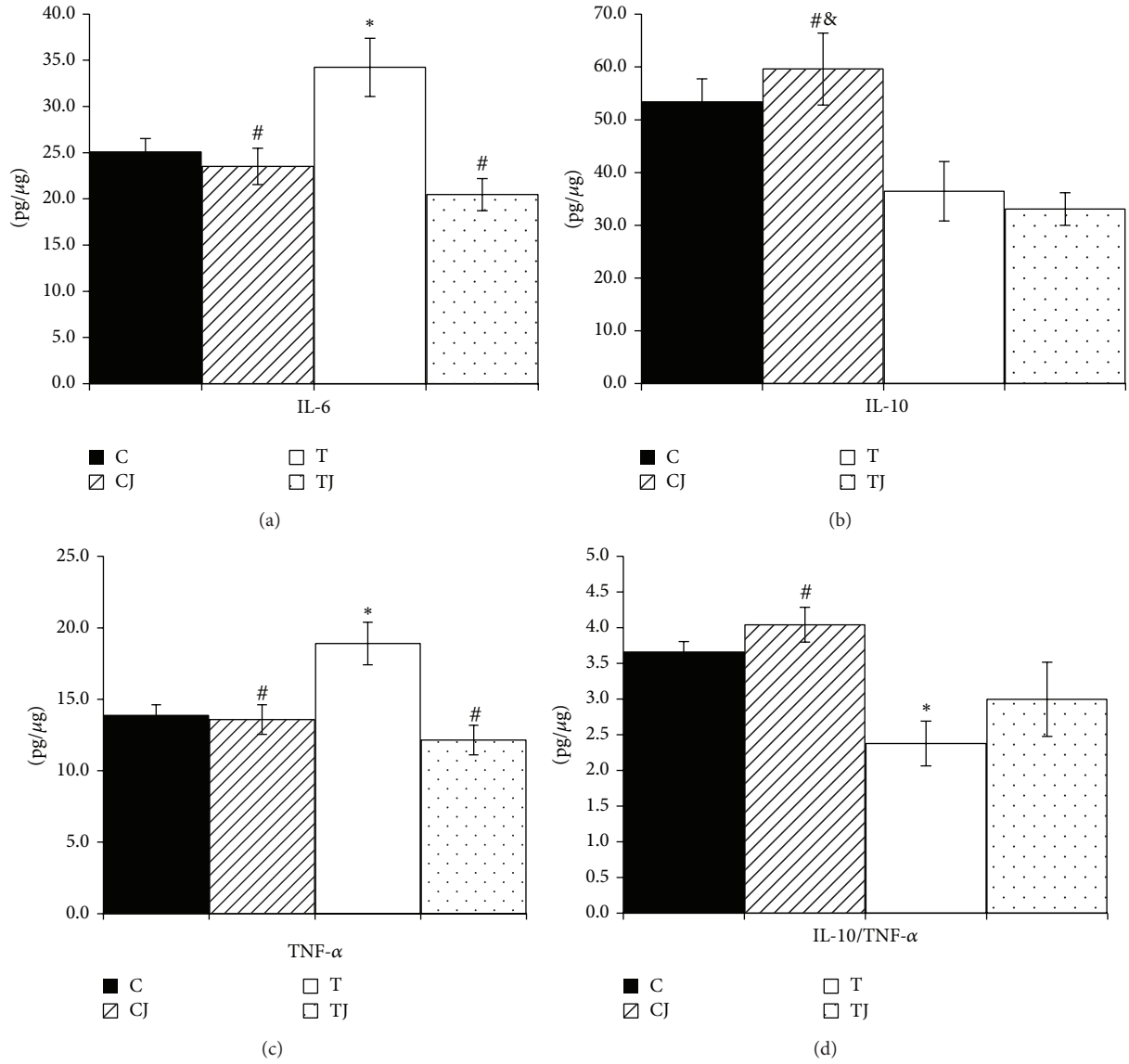

Figure 3: IL-6 protein expression (a), IL-10 (b), TNF-α (c), and IL-10/TNF-α ratio (d) in 21-day-old offspring colon. C: offspring of dams fed control diet; CJ: offspring of dams fed control diet supplemented with 0.5% freeze-dried Jussara powder; T: offspring of dams fed diet enriched with hydrogenated vegetable fat, TFAs; TJ: offspring of dams fed diet enriched with TFAs supplemented with 0.5% freeze-dried Jussara powder. Data are means ± SEMs of 7–11 determinations per group. $^{*}P < 0.05$ versus C. $^{\#}P < 0.05$ versus T. $^{\&}P < 0.05$ versus TJ.

the maternal diet during pregnancy and lactation in MMTV-Wnt1-transgenic mice does not affect BW of the offspring at weaning. Furthermore, authors demonstrated that 2% Jussara supplementation in adult ApoE-deficient mice leads to no change in BW during the entire experimental period [46].

Our findings also indicate that the addition of Jussara to the maternal diet restores total cholesterol to a normal range and reduces serum TAG and glucose in 21-day-old offspring. Similar effects have been reported by De Souza et al. [47] after supplementation with 2% açaí for 6 weeks, with decreased total cholesterol observed in female Fischer rats. In fact, studies have indicated that the beneficial effect of similar fruit on the lipid profile is linked to diverse components contained in the fruit, such as *cis*-unsaturated fatty acids, polyunsaturated fatty acids (PUFAs), polyphenols, and dietary fiber. These components are associated with reduced intestinal absorption of fatty acids, greater balance in the synthesis and absorption of sterols, and increased expression of genes involved in cholesterol metabolism and excretion in the adult animals [48–50].

Moreover, there is evidence that phenolic compounds, particularly the flavonoids, induces Glut 4 in the adipose tissue and skeletal muscle and can improve glucose homeostasis and lipid metabolism via AMP-activated protein kinase activation in adult animal models [51].

Our study found that the Jussara supplementation in maternal diet led to a reduction in proinflammatory cytokines (IL-6, TNF-α) and receptors (TNFαR, IL-6R, and

TLR-4 mRNA expression) induced from TFAs to normal levels, accompanied by an increase in anti-inflammatory cytokines (IL-10, IL-10/TNF-α ratio) and *Lactobacillus* spp. genomic DNA levels in the colon of offspring.

Indeed, TFAs are known for their ability to increase expression of inflammatory markers such as IL-6 and TNF-α [4, 52] and there is evidence that exposure to a high-fat diet increases inflammation in the colon [14, 15, 53] while high-polyphenol diet reduces this process [54]. Previous studies have shown that in adult offspring of mothers fed TFAs during pregnancy and lactation, high levels of LPS activate TLR-4 and mediate low-grade inflammation [7]. Additionally, as described in other studies, changes in the composition of the microbiota and the subsequent alteration of membrane permeability damage intestinal barrier integrity. This damage can cause an increase in bacterial translocation and uptake of LPS, resulting in TLR4-mediated inflammatory responses in the offspring [16, 55]. Thus, this suggests a potential mechanism by which TFAs increase the inflammatory status of the offspring colon.

In accordance with our results, we believe that the anti-inflammatory effect of Jussara also could be associated with the high nutritional value, *cis*-unsaturated fatty acids, PUFAs, bioactive compounds levels as phenolics (415 mg GAE/100 g f.m.), particularly anthocyanins (239.16 ± 7.6 mg C3R/100 g), and dietary fiber presence in the fruit of the Jussara palm [35, 36].

PUFAs can influence synthesis of the proinflammatory cytokines TNF-α and IL-6 in order to downregulate inflammatory transcription factors by actions upon intracellular signaling through the inhibition of NF-κB pathway [56–58]. In this sense, the study performed by Fong et al. [59] in female rats fed a diet containing DHA, polyunsaturated long chain fatty acids (DHA group), during pregnancy and lactation, found decreased expression of TNF-α and IL-6 in 21-day-old offspring.

Likewise, polyphenols, especially anthocyanin, have been associated with the modulation of oxidative stress and inflammation in some studies from the use of similar fruits or isolation form by inhibiting NF-κB activation [33, 60, 61].

Lee et al. [62] found that fruits containing different major anthocyanins showed similar anti-inflammatory effects in macrophages. Xie et al. [63] demonstrated that the diet containing 5% freeze-dried açaí (*Euterpe oleracea* Mart.) juice powder reduced TNF-α and IL-6 in adult ApoE-deficient mice model. The same authors reported that polyphenols isolated from the açaí pulp reduces these LPS-induced proinflammatory cytokines by inhibiting NF-κB in macrophages [64].

The intestinal microbiota modulation has been considered as a possible mechanism by which polyphenols, particularly anthocyanins, may exert their benefic effect [65]. In recent study, high-polyphenols apple, was associated with reduction of inflammation markers and modulation in intestinal microbiota in healthy adult mice [66]. Additionally, Neyrinck et al. [67] demonstrated that the pomegranate extract, rich in phenolic compounds, modulates the gut microbiota in favor of *Bifidobacterium* spp. and downregulated IL-6 in the colon of adult mice. These authors suggest the influence of the intestinal microbiota to reduce proinflammatory cytokines by polyphenol in mice. Similarly, our findings suggest that the gut microbiota modulation have an important role in benefic effect of Jussara in offspring.

Dietary fiber has also been associated with benefic changes in intestinal microbiota, especially in the amount of bifidobacteria and lactobacilli with a consequent enhancement in colonic barrier functions [30, 68]. Increases in lactobacilli and reductions in colonic paracellular permeability have been linked to reductions in bacterial translocation and absorption of LPS, resulting in the downregulation of TLR-4-mediated inflammatory responses [16]. Recently, Arora et al. [69] demonstrated benefits in intestinal histology, reducing endotoxemia and inflammation in Female Wistar rats exposed to *Lactobacillus plantarum*. Peña and Versalovic [70] also reported an anti-inflammatory effect of *Lactobacillus rhamnosus* GG in a macrophage model, with inhibition of TNF-α production and a reduction in the TNF-α/IL-10 ratio.

Thus, it is possible that the increase in *Lactobacillus* spp. in Jussara-supplemented groups plays an important role in the downregulation of proinflammatory cytokines and the upregulation of anti-inflammatory interleukin markers in the colon of 21-day-old offspring. This effect could be associated with the fortification of the intestinal barrier integrity and intestinal mucosal permeability, which could result in reduced LPS translocation.

Therefore, we demonstrated that Jussara supplementation during pregnancy and lactation was a natural alternative to reduce of inflammation biomarkers in colon in 21-day-old offspring without altering the normality status.

5. Conclusion

In summary, we showed that supplementation of the maternal diet with the 0.5% Jussara during pregnancy and lactation reverses the adverse effects of perinatal TFAs. The maternal intake of Jussara in perinatal period improves lipid profiles, glucose levels, body composition, and gut microbiota and reduces low-grade inflammation in the colon of 21-day-old offspring. These effects are most likely a result of better fatty acid balance, the presence of fibers and phenolic compounds in Jussara favoring colonic bacterial population, and possibly the fortification of the intestinal barrier integrity, which could result in reduced LPS translocation. These findings support our hypothesis on the potential role of Jussara supplementation in modulating the adverse inflammatory effects of maternal TFA intake in offspring. Our results could contribute to the control of inflammation and the prevention of chronic disease development until adulthood.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

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Research Article

Nutritional Recovery Promotes Hypothalamic Inflammation in Rats during Adulthood

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We evaluated whether protein restriction in fetal life alters food intake and glucose homeostasis in adulthood by interfering with insulin signal transduction through proinflammatory mechanisms in the hypothalamus and peripheral tissues. Rats were divided into the following: a control group (C); a recovered group (R); and a low protein (LP) group. Relative food intake was greater and serum leptin was diminished in LP and R compared to C rats. Proinflammatory genes and POMC mRNA were upregulated in the hypothalamus of R group. Hypothalamic NPY mRNA expression was greater but AKT phosphorylation was diminished in the LP than in the C rats. In muscle, AKT phosphorylation was higher in restricted than in control animals. The HOMA-IR was decreased in R and C compared to the LP group. In contrast, the K_{itt} in R was similar to that in C and both were lower than LP rats. Thus, nutritional recovery did not alter glucose homeostasis but produced middle hyperphagia, possibly due to increased anorexigenic neuropeptide expression that counteracted the hypothalamic inflammatory process. In long term protein deprived rats, hyperphagia most likely resulted from increased orexigenic neuropeptide expression, and glucose homeostasis was maintained, at least in part, at the expense of increased muscle insulin sensitivity.

1. Introduction

Epidemiological and animal studies support a relationship between poor fetal growth and the subsequent development of obesity, type 2 diabetes, and metabolic syndrome [1–4]. Such developmental programming has been explained by the "thrifty phenotype" hypothesis, which proposes that poor fetal nutrition can result in reprogramming of the fetus, which allows the offspring to maximize the body's capacity for energy storage under conditions of poor nutrition once out of the womb. However, this phenotype would be detrimental under conditions where normal or excessive nutrition are present and would thus promote obesity [2, 5].

Body weight, food intake, and metabolism are regulated by the hypothalamus, which processes central and peripheral signals. Within the hypothalamus, neurons residing in the ARC (arcuate nucleus), PVN (paraventricular), and PF/LH (perifornical/lateral hypothalamus) axis communicate with each other and are subject to the influence of several peripheral factors, including leptin and insulin [6]. The effect of these hormones on food intake occurs in part by convergence on a specific set of neurons within the ARC [7, 8] that contains neuronal populations expressing orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP) and the anorectic proopiomelanocortin (POMC). Increased

NPY synthesis and secretion and reduced expression of POMC and its cleaved product α-MSH [9] are characteristic of various models of obesity [10, 11]. These changes may be due to disturbances in the production and release of leptin and insulin. Inappropriate concentrations of or a shift in these hormones due to poor fetal nutrition during a critical window of neuronal development and feeding pathway differentiation may have permanent structural consequences [12]. Moreover, increased hypothalamic inflammation may also contribute to increased susceptibility to obesity in rats exposed to early malnutrition. It has been shown that a low protein diet during lactation interferes with the innate immune response in adulthood, imprinting permanent alterations on cytokine production. These animals exhibited high circulating levels of tumor necrosis factor (TNF)-α and an increased expression of TNF-α mRNA in the spleen and liver [13]. TNF-α induces hypothalamic and peripheral insulin resistance in rodents [14–16] and alters insulin sensitivity and glucose homeostasis in humans [17, 18]. In obese mice, the inhibition of hypothalamic inflammation by immunoneutralizing antibodies against TNFα and Toll-like receptor (TLR) 4 improved insulin signal transduction in the liver [16]. In previous studies [19], we verified that adult rats were exposed to a low protein diet during intrauterine life and lactation and then fed a regular diet after weaning exhibited elevated food intake but did not express the obesity phenotype or glucose intolerance. It has been proposed that the degree of mismatching between the pre- and postnatal environment is a major determinant of subsequent disease [20, 21] and that the lactation period is a critical time for increasing the risk of obesity and insulin resistance, even in obesity-resistant animals [22]. Thus, we evaluated whether protein restriction in fetal life alters food intake and glucose homeostasis in adulthood by interfering with insulin signal transduction in the hypothalamus and peripheral tissues through proinflammatory mechanisms.

2. Materials and Methods

2.1. Animals and Diets. All experimental procedures involving rats were performed in accordance with the guidelines of the Brazilian Society of Science in Animals of Laboratory (SBCAL) and were approved by the ethics committee at the Federal University of Mato Grosso (protocol No. 23108.051511/10-0). Male and virgin female Wistar rats (85–90 days old) were obtained from the university's breeding colony. Mating was accomplished by housing females with males overnight, and pregnancy was confirmed by examining vaginal smears for the presence of sperm. Pregnant females were separated at random and fed from day 1 of pregnancy until the end of lactation with an isoenergetic diet containing either 6% protein (low protein diet, LP) or 17% protein (control diet). The protein in the LP diet was replaced by the same amount of carbohydrate, as described previously [19]. Spontaneous delivery took place at day 22 of pregnancy, and at 3 days of age, large litters were reduced to eight pups each to ensure a standard litter size per mother. After birth, the males were divided into three groups: (1) a control group (C) consisting of rats born to and suckled by dams fed the control diet during pregnancy, lactation, and after weaning; (2) a recovered group (R) consisting of the offspring of dams fed an LP diet during pregnancy but fed the control diet during lactation and after weaning; and (3) an LP group consisting of the offspring of dams fed a LP diet during pregnancy and lactation and after weaning. During the experimental period, the rats were fed *ad libitum* with their respective diets and had free access to water. They were kept under standard lighting conditions (12 hours light: dark cycle) at 24°C. Food intake and body weight were recorded weekly, and the data are expressed as absolute and relative values. Absolute food intake refers to food consumed during ten weeks of the experimental period. To assess the relative food intake, the food intake was normalized per 100 g body weight at week ten of the experimental period. At the end of the experimental period, the rats were subjected to glucose and insulin tolerance test. After the tests, the rats were killed by decapitation, and their blood was collected to perform biochemical and hormonal analyses of the hypothalamus, liver, and muscle.

2.1.1. Glucose Tolerance Test. After 12 hours of fasting, glucose (200 g/L) was administered intraperitoneally at a dose of 2 g/kg of body weight. Blood samples were obtained from an incision at the tip of the tail 0, 30, 60, 90, and 120 minutes after glucose administration to determine serum glucose concentrations (Accu-Chek portable glucose meter, Roche Diagnostics, Germany). The glucose response during the glucose tolerance test was calculated by estimating the total area under the glucose (ΔG) curve using the trapezoidal method [23].

2.1.2. Insulin Tolerance Test. After 12 hours of fasting, insulin (regular) was administered intraperitoneally at a dose of 1.5 U/kg of body weight. Blood samples were obtained from a cut at the tip of the tail 0, 5, 10, and 15 minutes after insulin administration to determine serum glucose concentrations (Accu-Chek portable glucose meter,Roche Diagnostics, Germany). Glucose responses during the insulin tolerance test were evaluated by the constant of the disappearance of plasma glucose (K_{itt}), which was calculated from the slope of the decrease in log-transformed plasma glucose between 0 and 15 minutes [24] after insulin administration, when the glucose concentration declined linearly.

2.2. Organ Weighs and Liver Glycogen Content. After medial laparotomy, epididymal white adipose and liver tissue were quickly removed and their fresh weight was determined. Liver aliquots were frozen immediately in liquid nitrogen and stored at −80°C to determine hepatic fat [25] and glycogen [26] contents.

2.3. Biochemical and Hormonal Profile. Blood samples were collected and centrifuged to 13.000 RPM for 30 minutes. Sera were stored at −80°C for the subsequent measurement of serum albumin concentrations by the colorimetric method [27] to characterize the nutritional status of the animals. Commercial ELISA kits were used to measure serum

insulin (rat/mouse insulin—Cat.# EZRMI-13 K—Millipore) and leptin (rat leptin—Cat.# EZRL-83 K—Millipore) concentrations.

2.4. RNA Extraction, Real Time PCR and PCR Array. Hypothalamic total RNA was extracted using Trizol reagent (Life Technologies, Gaithersburg, MD, USA), according to the manufacturer's recommendations. The RNA integrity was checked by agarose gel electrophoresis. The synthesis of cDNA was conducted using 3 μg of total RNA and the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Samples obtained from three hypothalami from C, R, and LP groups were analyzed using a real-time PCR array (Rat Inflammatory Cytokines Receptors RT^2 Profiler-SuperArray Bioscience Corp., Frederick, MD, USA) containing 84 genes related to this condition plus five housekeeping genes. Controls are also included on each array for genomic DNA contamination, RNA quality, and general PCR performance as shown in http://www.sabiosciences.com/rt_pcr_product/HTML/PARN-011A.html. To confirm the data obtained from PCR array, some genes were selected to perform an individual real-time PCR analysis. Thus, the optimal concentration of cDNA and primers and the maximum efficiency of amplification were obtained through five-point, twofold dilution curve analysis for each gene. Each PCR contained 20–50 ng of reverse-transcribed RNA and was run according to the manufacturer's recommendation using the TaqMan PCR Master Mix (Applied Biosystems). Intron-skipping primers were obtained from Applied Biosystems (TNFα Rn00562055_m1, NPY Rn00561681_m1, IL1β Rn00580432_m1, POMC Rn00595020_m1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH number 4352338E) primers were used as controls. No significant change in GAPDH expression was detected in the different experimental conditions. The real-time PCR analysis was conducted on ABI Prism 7500 detection system (Applied Biosystems). The data were analyzed using the system Sequence Detector 1.7 (Applied Biosystems).

2.5. Immunoblotting. For experiments that measured insulin signaling through AKT phosphorylation, the abdominal cavities of anesthetized rats were opened, and the animals received a bolus injection of saline (100 μL) (-) or insulin (100 μL, 10^{-6} M) intravenously in a cava vein. Liver, soleus muscle, and hypothalamus specimens were removed 45 seconds, 90 seconds, and 15 minutes, respectively, after the insulin injection and immediately homogenized by sonication (15 s) in a freshly prepared antiprotease cocktail (10 mmol/L imidazole, pH 8.0, 4 mmol/L EDTA, 1 mmol/L EGTA, 0.5 g/L pepstatin A, 2 g/L aprotinin, 2.5 mg/L leupeptin, 30 mg/L trypsin inhibitor, 200 μmol/L DL-dithiothreitol, and 200 μmol/L phenylmethylsulfonyl fluoride). After sonication, an aliquot of extract was collected, and the total protein content was determined by the dye-binding protein assay kit (Bio-Rad Laboratories, Hercules, CA). The samples were incubated for 5 minutes at 80°C with 4x concentrated Laemmli sample buffer (1 mmol sodium phosphate/L, pH 7.8, 0.1% bromophenol blue, 50% glycerol, 10% SDS, and 2% mercaptoethanol) and then run on 10% polyacrylamide gels at 120 V for 30 minutes. The electrotransfer of proteins to nitrocellulose membranes (Bio-Rad) was performed for one hour at 120 V (constant) in buffer containing methanol and SDS. After checking the efficiency of transfer by staining with Ponceau S, the membranes were blocked with 5% skimmed milk in TTBS (10 mmol Tris/L, 150 mmol NaCl/L, 0.5% Tween 20) overnight at 4°C. AKT and pAKT were detected on the membranes after a two-hour incubation at room temperature with primary antibodies (AKT sc1618, pAKT sc7985-R, Santa Cruz Biotechnology, Santa Cruz, CA) (diluted 1 : 500 in TTBS containing 3% dry skimmed milk). The membranes were then incubated with a secondary specific immunoglobulin G antibody (diluted 1 : 5000 in TTBS containing 3% dry skimmed milk) for two hours at room temperature. Enhanced chemiluminescence (SuperSignal West Pico, Pierce) after incubation with a horseradish peroxidase-conjugated secondary antibody was used for detection by autoradiography. Band intensities were quantified by optical densitometry (Scion Image, Frederick, MD).

2.6. Statistical Analysis. The results are presented as the means and SEM for the number of rats (n) indicated. Bartlett's test for the homogeneity of variances was initially used to check the fit of the data to the assumptions for parametric analyses of variance. When necessary, data were log-transformed to correct for variance heterogeneity or nonnormality [28]. These data were analyzed by one-way analyses of variance, followed by the LSD test for individual differences between groups. P values less than 0.05 were considered to indicate statistically significant differences.

3. Results

Absolute food intake was similar between R and C rats and higher in both of these groups compared with LP ($P < 0.0001$). When expressed per gram of body weight, food intake was significantly greater in LP rats than in R rats ($P < 0.001$), while the latter group had significantly higher food intake than C rats ($P < 0.02$). Although R rats had a greater final body weight and epididymal white adipose tissue (EWAT) weight than LP rats ($P < 0.0001$) at the end of the experimental period, their weights were still significantly lower than that of C rats ($P < 0.05$). The R and C rats had similar liver weights, and in both cases these were significantly higher than those of LP rats ($P < 0.0001$). Liver glycogen content was similar in R and LP rats, and both groups exhibited greater liver glycogen concentrations than the C group ($P < 0.05$ and $P < 0.01$, resp.). Serum albumin concentrations do not differ between the R and C rats, and these values were significantly higher than those of LP rats ($P < 0.0001$). The basal serum glucose level was not different among three groups of rats. The basal serum insulin concentration was higher in R rats than in LP rats ($P < 0.0001$) but lower in R rats than in C rats ($P < 0.01$). Serum leptin concentrations did not differ between R and LP

Table 1: Absolute and relative food intake, final body weight, epididymal white adipose tissue (EWAT) weight, liver weight, liver glycogen content, serum albumin, serum glucose, serum insulin, and serum leptin concentrations of control (C), recovered (R), and low protein (LP) rats. Values presented are the mean ± SEM for rats per group. Mean values with unlike superscript letters were significantly different ($P < 0.05$, LSD test).

Parameters	Groups		
	C	R	LP
Food intake (g)	375 ± 19^{b} (15)	351 ± 11^{b} (15)	146 ± 11^{a} (14)
(g/100 g BW)	33.7 ± 0.8^{a} (15)	39.2 ± 1.6^{b} (12)	48.4 ± 2.4^{c} (11)
Body weight (g)	483 ± 11^{c} (15)	444 ± 12^{b} (15)	171 ± 36^{a} (12)
EWAT weight (g)	12.9 ± 0.6^{c} (10)	10.7 ± 0.8^{b} (16)	2.2 ± 0.3^{a} (13)
Liver weight (g)	15.43 ± 0.77^{b} (3)	13.63 ± 1.26^{b} (8)	6.55 ± 0.29^{a} (8)
Liver glycogen content (mg/100 mg)	0.082 ± 0.002^{a} (5)	0.127 ± 0.023^{b} (4)	0.158 ± 0.014^{b} (5)
Serum albumin (g/dL)	3.50 ± 0.04^{b} (7)	3.49 ± 0.03^{b} (7)	3.04 ± 0.02^{a} (7)
Serum glucose (mmol/L)	7.3 ± 0.7 (5)	6.3 ± 0.9 (4)	4.9 ± 1.0 (4)
Serum insulin (ng/mL)	1.7 ± 0.3^{c} (5)	1.0 ± 0.1^{b} (4)	0.11 ± 0.03^{a} (4)
Serum leptin (ng/mL)	4.03 ± 0.05^{b} (4)	3.22 ± 0.28^{a} (4)	2.96 ± 0.16^{a} (4)

rats, and these values were significantly lower than those of C rats ($P < 0.01$) (Table 1).

Mean total areas under the ΔG in response to an intraperitoneal glucose load were similar in R and C rats and both were higher than LP rats ($P < 0.03$) (Figure 1(a)). Insulin resistance, calculated by the HOMA-IR index, decreased in R and C groups compared to the LP group ($P < 0.0001$) (Figure 1(b)). In contrast, the glucose disappearance rate during the intraperitoneal insulin tolerance test (K_{itt}) in R rats was similar to that observed in C rats, and both of these values were lower than the disappearance rate in LP rats ($P < 0.0001$) (Figure 1(c)).

Hypothalamic (Figure 2(a)) and hepatic (Figure 2(c)) AKT content detectable by immunoblotting was not significantly different between the groups. However, after the administration of insulin, the AKT phosphorylation increments in the hypothalamus (Figure 2(b)) and liver (Figure 2(d)) were similar in R and C rats, and the values of these groups were higher than the values of the LP group ($P < 0.05$). In muscle, both the AKT content (Figure 2(e)) and the magnitude of AKT phosphorylation increments (Figure 2(f)) were similar between R and LP groups, and both were higher than the C group ($P < 0.03$ and $P < 0.01$, resp.).

Of the 84 genes evaluated in the hypothalamus by PCR array (Rat Inflammatory Cytokines Receptors RT^2 Profiler-SuperArray Bioscience Corp., Frederick, MD, USA), 72 genes (85%) showed a significant change in their level of expression in the R and LP groups compared with the C group. Of the 72 genes changed, 52 (72%) were at least 2.5-fold upregulated in the R group and 59 (82%) were 2.5-fold downregulated in the LP group compared to the C group (Figure 3(a)). Real-time PCR was conducted to validate the findings of the PCR array, and the genes selected for analysis were TNFα and ILβ. TNFα expression was similar between the C and LP groups and smaller in the R group than in the C and LP groups ($P < 0.5$) (Figure 3(b)), whereas ILβ did not differ among the three groups (Figure 3(c)).

Greater NPY mRNA expression was detected in the hypothalamus of LP rats than in those of C rats ($P < 0.02$). NPY mRNA expression was not significantly different between the R rats and either the C or the LP rats (Figure 4(a)). The R group showed higher POMC mRNA expression compared with both the LP and the C groups ($P < 0.01$ and $P < 0.02$, resp.) (Figure 4(b)).

4. Discussion

In the present study, we showed that nutritional recovery only attenuated the typical hyperphagia associated with protein malnutrition. Long term feeding regulation is provided by the main circulating hormones leptin and insulin [29], and leptin circulating levels are proportional to total fat mass [30, 31]. In this study, the low visceral fat content correlated with reduced serum leptin levels in recovered and low protein rats. The expected upregulation of hypothalamic NPY mRNA expression and downregulation of hypothalamic POMC mRNA expression in response to low circulating leptin levels [32, 33] was observed in our low protein rats. Although the recovered group also exhibited reduced serum leptin levels, these animals showed hypothalamic POMC mRNA

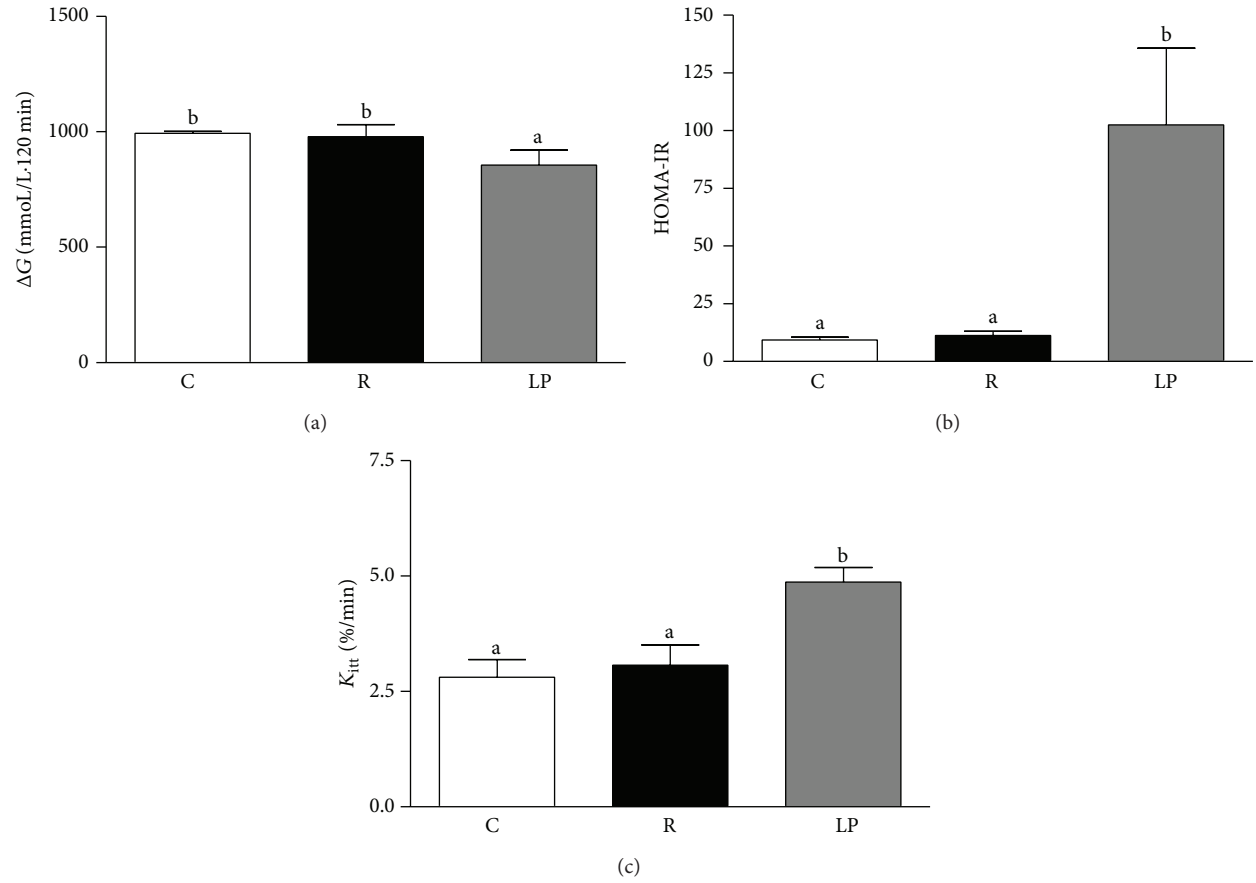

Figure 1: Total areas under the glucose (ΔG) curve obtained from the intraperitoneal glucose-tolerance test (a), homeostasis model assessment of insulin resistance (HOMA-IR) index (b), and glucose disappearance rates (K_{itt}) (c) in control (C), recovered (R), and low protein (LP) rats. The bars represent means ± SEM. Mean values with unlike superscript letters were significantly different ($P < 0.05$, LSD test).

overexpression, most likely due to the higher serum insulin levels in the recovered group compared with the low protein group. Insulin administration increases POMC mRNA expression while reducing NPY expression and protecting against diet-induced obesity [34]. Thus, increased expression of this anorexigenic neuropeptide may have contributed to the attenuation of hyperphagia. However, high hypothalamic TNFα mRNA expression in these animals indicates a hypothalamic inflammatory process that could contribute to incomplete reversion of hyperphagia and development of late obesity.

Inflammation in the hypothalamus leads to insulin resistance, which may play a role in the development of obesity and act as a molecular link between obesity and type 2 diabetes [35–37]. In the present study, nutritional recovery upregulated, whereas low protein diet downregulated, proinflammatory cytokines mRNA expression. Phosphatidylinositol 3-kinase (PI3K)/AKT is an important pathway to control energy homeostasis [38], as it is responsible for the anti-inflammatory response, liver glycogen synthesis, and regulation of insulin-stimulated transport of GLUT4 in muscle [39]. Thus, we initially evaluated hypothalamus AKT signaling and verified that recovered animals did not show impairment in hypothalamic AKT protein expression or phosphorylation. Interestingly, low protein rats that did not exhibit a hypothalamic inflammation signal showed unaltered AKT expression but reduced AKT phosphorylation. As the basal AKT phosphorylation did not differ among groups (data not shown), it is plausible that reduced insulinemia contributed to lower AKT signaling in the hypothalamus of our low protein rats.

Hypothalamic inflammation plays an important role in the development or progression of the hyperglycemic phenotype [40] because glucose homeostasis is controlled by the brain-liver axis [16]. The next step was to evaluate the AKT signaling in the liver, and coincidently, we verified the same pattern in the hypothalamus. The hepatic insulin resistance assessed by AKT phosphorylation level was confirmed by elevated HOMA-IR in low protein rats. The paradoxical association between hepatic insulin resistance and high glycogen levels can be explained by reduced glucose-6-phosphatse activity typically observed in malnourished animals [41]. In low protein rats, the liver insulin resistance was counteracted by the enhanced muscle AKT phosphorylation and expression that resulted in peripheral insulin sensitivity, reflected in the increased K_{itt} value. This effect resulted in better glucose tolerance, judging by the low ΔG during ipGTT. The persistently high muscle AKT phosphorylation and expression observed in recovered animals may have

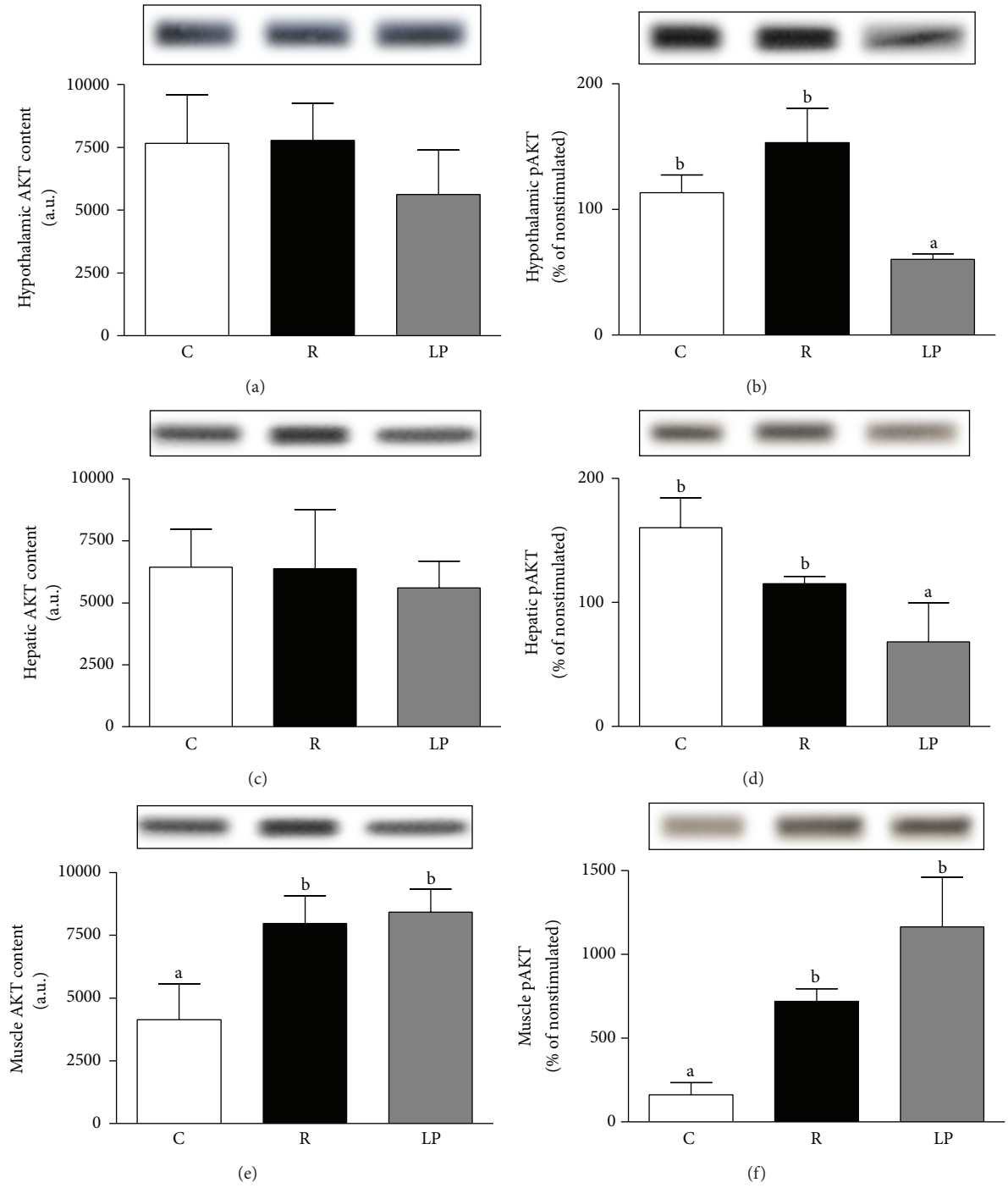

Figure 2: AKT content and AKT phosphorylation in hypothalamus ((a) and (b)), liver ((c) and (d)), and skeletal muscle ((e) and (f)) of control (C), recovered (R), and low protein (LP) rats. AKT phosphorylation data represent the percentage of nonstimulated values. The rats were injected with saline (not shown) or insulin, and 45 s, 90 s, and 15 minutes later, liver, hind-limb skeletal muscle, and hypothalamus, respectively, were excised and homogenized as described in the Materials and Methods section. Data are presented as means ± SEM. Mean values with unlike superscript letters were significantly different ($P < 0.05$, LSD test).

resulted from incomplete restoration of body weight and serum insulin levels. Lower body weight associated with decreased serum insulin levels apparently has the opposite, compensatory effect [42].

In conclusion, our results are consistent with the hypothesis that protein deprivation produces increased food intake that is partially restored by nutritional recovery. The data also provide direct evidence of maintenance of glucose homeostasis in both low protein and recovered rats. In recovered rats, middle hyperphagia may have resulted from increased anorexigenic neuropeptide expression that counteracted the hypothalamic inflammatory process. In long term protein

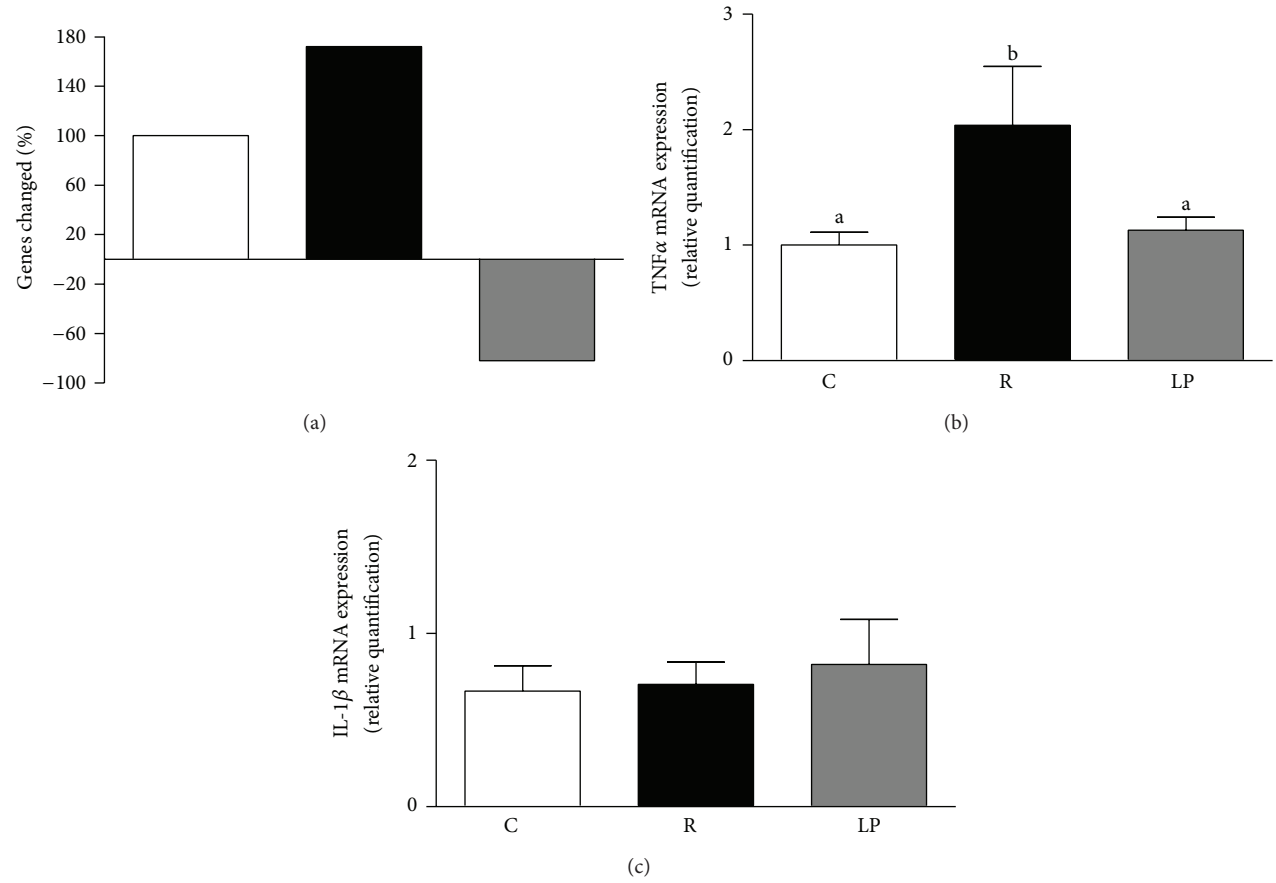

Figure 3: Fold change genes modulated by the experimental treatments evaluated in hypothalamus by PCR array (a). TNFα (b) and IL-1β (c) mRNA expression in hypothalamus of control (C), recovered (R), and low protein (LP) rats. Data are presented as means ± SEM. Mean values with unlike superscript letters were significantly different ($P < 0.05$, LSD test).

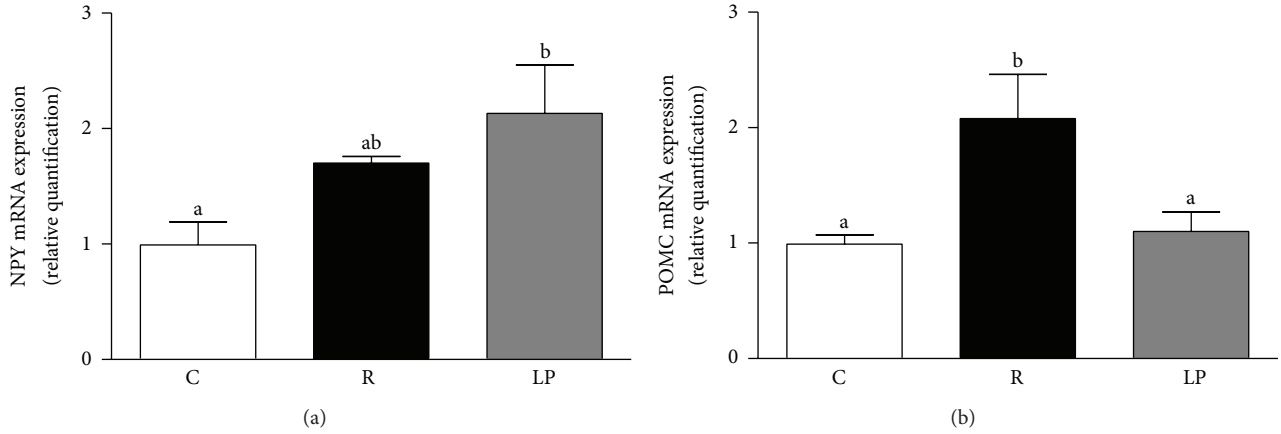

Figure 4: NPY (a) and POMC (b) mRNA expression in hypothalamus of control (C), recovered (R), and low protein (LP) rats. Data are presented as means ± SEM. Mean values with unlike superscript letters were significantly different ($P < 0.05$, LSD test).

deprived rats, hyperphagia appears to have been produced by increased orexigenic neuropeptide levels, and glucose homeostasis was maintained, at least in part, at the expense of increased muscle insulin sensitivity.

Conflict of Interests

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

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Review Article

Flavocoxid, a Nutraceutical Approach to Blunt Inflammatory Conditions

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Flavonoids, from *Scutellaria baicalensis* (Chinese skullcap) and *Acacia catechu* (black catechu), have been shown to exert a variety of therapeutic effects, including anti-inflammatory, antiviral, antibacterial, and anticancer activities. Flavocoxid is a mixed extract containing baicalin and catechin and it acts as a dual balanced inhibitor of cyclooxygenase-1 (COX-1) and COX-2 peroxidase enzyme activities with a significant inhibition of 5-lipoxygenase (5-LOX) enzyme activity *in vitro*. Flavocoxid downregulates gene or protein expression of several inflammatory markers and exerts also strong antioxidant activity in several experimental models. Controlled clinical trials and a postmarketing study have clearly shown that flavocoxid is as effective as naproxen in managing the signs and symptoms of osteoarthritis of the knee and it has better upper gastrointestinal, renal, and respiratory safety profile than naproxen. Flavocoxid may therefore provide a potential therapeutic approach to the treatment of chronic inflammatory conditions.

1. Introduction

Inflammation is considered the main response of the body evoked to deal with injuries and its hallmarks include swelling, redness, pain, and fever [1]. Several mediators regulate the events of acute inflammation, influence vascular changes, and provoke inflammatory cell recruitment [1–3]. The inflammatory response is a complex self-limiting process precisely regulated to prevent extensive damage of the host. When the self-limiting nature of this protective mechanism is inappropriately regulated, it is transformed to a detrimental chronic state of inflammation. Several diseases are associated with chronic inflammation, including osteoarthritis, atherosclerosis, diabetes, obesity, Crohn's disease, and cancer [1–3]. Although the today used steroidal anti-inflammatory drugs (SAID) and nonsteroidal anti-inflammatory drugs (NSAIDs) effectively manage the acute inflammatory reaction, in chronic inflammatory states the long-term treatment is followed by severe adverse effects. This justifies the search for innovative and safe anti-inflammatory agents. Attractive research candidates are plant constituents. Recently, there has been interest in the potential of flavonoids to modulate the chronic inflammatory reaction. Flavonoids are major constituents of fruits, vegetables, and beverages, such as wine, tea, cocoa, and fruit juices. Flavonoids have a similar structure consisting of two aromatic rings (A and B), which are bound together by three carbon atoms, forming an oxygenated heterocycle (ring C; Figure 1). According to changes in the chemical structure, flavonoids are divided into seven classes: flavonols, flavones, flavanones, flavanonols, flavanols, anthocyanidins, and isoflavones [4]. The flavanols, also referred to as flavan-3-ols, are mainly present in green and black tea, red wine, chocolate, and fruits such as apples, grapes, and strawberries. Typical dietary flavanols include catechin, epicatechin, epigallocatechin (EGC), and epigallocatechin gallate (EGCG). Flavones, such as apigenin and luteolin, are found in parsley, chives, artichoke, and celery. Dietary flavanones include naringenin, hesperetin, and taxifolin and

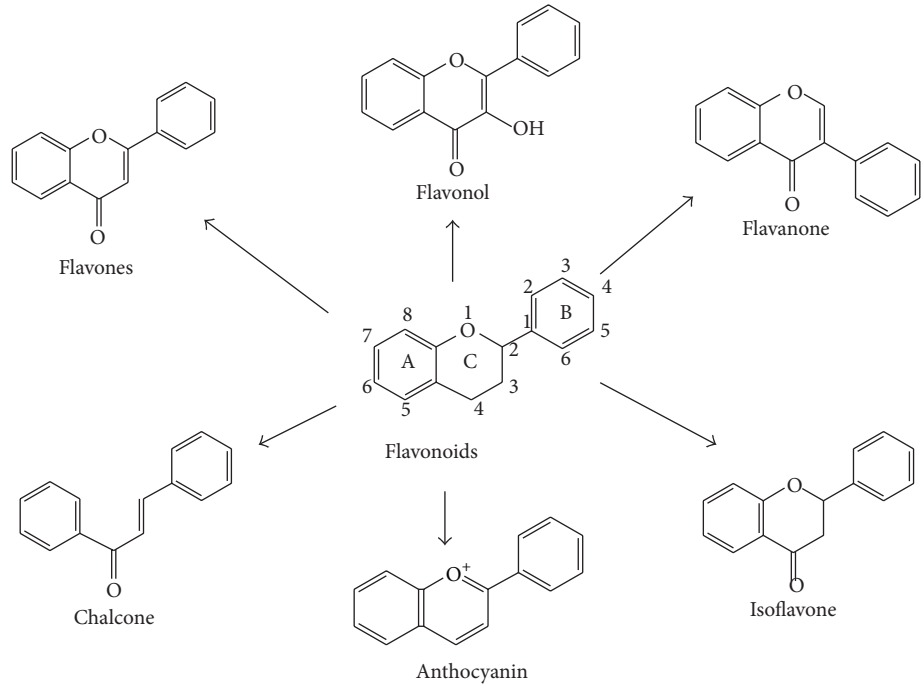

Figure 1: Basic chemical structures of different natural occurring flavonoids.

are found mainly in citrus fruit and tomatoes. Finally, isoflavones such as genistein and daidzein are a subclass of the flavonoids family found in soy and soy products. They have a large structural variability and more than 600 isoflavones have been identified to date and are classified according to oxidation level of the central pyran ring (Figure 1). *Acacia catechu*, also called black catechu, is a traditional medicinal plant commonly used in Indian subcontinent and Southeast Asia, with antipyretic, antidiarrheal, hypoglycaemic, and hepatoprotective activities [5, 6]. The major chemical components in an *Acacia catechu* extract are catechin and epicatechin-3-O-gallate. Catechin reduces inflammation and blunts the production of proinflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), as demonstrated in rats with experimental arthritis [7]. *Scutellaria baicalensis*, a traditional Chinese herbal medicine, also called Chinese skullcap, has been used to treat respiratory inflammatory diseases [8–12], viral infections [13–15], cardiovascular diseases [16–18], and cancer [19, 20]. Baicalin, extracted by *Scutellaria baicalensis*, inhibits cyclooxygenase COX-1 and COX-2 peroxidase and 5-lipoxygenase (5-LOX) enzyme activities, decreases production of proinflammatory eicosanoids, and attenuates edema in an *in vivo* model of inflammation [21]. The anti-inflammatory activity of baicalin was also associated with a binding inhibition of chemokines, such as macrophage inflammatory protein (MIP)-1β, monocyte chemotactic protein-2 (MCP-2), causing a reduced capacity of the chemokines to induce cell migration [21]. The flavonoids, baicalin and catechin, modulate the activities of arachidonic acid, metabolizing enzymes involved in this pathway, and iNOS (NO producing enzyme). Flavocoxid is a mixture of the flavonoid molecules catechin, from *Acacia catechu*, and baicalin, extracted from *Scutellaria baicalensis*, concentrated to greater than 90% purity (Figure 2). Thanks to its components flavocoxid, in addition to anti-inflammatory properties, may also act as an antioxidant, reducing reactive oxygen species including hydroxyl radical, superoxide anion radical, and hydrogen peroxide. The combined effect of the 2 flavonoids is greater than the isolated molecules, as shown in a recent paper [22]. As a consequence of NF-κB modulation, flavocoxid reduces COX-2, 5-LOX, iNOS, and TNFα production, and it also blunts the formation of COX-2, 5-LOX, and iNOS metabolites, as PGE2, LTB4, and nitrates [22–24]. These effects provide a rationale for the use of a dual inhibitor in acute and chronic inflammatory conditions. Flavocoxid, marketed as Limbrel in the USA, is a USFDA-regulated prescription, and it is expected to have significant therapeutic efficacy in the managing of chronic inflammation. This review will focus on the preclinical pharmacology, toxicology, and clinical pharmacology of this new attractive compound.

2. Preparation of Flavocoxid

Flavocoxid is prepared from roots of *Scutellaria baicalensis* and *Acacia catechu* (US patent number 7,514,469). The roots of *Scutellaria baicalensis* are extracted with 70% ethanol and then recrystallized with an ethanol/water solvent [23]. The *Scutellaria baicalensis* extract contains baicalin as the major component and additional minor free-B-ring flavonoids. In the roots of *Acacia catechu*, (+)-catechin is the major component at a content of 80%. Minor amount of its enantiomer epicatechin and other minor amounts of flavans are present [23]. Analysis of the extracts is carried

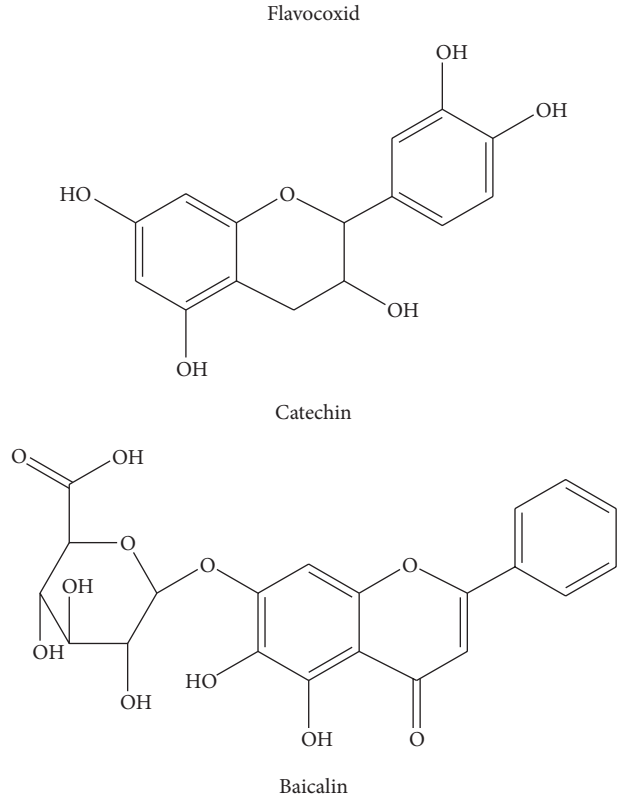

Figure 2: Flavocoxid components: catechin and baicalin.

out separately by high performance liquid chromatography with photodiode array detector (HPLC/PDA) and liquid chromatography-mass spectrometry (LC/MS) and indicates a major compound, baicalin, from *Scutellaria baicalensis* extract and (+)-catechin from *Acacia catechu* extract by comparison with known standards. The presence of these compounds is then confirmed by carbon nuclear magnetic resonance (^{13}C-NMR) and proton nuclear magnetic resonance (^{1}H-NMR) analysis, respectively. The final flavocoxid formulation (Figure 2) is a mixture of >90% purified baicalin and catechin with the remainder being excipient (5-6%) and water (3%). Confirmation of the combined flavonoids content can be obtained by HPLC analysis. Both flavonoids are detected using UV detector at 275 nm and identified based on retention time by comparison with known flavonoids standards [23]. These ingredients are generally recognized as safe (GRAS). For an ingredient to be recognized as GRAS by the US Food and Drug Administration (FDA), it requires technical demonstration of nontoxicity and safety, general recognition of safety through widespread usage, and agreement of that safety by experts in the field.

3. Effects of Flavocoxid on Arachidonic Acid Formation and Metabolism

A series of *in vitro* and *in vivo* experiments have been carried out in order to dissect out the exact mechanism of action of flavocoxid. First of all, flavocoxid was tested in peritoneal macrophages (MΦ) stimulated with lipopolysaccharide (LPS) to investigate a possible effect on phospholipase A_2 (PLA_2) activity [24]. Flavocoxid did not significantly modify cell viability at 200 and 500 μg/mL, but it markedly inhibited PLA_2 activity (IC_{50} = 60 μg/mL) at doses of 50, 100, 200, and 500 μg/mL [24]. This finding indicates that flavocoxid modulates the generation of AA from membrane phospholipids caused by tissue damage during chronic inflammation. The effects of flavocoxid on COX-1 and COX-2 enzyme activities were also investigated in dedicated *in vitro* enzyme assays [24]. COX proteins have two different enzymatic moieties for AA metabolism: the cyclooxygenase (CO) one and the peroxidase one (PO). The CO activity converts AA to PGG_2 and the PO activity transforms PGG into PGH_2. Finally, cell synthases and isomerases convert PGH_2 to thromboxanes (TXB), prostaglandins (PG), and prostacyclin (PGI). Experiments were carried out to investigate the specific inhibitor effects of flavocoxid on CO and PO enzyme moieties of either COX-1 or COX-2. The compound had no significant anti-CO COX-2 activity up to 50 μg/mL. In addition, flavocoxid showed CO COX-1 IC_{50} of 25 μg/mL, while indomethacin has a CO COX-1 IC_{50} of 0.012 μg/mL, thus suggesting that flavocoxid has little anti-CO activity on both COX enzymes compared to well-known anti-inflammatory agents. Imbalances in COX-2 versus COX-1 inhibition caused by selective COX-2 lead to production of several AA metabolites responsible for an enhanced risk of edema, hypertension, and myocardial infarction [25]. Flavocoxid produced a balanced inhibition of both COX-1 and COX-2 PO activities, with IC_{50} of 12.3 and 11.3 μg/mL, respectively. This clearly indicates that flavocoxid exerts its activity via modulation of the PO activity of these enzymes. Currently available NSAIDs and COX-2 inhibitors do not influence the 5-LOX pathway and therefore do not block the production of the dangerous leukotrienes (LTs) that cause vasoconstriction and leukocyte attraction and accumulation. Furthermore COX-1 and COX-2 blockade by NSAIDs or selective COX-2 inhibitors causes a shunting of the AA metabolism towards the 5-LOX pathway, thus inducing an overproduction of these fatty acid mediators which can cause multiple organ damage [26]. Enhanced levels of LTs are also measured in a variety of pathological conditions such as asthma, gastric ulceration, renal insufficiency, and cardiovascular complications [27, 28]. Flavocoxid was incubated along with purified 5-LOX enzyme in the presence of an oxygen sensing chromagen *in vitro* to study the formation of unstable hydroperoxyeicosatetraenoic acids (HPETEs) intermediates in the synthesis of LTs. Flavocoxid inhibited the 5-LOX enzyme showing an IC_{50} of 110 μg/mL. Phenidone, a well-known 5-LOX inhibitor used as a positive control in theses assays, had an IC_{50} of 1.3 μg/mL. No other NSAIDs or selective COX-2 inhibitors, including rofecoxib, valdecoxib, diclofenac, meloxicam, and aspirin, displayed an anti-5-LOX activity. All these findings, taken together, indicate that flavocoxid also reduces the production of LTBs from 5-LOX and may avoid the deleterious accumulation of these lipid mediators caused by the NSAIDs-induced 5-LOX shunt. Nonenzymatic lipid peroxidation is another important pathway of the AA metabolism. Reactive oxygen

species (ROS) may react with exaggerated AA levels leading to the production of F2-isoprostanes and 4-hydroxynonenal (HNE) together with enhanced malondialdehyde levels. All these markers of oxidative stress are elevated during chronic inflammation and contribute to the pathological cascade leading to organ damage and dysfunction. Therefore, it is relevant for an anti-inflammatory agent to possess an antioxidant effect. Experiments were carried out to analyze this issue, and the *in vitro* antioxidant activity of flavocoxid was evaluated using oxygen radical absorbance capacity (ORAC) procedures and was compared with that of well-known antioxidants such as vitamin C and vitamin E. The ORAC analysis provides a measure of the scavenging capacity of antioxidants against the peroxyl radical. Trolox, a water-soluble vitamin E analog, is used as the calibration standard, and the ORAC result is expressed as μmolTE/g dry weight. The $ORAC_{total}$ for flavocoxid (3719 μmolTE/g) was significantly greater than that of either vitamin C (2000 μmolTE/g) or vitamin E (1100 μmolTE/g). Flavocoxid showed also high values for the ferric reducing/antioxidant power (FRAP), the peroxynitrite radical averting capacity (NORAC), and the Trolox equivalent antioxidant capacity (TEAC), thus clearly showing that this compound exerts a strong antioxidant activity [24].

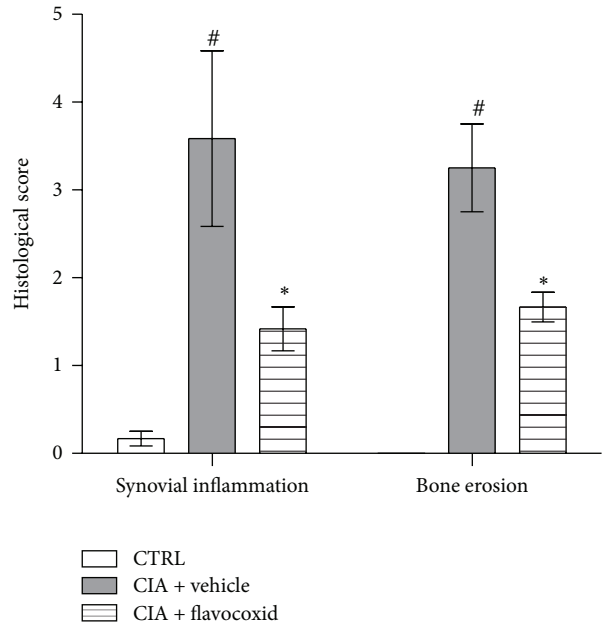

Figure 3: Effects of flavocoxid in a collagen-induced arthritis model. $^{\#}P < 0.001$ versus ctrl; $^{*}P < 0.005$ versus CIA + vehicle.

4. Effects of Flavocoxid on Proinflammatory Gene Expression

The effects of flavocoxid on gene and protein expression of inflammatory markers were studied in rat peritoneal macrophages stimulated with *Salmonella enteritidis* LPS [29]. In fact it is worthy of interest to identify for an anti-inflammatory agent a mechanism of action at the level of inflammatory gene and protein expression. Peritoneal macrophages had a constitutive expression of COX-1 and LPS did not modify it. By contrast LPS stimulation of peritoneal macrophages resulted in a marked increase in both COX-2 and 5-LOX expression [29]. Flavocoxid attenuated, in a concentration dependent manner, the increase in COX-2 and 5-LOX expression [29]. Together with LPS-induced COX-2 and 5-LOX activation, the generation of PGE_2 and LTB_4 was markedly augmented. Flavocoxid significantly reduced the increase in PGE_2 and LTB_4. In addition, LPS-primed macrophages had an enhanced mRNA expression for iNOS and augmented nitrate content; flavocoxid significantly blunted, in a concentration dependent manner, the increase in iNOS and nitrate production. These results [22, 24, 29] suggest that flavocoxid inhibits COX-2, 5-LOX, and iNOS gene activation and abrogate the biosynthesis of related inflammatory mediators. Among these mediators an important role is played by TNF-α, a pleiotropic proinflammatory cytokine [30], markedly induced in peritoneal macrophages stimulated with LPS. Flavocoxid caused a significant and concentration dependent reduction in the levels of TNF-α mRNA and in the formation of the mature protein. Nuclear factor kappa-B (NF-κB) plays a prominent role in the inflammatory cascade [31]; it is an important transcription factor complex that regulates the expression of several genes involved in immune and inflammatory responses during chronic human diseases [32, 33]. In unstimulated cells, NF-κB is constitutively localized in the cytosol as a heterodimer by physical association with an inhibitory protein, called IκBα. Following activation, the NF-κB heterodimer is rapidly translocated to the nucleus where it activates the transcription of target genes, including the genes encoding for proinflammatory cytokines, adhesion molecules, chemokines, and inducible enzymes (such as COX-2, 5-LOX, and iNOS). Flavocoxid was shown to reduce IκBα loss from the cytoplasm and to blunt NF-κB binding to DNA in LPS-stimulated macrophages [29]. Therefore flavocoxid, acts at gene and protein expression level through NF-κB activity inhibition, blocking the auto-amplifying loop during the inflammatory response.

5. Effects of Flavocoxid on Experimental Models of Inflammation

Flavocoxid was studied in animal experimental models of inflammation aimed to test its efficacy. In a first experiment, collagen-induced arthritis (CIA) was induced in DBA/1 mice by an intradermal injection of an emulsion containing bovine type II collagen in complete Freund's adjuvant. CIA animals were then randomized to receive vehicle or flavocoxid (20 mg/kg) and the treatment lasted 45 days (Figure 3, data on file). Flavocoxid reduced PGE_2 and LTB_4 levels, significantly ameliorated the clinical signs of arthritis, improved the histological damage, decreased the cartilage expression and the circulating levels of several markers of severity disease including TNF-α, IL-6, high mobility group box-1 (HMBG-1), and also caused an enhanced expression of the anti-inflammatory cytokine IL-10. Interestingly, flavocoxid positively modulated the balance between receptor activator

of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG), a cytokine system involved in bone and cartilage remodeling. Collectively these experimental findings demonstrate that flavocoxid is a valuable therapeutic agent in the treatment of inflammatory conditions, including arthritis and osteoarthritis.

The dual inhibitor was also tested in acute inflammatory diseases such as acute pancreatitis, an autodigestive inflammatory disease, that causes acinar cell damage and finally culminates in hemorrhagic necrosis of the pancreas and eventually multiple organ failure [34]. A large body of evidence suggests that upregulation of inflammatory mediators, including COX-2, 5-LOX, cytokines, and chemokines, orchestrates this pathological process [35]. Acute pancreatitis can be induced in rats by injection of cerulein, a secretagogue agent. Flavocoxid was investigated for its effects in cerulein-induced pancreatitis [36] at a dose of 20 mg/kg; it inhibited COX-2 and 5-LOX expression and reduced serum levels of lipase and amylase and the degree of pancreatic edema. Administration of flavocoxid also blunted the increased pancreatic TNF-α mRNA expression, serum LTB_4 and PGE_2 levels, and protected against histological damage in terms of vacuolization and leukocyte infiltration. These interesting findings may provide a potential therapeutic approach to the treatment of patients at high risk of developing this life-threatening condition.

Flavocoxid was also tested in a degenerative chronic disease, Duchenne muscle dystrophy (DMD), a progressive muscle-wasting disease leading to death, usually in early adulthood [37]. The disease results from absence of the protein dystrophin, which is an essential component of the dystrophin-glycoprotein complex that maintains membrane integrity of muscle fibers by linking cytoskeleton to extracellular matrix. Muscle degeneration in muscle dystrophy is exacerbated by the endogenous inflammatory response and increased oxidative stress, and NF-κB plays a pivotal role in orchestrating this inflammatory cascade [38, 39]. The effects of flavocoxid were studied in a comparison study with methylprednisolone, the gold standard treatment for DMD patients, using the mdx mice, the murine model of DMD [37]. Five-week-old mdx mice were treated for 5 weeks with flavocoxid, methylprednisolone, or vehicle. Flavocoxid was more effective than methylprednisolone in ameliorating functional properties both *in vivo* and *in vitro*; in reducing serum creatine kinase (CK), a marker of muscle necrosis; in blunting the expression of oxidative stress markers and inflammatory mediators. Furthermore more efficiently than methylprednisolone, flavocoxid inhibited NF-κB and mitogen-activated protein kinases (MAPKs) signal pathways, decreased muscle necrosis, and augmented muscle regeneration [37]. This experiment suggests that flavocoxid counteracts the chronic inflammatory cascade that contributes to muscle necrosis and degeneration in DMD showing a degree of activity higher than that of methylprednisolone. Since methylprednisolone possesses also important side effects, flavocoxid with a better safety-efficacy profile might represent a valuable alternative in the treatment of DMD. However this hypothesis deserves to be confirmed in a clinical setting.

In another experimental model of chronic inflammation, benign prostatic hyperplasia (BPH), we tested the effects of flavocoxid since COX and 5-LOX are significantly elevated in the overgrowing prostate [40]. Therefore a "dual inhibitor" of the COX and 5-LOX enzymes might be of benefit in this disease. Rats were treated, daily, with testosterone propionate (3 mg/kg/sc) or its vehicle for 14 days. Testosterone administered animals were randomized to receive vehicle (1 mL/kg, ip) or flavocoxid (20 mg/kg, ip) for 14 days. Flavocoxid reduced prostate weight and hyperplasia, blunted the augmented expression of COX-2 and 5-LOX as well as the increased production of PGE_2 and LTB_4, enhanced the proapoptotic Bax and caspase-9, and decreased the antiapoptotic Bcl-2 mRNA. Flavocoxid reduced also the epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) expression. The data obtained from this study would indicate that a "dual inhibitor" of the COX and 5-LOX enzymes, such as flavocoxid, might represent a rationale therapeutic approach to reduce benign prostate growth [40].

6. Toxicity Studies on Flavocoxid

A toxicological testing of the relative pure combination of baicalin and (+)-catechin has been performed *in vitro* and in experimental animals [41, 42]. THP-1 cells, a human immortalized monocyte cell line, were used to assess cytotoxicity in an LDH assay. When cells were grown to confluent monolayer and then exposed to increasing concentration of several NSAIDs, celecoxib, and the baicalin/(+)-catechin combination, only indomethacin and celecoxib showed significant cytotoxicity above 50 μg/mL. Flavocoxid showed low cytotoxicity at the highest test concentration (100 μg/mL) [41]. Acute (2000 mg/kg/day for 14 consecutive days) and subchronic (50, 250, and 500 mg/kg/day) toxicity study in mice revealed no abnormalities in any toxicological end points examined including animal body weight, gross organ pathology and tissue histology, and blood chemistry or serology [42]. Flavocoxid was also administered in Fisher 344 rats, a model for gastric toxicity of NSAIDs, and showed no sign of ulceration [41]. The baicalin/(+)-catechin combination was tested at a 10 μM concentration in a liver microsomal assay with endoplasmic reticulum fractions using spectrophotometric quantization of 7-benzyloxy-4-(trifluoromethyl)-coumarin as the substrate for CYP450 profiling. Flavocoxid showed only moderate inhibition of CYP1A2 (23%) at a 10 μM concentration and low inhibition for all other CYP isoforms (11/16%). The combined extract also showed no mutagenicity in the AMES test [41]. An additional 90-day oral toxicity study was carried out in Hsd : SD rats to determine the potential of the baicalin/(+)-catechin combination to produce gastric toxicity [42]. Flavocoxid was administered at the dose levels of 250, 500, and 1000 mg/kg/day. There were no flavocoxid-related adverse events, including mortality, changes in body weight and food consumption, neurological effects (assessed by the Functional Observational Battery and motor activity), organ weight changes, or histopathological alterations. In addition flavocoxid caused no change in sperm

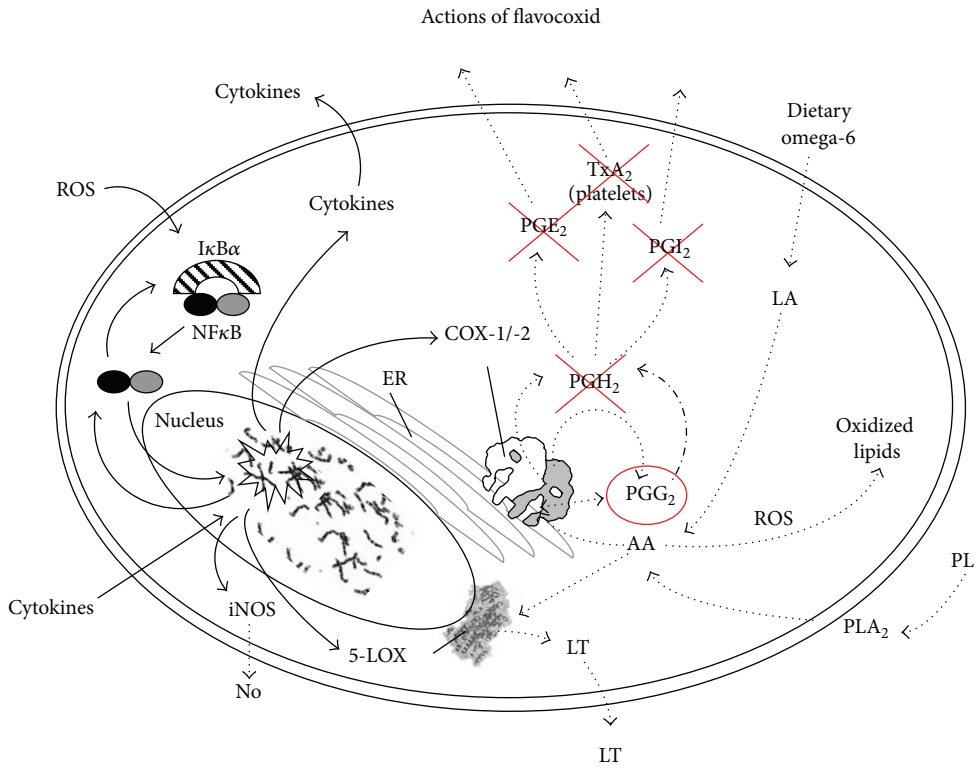

Figure 4: Mode of action of flavocoxid on cellular pathways.

count and comparable estrus aging [42]. This study identified a dose of 1000 mg/kg/day/as the no-observed-adverse-effect level (NOAEL). This promising preclinical safety profile encourages the use of flavocoxid in humans.

7. Clinical Evidence Supporting Flavocoxid Efficacy and Safety

A study was designed to compare the effectiveness and safety of flavocoxid to naproxen in subjects with moderate to severe osteoarthritis (OA) of the knee [43, 44]. This was a randomized, multicenter, and double-blind study involving 220 subjects with OA of the knee that were randomized to receive either flavocoxid (500 mg twice daily) or naproxen (500 mg twice daily for 12 weeks). Primary outcome measures included the Western Ontario and McMaster Universities Osteoarthritis index (WOMAC) and subscales and timed walk. More than 90% of the subjects in both groups had significant reduction in the signs and symptoms of OA. No statistical significant difference in efficacy between flavocoxid and naproxen was observed [43, 44]. Flavocoxid treated patients had also significantly fewer upper gastrointestinal, renal, and respiratory adverse events (AEs). These results indicate that flavocoxid is effective as naproxen in the management of OA of the knee and shows a better safety profile than naproxen. An additional study in healthy volunteers found that flavocoxid does not affect the primary or extrinsic pathway of hemostasis and, by not inhibiting the anticoagulation effects of aspirin, may have utility in cardiovascular patients with chronic inflammation [45]. An open-label, postmarketing study (GOAL: Gauging Osteoarthritis [OA] with Limber) was performed to determine the overall efficacy and gastrointestinal tolerability of flavocoxid [46]. A total of 1067 patients at 41 rheumatology practices were enrolled and prescribed flavocoxid 500 mg b.i.d. for 60 days. The Physician Global Assessment of Disease (PGAD) visual analog scale (VAS) was used as a global measure to assess the signs and the symptoms of OA including joint discomfort, functional stiffness, functional mobility, and quality of life. Furthermore both overall tolerability and upper GI tolerability were assessed by individual questions scored on a 5-part Likert scale. Physicians were also asked to monitor any interruption in or cessation of use of flavocoxid due to the GI symptom as well as changes in the use of gastroprotective medications. A close monitoring of adverse events (AEs) was also carried out. In the 1005 patients who completed all the follow-up visits there was a significant improvement in VAS score. The most important improvement was observed in patients with moderate to severe OA and in those subjects that were historically nonresponders to NSAIDs. A low incidence of o AEs was also observed with a good overall and GI tolerability. In addition the use of flavocoxid resulted in a >30% reduction in or cessation of the gastroprotective medications including proton pump inhibitors or histamine-2 receptor antagonists [46]. Overall this study, even if it was open label and not rigorously controlled, shows that flavocoxid possesses a significant efficacy in the management of OA with a good safety profile [46].

8. Conclusions

Steroidal anti-inflammatory drugs and nonsteroidal anti-inflammatory drugs are effective in the management of the acute inflammatory reaction, but they do not influence successfully chronic inflammatory states and possess severe adverse effects. Flavocoxid is a mixture of the flavonoids molecules catechin, from *Acacia catechu*, and baicalin, extracted from *Scutellaria baicalensis*, concentrated to greater than 90% purity. Flavocoxid hind PLA_2 causes a balanced inhibition of the COX-1 and COX-2 peroxidase moieties and decreases the generation of LTBs from 5-LOX, avoiding the deleterious accumulation of these lipid mediators caused by the NSAIDs-induced 5-LOX shunt. It has also a strong antioxidant activity, and through NF-κB activity inhibition blocks the amplifying loop of the inflammatory response and acts at the level of gene and protein expression (Figure 4). It exerts beneficial effects in several experimental models of inflammation. *In vitro* toxicity testing and acute and subchronic toxicity animal studies indicate for flavocoxid an optimal preclinical safety profile. Finally, clinical trials and a postmarketing study show that flavocoxid has a significant efficacy in management of OA and a good overall and GI tolerability. Flavocoxid may therefore provide a potential therapeutic approach to the treatment of acute and chronic inflammatory conditions.

Conflict of Interests

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

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Research Article

Metformin Attenuates Experimental Autoimmune Arthritis through Reciprocal Regulation of Th17/Treg Balance and Osteoclastogenesis

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Metformin is widely used to suppress certain functions of the cells found in diseases including diabetes and obesity. In this study, the effects of metformin on downregulating IL-17-producing T (Th17) cells, activating and upregulating regulatory T (Treg) cells, suppressing osteoclastogenesis, and clinically scoring collagen-induced arthritis (CIA) were investigated. To evaluate the effect of metformin on CIA, mice were orally fed with either metformin or saline as control three times a week for nine weeks. Histological analysis of the joints was performed using immunohistochemistry and Th17 cells and Treg cells of the spleen tissue were examined by confocal microscopy staining. Metformin mitigated the severity of CIA, reduced serum immunoglobulin concentrations, and reciprocally regulated Th17/Treg axis. Also, metformin treatment of normal cells cultured in Th17 conditions decreased the number of Th17 cells and increased the number of Treg cells. Metformin decreased gene expression and osteoclastogenic activity in CIA and normal mice. These results indicate that metformin had immunomodulatory actions influencing anti-inflammatory action on CIA through the inhibition of Th17 cell differentiation and the upregulation of Treg cell differentiation along with the suppression of osteoclast differentiation. Our results suggest that metformin may be a potential therapeutic for rheumatoid arthritis.

1. Introduction

Rheumatoid arthritis (RA) is a multisystem autoimmune disease of unknown etiology. The characteristic pathology of affected joints is the inflammation of hyperplastic synovial membrane that can result in destruction of adjacent cartilage and bone [1]. Though the exact molecular pathogenesis of RA remains elucidated, evidence suggests that interleukin- (IL-) 17-producing T cell, Th17, is a central player. IL-17 is known to act synergistically with tumor necrosis factor- (TNF-) α and IL-1β, which are abundant proinflammatory cytokines found in arthritic joints enhancing the activation of fibroblasts, chondrocytes, and osteoclasts [2]. In contrast, regulatory T (Treg) cell has been shown to have an anti-inflammatory role where its regulatory function is known to be deranged in RA patients. Th17 and Treg have been reported to have plasticity and can be converted to each other according to the cytokine milieu when the cells encounter [3]. Therefore, a therapeutic

approach that can increase Treg cell while diminishing Th17 cell simultaneously may be very promising in RA treatment.

Metformin was originally introduced as an antidiabetic medication. Earlier studies have suggested that the majority of pharmacologic effect of metformin is dependent on its ability to activate AMP-activated protein kinase (AMPK), a major cellular regulator of lipid and glucose metabolism [4]. Metformin inhibits mitochondrial respiratory chain resulting in energy deficiency noted as an increase in AMP, activating AMPK and protein kinase A (PKA).

Activated AMPK and PKA inhibit lipid synthesis and gluconeogenesis, respectively [5]. AMPK activation also promotes glucose uptake and fatty acid oxidation in muscle, enhancing the lowering effect of glucose [6]. In addition to the glucose lowering effect, metformin has been reported to exert an anti-inflammatory effect, which is also mediated by metformin-activated AMPK. Metformin-activated AMPK suppresses mammalian target of rapamycin (mTOR), which regulates T cell effector differentiation *in vitro* and *in vivo*. AMPK has been shown to be associated with Th17 cell suppression by inhibiting mTOR and signal transducer and activator of transcription 3 (STAT3), suggesting the therapeutic potential of AMPK agonist [7].

While Th17 cells are responsible for perpetuating chronic inflammation in RA, osteoclasts resorb bone resulting in subsequent joint destruction [8]. The role of mTOR in osteoclastogenesis has been reported where a downstream molecule of the mTOR pathway (S6K) conveys cell survival signal in osteoclasts [9]. Recently, Indo et al. reported that AMPK-mediated inhibition of mTOR and hypoxia-induced factor (HIF)-1α negatively regulated osteoclastogenesis [10].

These regulatory effects of AMPK on Th17 cells and osteoclasts have prompted the investigation on the effect of the AMPK agonist, metformin, on autoimmune arthritis. In the present study, the suppression of metformin in a collagen-induced arthritis (CIA) mouse model was demonstrated by focusing on the suppression of Th17 differentiation and enhancing Treg differentiation. Phosphorylation of STAT3 was diminished by metformin while phosphorylation of STAT5 increased, which seems to contribute to the alteration of Th17/Treg population. In addition, osteoclastogenesis was suppressed via inhibition of mTOR and AMPK activation by metformin.

2. Materials and Methods

2.1. Mice. 12-week-old male C57BL/6 mice (Orient Bio, Korea) were maintained under specific pathogen-free conditions and fed standard laboratory mouse chow (Ralston Purina, St. Louis, MO) and water *ad libitum*. All experimental procedures were examined and approved by the Animal Research Ethics Committee of the Catholic University of Korea, which conforms to all National Institutes of Health of the USA guidelines. All surgeries were performed under isoflurane anesthesia and all efforts were made to minimize suffering.

2.2. Induction of Arthritis and Treatment of Metformin. CIA was induced in C57BL/6 mice ($n = 20$). Mice were immunized into the base of the tail with 100 μg of chicken CII (Chondrex Inc., Redmond, WA, USA) in complete Freund's adjuvant (Chondrex Inc.). 100 μg of chicken CII in incomplete Freund's adjuvant (Chondrex Inc.) was injected at tail and one foot on day 14. CIA mice were orally fed 3 times a week for 9 weeks with 5 mg/mouse metformin (Sigma-Aldrich) or saline as a control beginning on day 7 after first immunization (permit number: CUMC-2013-0128-01). Arthritis in these mice was examined visually two times per week for the appearance of arthritis in the peripheral joints.

2.3. Clinical Scoring of Arthritis. The severity of arthritis was recorded using the mean arthritis index on scale of 0–4, as previously reported [11], as follows: (0), no evidence of erythema and swelling; (1), erythema and mild swelling confined to the midfoot (tarsals) or ankle joint; (2), erythema and mild swelling extending from the ankle to the midfoot; (3), erythema and moderate swelling extending from the ankle to the metatarsal joints; (4), erythema and severe swelling encompassing the ankle, foot, and digits. The severity of arthritis was analyzed by the sum of scores from all legs, assessed by two independent observers with no knowledge of the experimental groups.

2.4. Immunohistopathological Analysis of Arthritis. Joints of each mouse were fixed in 10% formalin, decalcified in 10% EDTA, and embedded in paraffin wax. The sections were stained with hematoxylin-eosin (H&E), safranin O, and toluidine blue to detect proteoglycans. For immunohistochemistry, the sections were performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) [12]. Inflammation was scored according to criteria previously reported. [13]. Tissues were stained anti-receptor activator of NF-κB (RANK), anti-receptor activator of NF-κB ligand (RANKL), anti-TNF-α, anti-TNF receptor-associated factor 6 (TRAF6), anti-IL-17, and anti-IL-6 (all from Santa Cruz Biotechnology Inc.) and anti-phosphorylated(p) STAT3 (Y705), anti-pSTAT3 (S727), and anti-pAMPK anti-pmTOR (all from Cell Signaling, Danvers, MA).

2.5. Confocal Microscopy of Immunostaining. Spleen tissues were obtained on day 35 after first immunization. The tissue was stained using PE-conjugated anti-CD4, FITC-conjugated anti-forkhead box P3 (Foxp3), APC-conjugated anti-CD25, FITC-conjugated anti-IL-17, FITC-conjugated anti-pSTAT3 (Y705), and FITC-conjugated anti-pSTAT3 (S727) (all from eBiosciences, San Diego, CA, USA). Stained sections were analyzed using a Zeiss microscope (LSM 510 Meta; Carl Zeiss, Oberkochen, Germany).

2.6. Measurement of Immunoglobulin (Ig) Concentrations. Anti-IgG, IgG1, and IgG2a were measured by mouse IgG, IgG1, and IgG2a ELISA quantitation kits (Bethyl Laboratories, Montgomery, TX) [12].

2.7. Flow Cytometry of Intracellular Cytokines. To analyze intracellular cytokines, splenocytes were stained with PerCP-conjugated anti-CD4, APC-conjugated anti-CD25,

Table 1: Mouse primer sequences.

Name	Forward $5' \rightarrow 3'$	Reverse $5' \rightarrow 3'$
Interleukin(IL)-17 A	CCT CAA AGC TCA GCG TGT CC	GAG CTC ACT TTT GCG CCA AG
Aryl hydrocarbon receptor (Ahr)	AGC AGC TGT GTC AGA TGG TG	CTG AGC AGT CCC CTG TAA GC
Runt-related transcription factor 1 (RUNX1)	TAC CTG GGA TCC ATC ACC TC	GAC GGC AGA GTA GGG AAC TG
RAR-related orphan receptor gamma T (RORgT)	TGT CCT GGG CTA CCC TAC TG	GTG CAG GAG TAG GCC ACA TT
Forkhead box P3 (Foxp3)	GGC CCT TCT CCA GGA CAG A	GCT GAT CAT GGC TGG GTT GT
Tartrate resistant acid phosphatase (TRAP)	TCC TGG CTC AAA AAG CAG TT	ACA TAG CCC ACA CCG TTC TC
Integrin $\beta 3$	CTG TGG GCT TTA AGG ACA GC	GAG GGT CGG TAA TCC TCC TC
Calcitonin receptor	CGG ACT TTG ACA CAG CAG AA	AGC AGC AAT CGA CAA GGA GT
Carbonic anhydrase II	TGG TTC ACT GGA ACA CCA AA	AGC AAG GGT CGA AGT TAG CA
Cathepsin K	CAG CAG AGG TGT GTA CTA TG	GCG TTG TTC TTA TTC CGA GC
Matrix metalloproteinases-9 (MMP-9)	CTG TCC AGA CCA AGG GTA CAG CCT	GAG GTA TAG TGG GAC ACA TAG TGG
HIF-la	AGG CCT AGA TGG CTT TGT GA	TAT CGA GGC TGT GTC GAC TG
AMPka	TGTTCCAGCA GATCCTTTCC	ATAATTGGGTGAGCCACAGC
β-Actin	GTA CGA CCA GAG GC A TAC AGG	GAT GAC GAT ATC GCT GCG CTG

FITC-conjugated anti-IL-17, and PE-conjugated anti-Foxp3 (eBiosciences), followed by fixation and permeabilization with a Foxp3 staining buffer kit (BD Bioscience) according to the manufacturer's instructions. Four hours before the staining, the cells were stimulated with phorbol myristate acetate (25 ng/mL) and ionomycin (250 ng/mL) (all from Sigma-Aldrich) and GolgiStop (BD Bioscience). All data was analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

2.8. Real-Time Quantitative Polymerase Chain Reaction (PCR). The mRNA expression levels were estimated using a LightCycler 2.0 instrument (Roche Diagnostic, Mannheim, Germany) with the version 4.0 software. All reactions were performed with the LightCycler FastStart DNA Master SYBR Green I (Takara, Shiga, Japan), following the manufacturer's instructions. The mRNA expression was normalized to that of β-actin. The primers sequences are shown in Table 1.

2.9. In Vitro Differentiation into Th17 Cells. To purify splenic CD4+ T cells, the splenocytes were incubated with CD4-coated magnetic beads and isolated using magnetic activated cell sorting (MACS) separation columns (Miltenyi Biotec). Isolated CD4+ T cells were stimulated with Th17 cell-polarizing conditions for 3 days: plate-bound anti-CD3 (0.5 μg/mL), anti-CD28 (1 μg/mL) (both from BD PharMingen, CA, USA); anti-interferon (IFN)-γ (2 μg/mL), anti-IL-4 (2 μg/mL), transforming growth factor (TGF)-β (2 ng/mL), and IL-6 (20 ng/mL) (all from R&D Systems, Minneapolis, MN, USA).

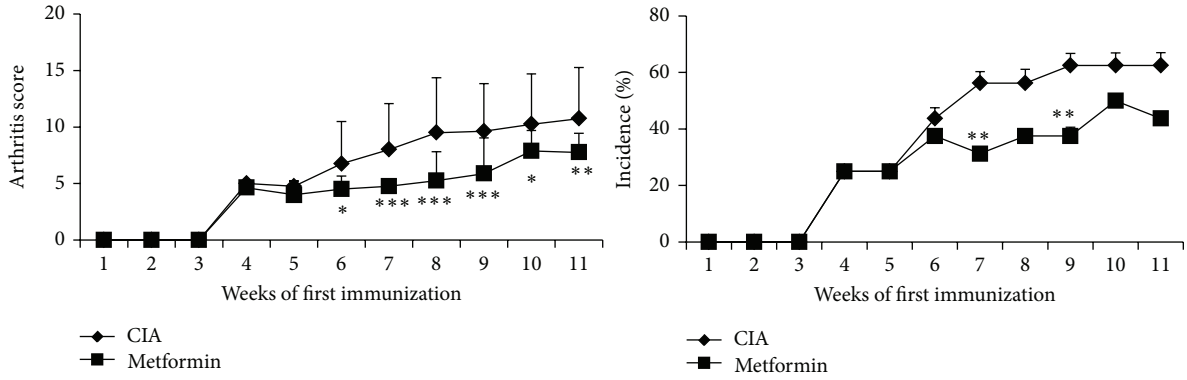
2.10. In Vitro Osteoclastogenesis. Osteoclasts were stimulated in the presence of macrophage colony-stimulating factor (M-CSF) (10 ng/mL) (R&D Systems) and receptor activator of nuclear factor kappa-B ligand (RANKL) (50 ng/mL) (PeproTech, London, UK) and absence or presence of metformin 1 mM. The medium was changed every two days. Osteoclasts were generated after 8–10 days [14].

2.11. Western Blot Analysis. The protein was separated by SDS-PAGE and transferred on nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Western blot was performed by SNAP i.d. protein detection system (Millipore). The hybridized bands were detected by enhanced chemiluminescence (ECL) detection kit (Thermo Scientific brand of Thermo Fisher Scientific, Inc.). The antibodies were as follows: anti-AMPK, anti-*p*AMPK, anti-mTOR, anti-*p*mTOR, anti-*p*STAT3 Y705, anti-*p*STAT3 S727, and anti-STAT3 (all from Cell Signaling); TRAF6 (Santa Cruz); and β-actin (Sigma).

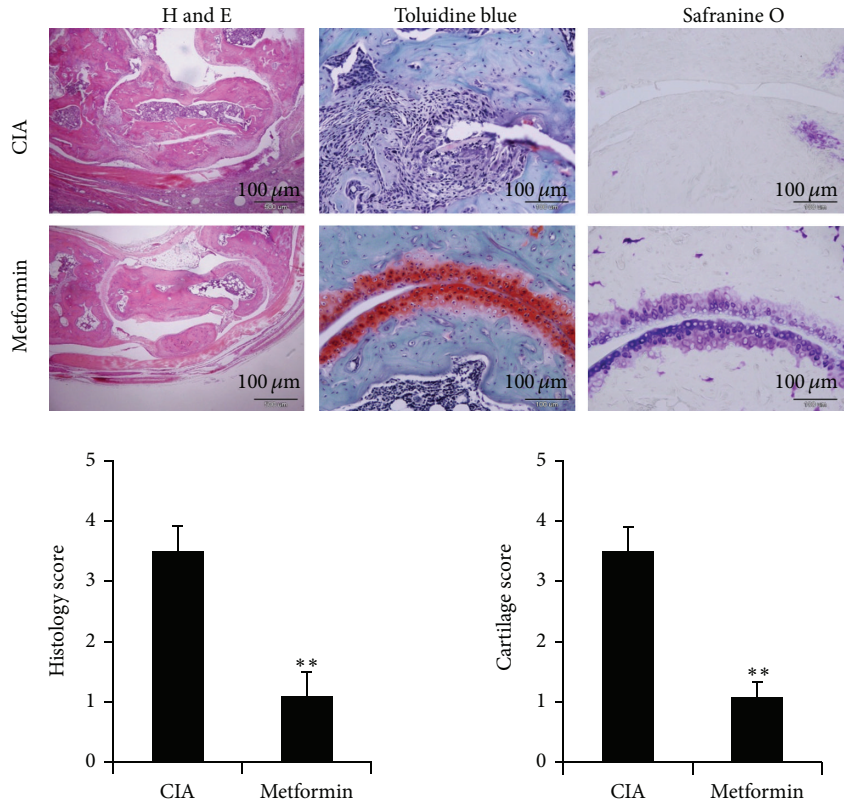
2.12. Statistical Analysis. All data were expressed as the mean ± SD. Statistical analysis was performed using SPSS 10.0 for Windows (IBM Corp., Armonk, NY). Comparing numerical data between groups was performed with 2-way ANOVA and nonparametric Mann-Whitney tests. Differences in the mean values of various groups were analyzed by using ANOVA with a post hoc test. P values < 0.05 were considered significant.

3. Results

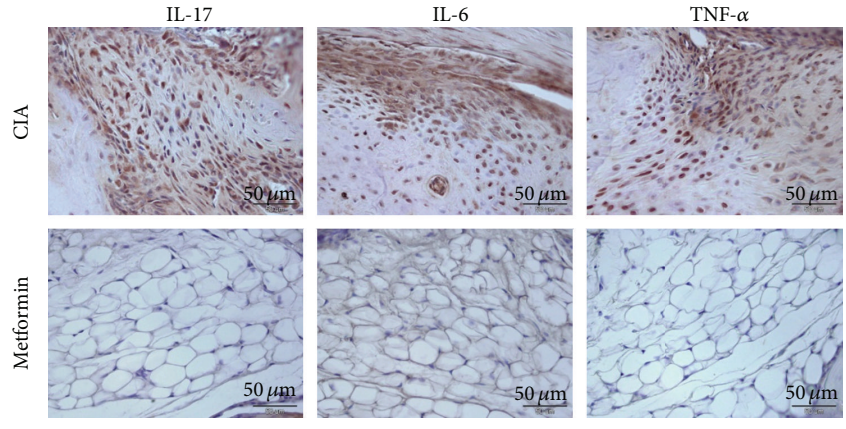
3.1. Metformin Ameliorates Collagen-Induced Arthritis. To investigate the antiarthritic effect of metformin, mice with CIA were orally fed with either metformin or saline three times a week from day 7 after first immunization. Metformin-treated CIA mice showed significantly reduced severity (Figure 1(a), left) and incidence of clinical arthritis (Figure 1(a), right). Histological and cartilage scores measured based on inflammatory cell infiltration and cartilage damage were also significantly lower in metformin-treated mice (Figure 1(b)). IL-17, IL-6, and TNF positive cells decreased in metformin-treated CIA mice (Figure 1(c)).



(a)

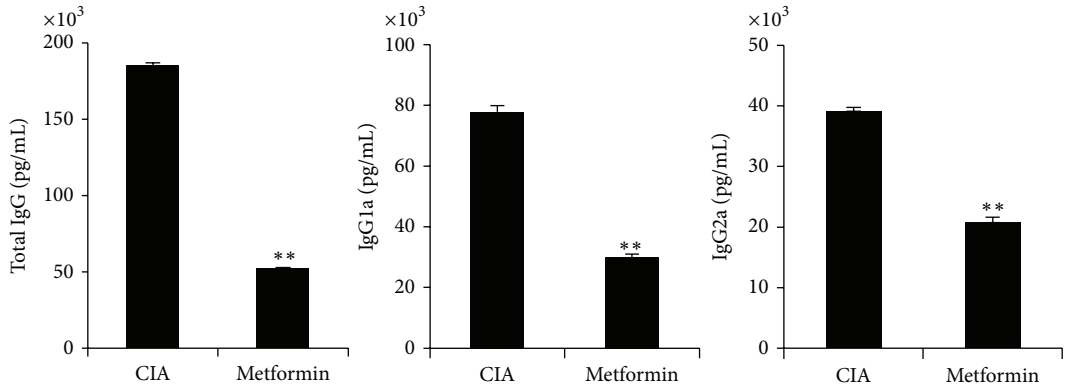


(b)



(c)

Figure 1: Continued.



(d)

Figure 1: Therapeutic effects of metformin in CIA model. CIA was induced in C57BL/6 mice. Metformin 5 mg/mice ($n = 10$) or saline ($n = 10$) was oral feed three times into CIA in a week. Mice were sacrificed on day 70 after first immunization. (a) Clinical arthritis scores were determined. (b) The joint tissues from CIA: metformin-treated CIA mice were stained with H&E, safranin O, and toluidine blue (original magnification, ×200). The average histopathological score is shown in bar graphs (below) (scale bar = 100 μm). (c) Immunohistochemical detection of IL-17, IL-6, and TNF-α was stained in the synovium of CIA and metformin-treated CIA. All tissues were counterstained with hematoxylin (original magnification, ×400). All images were obtained for each mouse ($n = 10$), showing representative images (scale bar = 50 μm). (d) Mice serum was obtained on day 30 after CII immunization. The serum obtained after first immunization. The levels of IgG, IgG1, and IgG2a antibodies were measured from each group. Mean ± SD of three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$).

Total IgG, IgG1, and IgG2a antibodies significantly decreased in metformin-treated CIA mice when compared to saline-treated controls (Figure 1(d)). Collectively, these results demonstrated that metformin attenuated CIA.

3.2. Analysis of the Th17, Treg Cell Population in CIA Mice. We investigated the effect of metformin on Th17, Treg population in CIA mice. First, we was analyzed for Th17 and Treg cells using tissue confocal staining. The numbers of CD4+IL-17+ T cells and CD4+ *p*STAT3+ (both at S727 and Y705) T cells were reduced in metformin-treated CIA mice more than in the controls. In contrast, the numbers of CD4+ CD25+ Foxp3+ Treg cells and CD4+ *p*STAT5+ T cells were higher in the metformin-treated group (Figure 2). Next, spleen cells isolated from each group were analyzed for the expression of IL-17 and Foxp3 using flow cytometry. The results showed that CD4+IL-17+Th17 cells were lower in the metformin-treated CIA, whereas CD4+CD25+ Foxp3+Treg cells were increased in metformin group (Figure 3(a)). As well, mRNA expressions of the Th17-related cytokines and transcription factors were significantly reduced in the splenocytes from metformin-treated CIA mice and there was greater expression of Foxp3 (Figure 3(b)). The *p*AMPK expression increased in metformin-treated CIA (Figure 3(c)).

3.3. Metformin Regulates Th17, Treg Cell Differentiation In Vitro. As the proportion of Th17 cell was decreased in metformin-treated CIA mice *in vivo*, the effect of metformin on Th17 cell and Treg cell differentiation *in vitro* was examined. CD4+ T cells isolated from healthy C57BL/6 mice were cultured in Th17 cell-polarizing condition in the presence or absence of metformin. The differentiation of Th17 cells was inhibited with metformin as there was a simultaneous increase in Treg differentiation (Figure 3(d)). In addition, metformin inhibited the expression levels of the Th17-associated genes, IL-17, Ahr, RUNX1, and RORγT, as it increased mRNA levels of Foxp3 (Figure 3(e)). Treatment with metformin increased the expression of *p*AMPK in IL-6 10 ng/mL stimulated CD4 T cell (Figure 3(f))). These results indicate that metformin suppresses Th17 cell differentiation while enhancing Treg differentiation and AMPK activation in a proinflammatory condition (Figure 6).

3.4. The Effect of Metformin on Osteoclastogenesis In Vivo. Metformin-treated CIA mice showed reduced bone erosion and cartilage destruction in the arthritic joints (Figure 1(b)). These results have prompted us to investigate direct effect of metformin on osteoclastogenesis. To determine effect of metformin on osteoclastogenesis, we were immunochemical staining for RANK and RANKL in joint tissues. As expected, the expression of RANK and RANKL decreased in the inflamed joint tissue of metformin-treated CIA (Figure 4(a)). The preosteoclasts derived from the control CIA mice and metformin-treated CIA mice were stimulated with M-CSF and RANKL. The results showed that the osteoclasts from metformin-treated CIA mice were less capable of differentiating into osteoclasts (Figure 4(b)). The expression levels of various osteoclastogenic markers, such as TRAP, MMP-9, integrin β3, calcitonin receptor, and cathepsin K, were decreased, also. The reduction in osteoclastic activity was observed following metformin-treated mice (Figure 4(c)).

3.5. Metformin Suppresses Osteoclast Differentiation by Inhibiting the STAT3, AMPK Pathway. To verify the observation that metformin reduced the expression of osteoclastogenic markers *ex vivo*, we investigated whether metformin could inhibit osteoclast differentiation *in vitro*. The results showed that metformin treatment inhibited osteoclast differentiation as determined by the TRAP staining assay

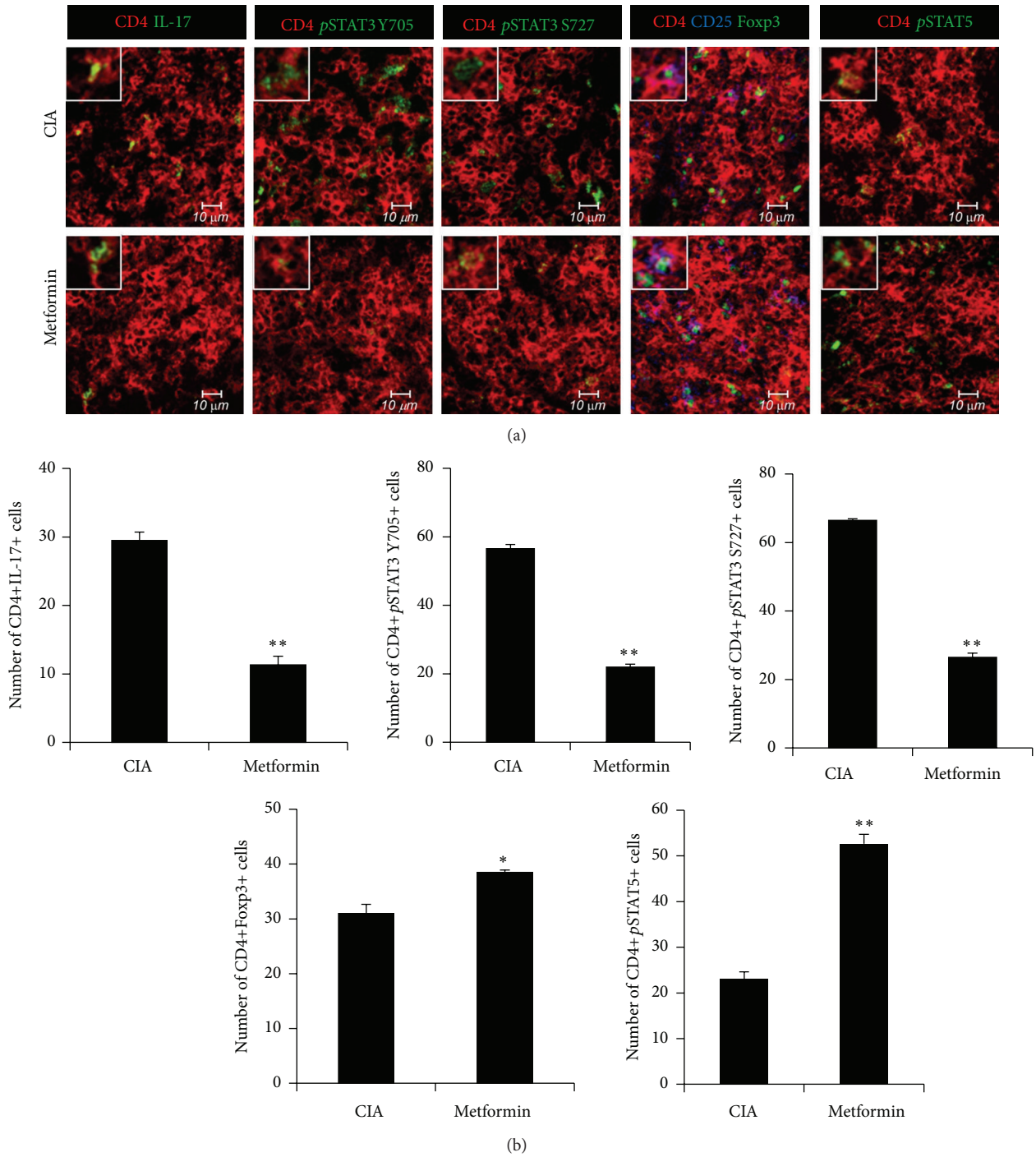
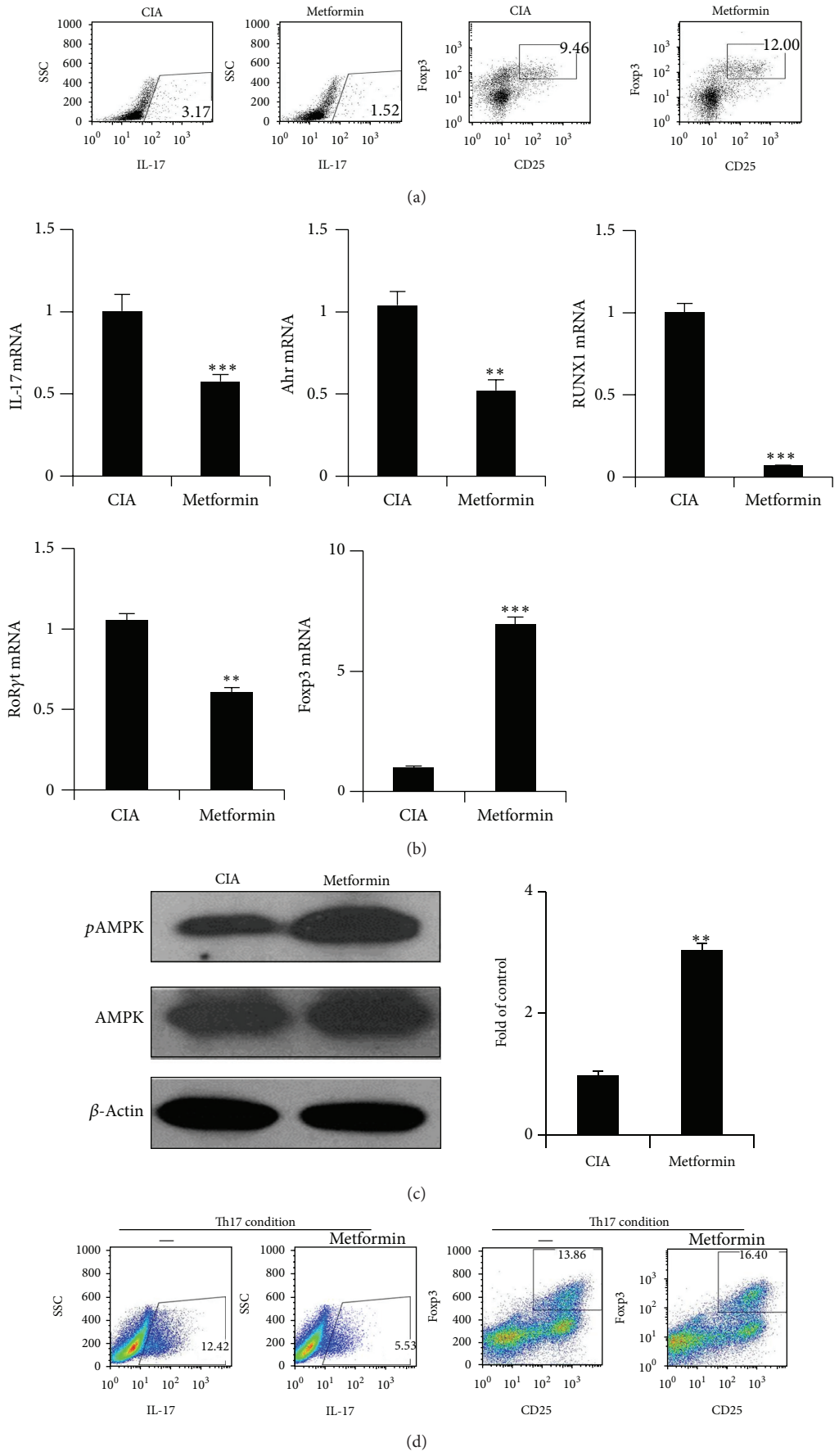


Figure 2: Regulation of Th17 cells and Foxp3+ regulatory T cells in CIA spleen. Spleen tissue was obtained from metformin-treated CIA and control CIA on day 35 after first immunization. (a) Confocal staining examined by antibodies: Th17 cell was stained with CD4 (red) and IL-17 (green). CD4+ CD25+ Foxp3+ regulatory T cells were stained with CD4 (red), CD25 (blue), and Foxp3 (green). For activated STATs analysis, the tissues were stained with CD4 and *p*STAT3 S727, *p*STAT3 Y705, or *p*STAT5. All images were performed for each mouse ($n = 5$), showing representative images (scale bar = 10 μm). (b) The mean values are presented in the form of a histogram by four individuals. Results are shown as mean ± SD ($n = 5$ mice per group). Mean ± SD of three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$).



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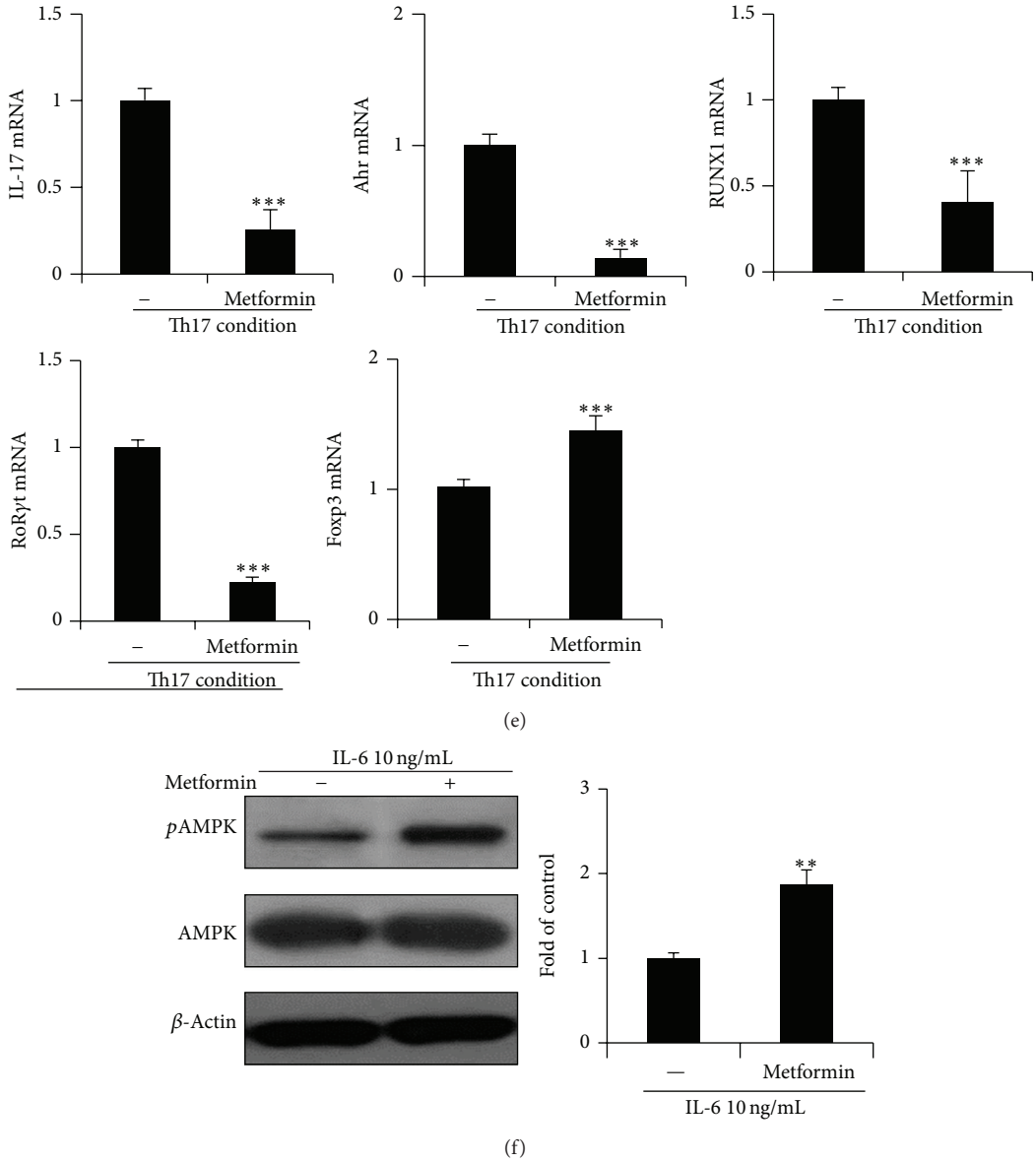


Figure 3: Treatment with metformin Treg cells and decreases Th17 cells in CIA mice and *in vitro* Th17 polarizing condition. (a–c) Splenocytes were also obtained from metformin-treated CIA ($n = 5$) and control CIA ($n = 5$) on day 35 after first immunization. (a) Both isolated CD4+ T cells were stained with anti-CD25, anti-Foxp3, and anti-IL-17 antibody. The proportions of CD4+IL-17+ T cells and CD4+CD25+ Foxp3+ regulatory T cells were analyzed using flow cytometry. (b) The gene levels of IL-17, Ahr, RUNX1, RORγT, and Foxp3 in splenocytes were determined by real-time PCR. (c) The expressions of phosphorylated AMPK were measured by western blot. The fold of control measured *p*AMPK/AMPK/*β*-actin ratio (right). (d–f) Isolated CD4+ T cells of C57BL/6 mice were cultured with under Th17 polarizing conditions in the presence or absence of 1 mM metformin for 3 days. (d) The cells were stained with anti-CD4, anti-CD25, anti-IL-17, and anti-Foxp3. (e) The mRNA expression levels of IL-17, Ahr, RUNX1, RORγT, and Foxp3 were determined by real-time PCR. (f) CD4+ T cells were stimulated with IL-6 10 ng/mL in the presence or absence of metformin 1 mM for 1 hour. The activation of *p*AMPK was measured by western blot. The representative results are shown in the right panel. Data are presented as the mean ± SD of four independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$).

(Figure 5(a)). We also measured osteoclastogenic markers mRNA expression levels in control osteoclasts and metformin-treated ones. The mRNA expression of the osteoclast-related markers, TRAP, cathepsin K, MMP-9, calcitonin receptor, carbonic II, and integrin 3*β*, decreased by meformin treatment. We also found that metformin suppressed the expression of HIF-1*α* mRNA whereas it induced expression of AMPK mRNA (Figure 5(b)). As the pharmacologic effect of metformin is largely dependent on AMPK, the activation of AMPK, mTOR, and STAT3 during osteoclastogenesis was addressed. Western blot analysis demonstrated that expressions of TRAF6, *p*mTOR, and *p*STAT3 (Y705 and S727) were reduced with metformin treatment. Also, *p*AMPK activation was increased (Figure 5(c)). Consistently,

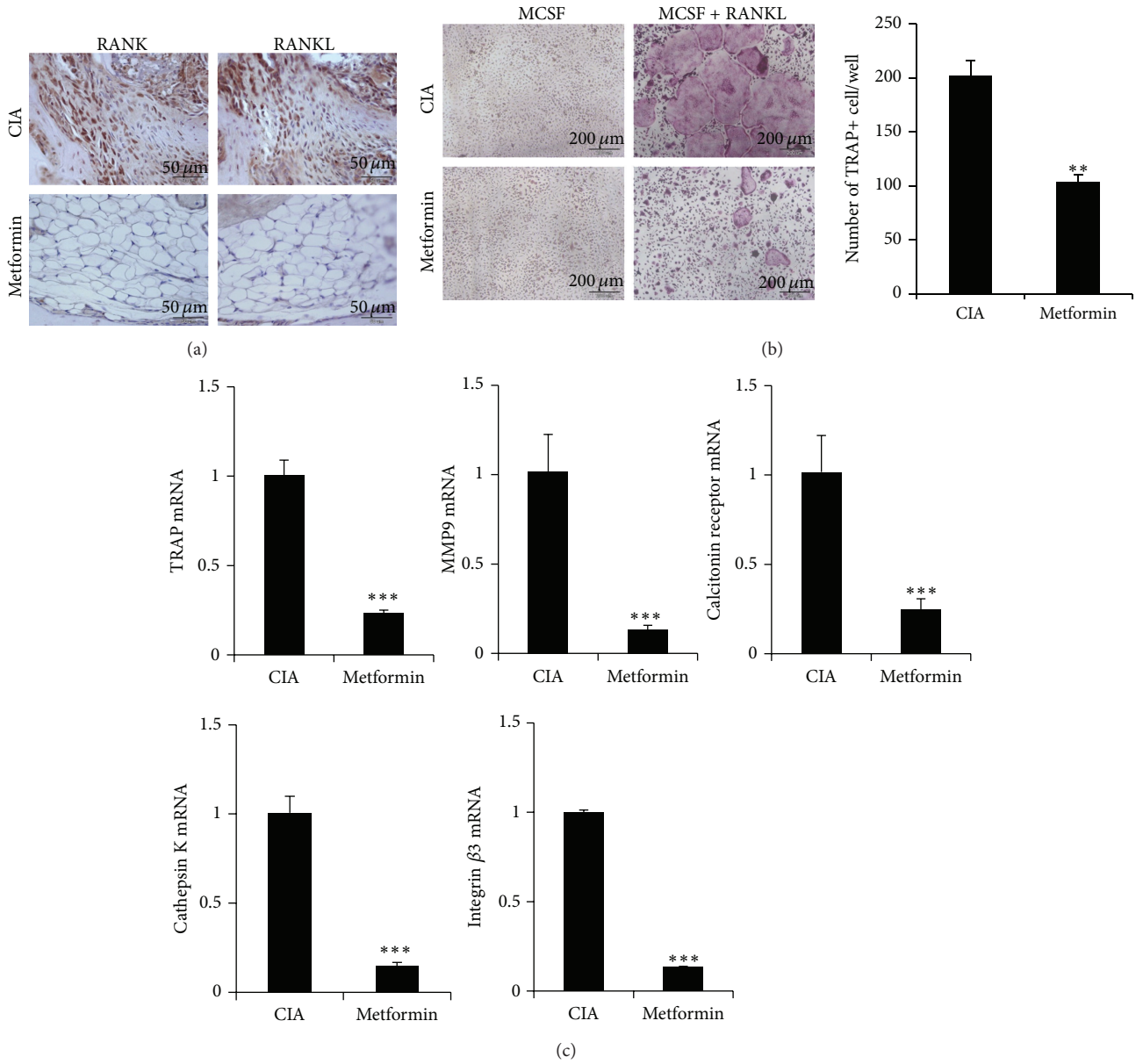


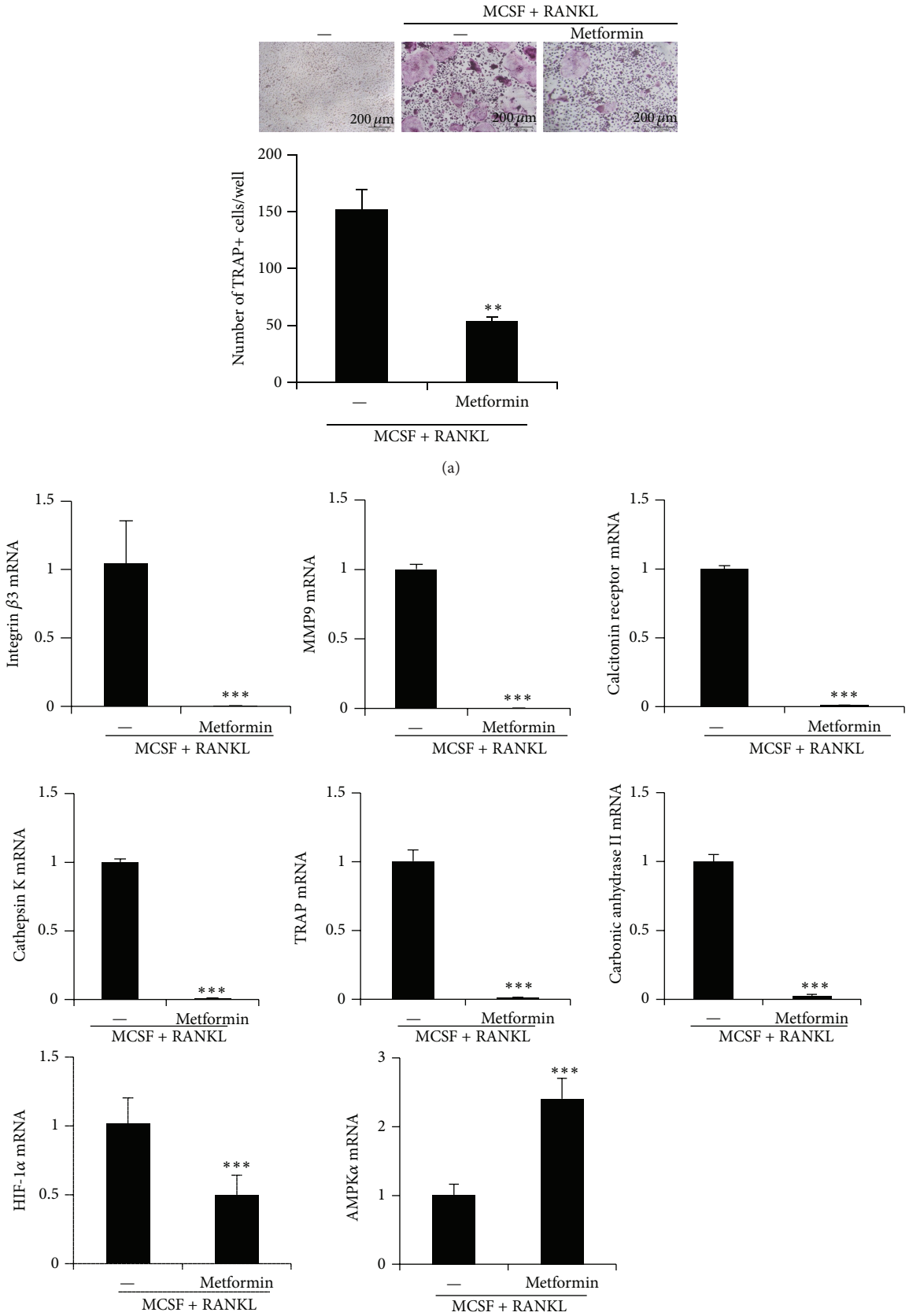
Figure 4: Metformin inhibits osteoclast formation in CIA mice. (a) Joint tissues of CIA control mice ($n = 5$) and metformin-treated CIA ($n = 5$) were stained with anti-RANKL and anti-RANK antibodies 70 days after the first immunization. All images were obtained for each mouse ($n = 5$), showing representative images (scale bar = 50 μm). (b) Isolated preosteoclasts from each group were cultured with 10 ng/mL M-CSF and/or 50 ng/mL RANK. Differentiated osteoclasts were stained with TRAP. The TRAP+ cells were indicated in graph (right) (scale bar = 200 μm). (c) The mRNA expressions of TRAP, MMP-9, calcitonin receptor, cathepsin K, and integrin $\beta 3$ as osteoclast markers were quantified by real-time PCR. Data are presented as the mean ± SD of four independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$).

immunohistochemical staining of arthritic joints revealed that expressions of these molecules were significantly lower in metformin-treated CIA mice (Figure 5). Collectively, these results suggest that metformin suppresses osteoclastogenesis via inhibition of mTOR and STAT3, which is mediated by metformin-activated AMPK.

4. Discussion

In the present study, we demonstrated that metformin suppressed CIA via reciprocal regulation of Th17 cell and Treg cell. Metformin-activated AMPK seems to be involved in this regulation through the inhibition of mTOR and its downstream molecules, HIF-1α and STAT3.

As Th17 receives the spotlight in the pathogenesis of RA, a number of medications that target Th17 have been developed. IL-6 is essential cytokine for Th17 cell differentiation signaling through JAK-STAT pathway [15]. However, tocilizumab, an IL-6 receptor blocking antibody, is currently being used, whereas tofacitinib, a JAK inhibitor, is under clinical trials. The molecules shown to inhibit STAT3 or RORγT have demonstrated promising results in animal models.



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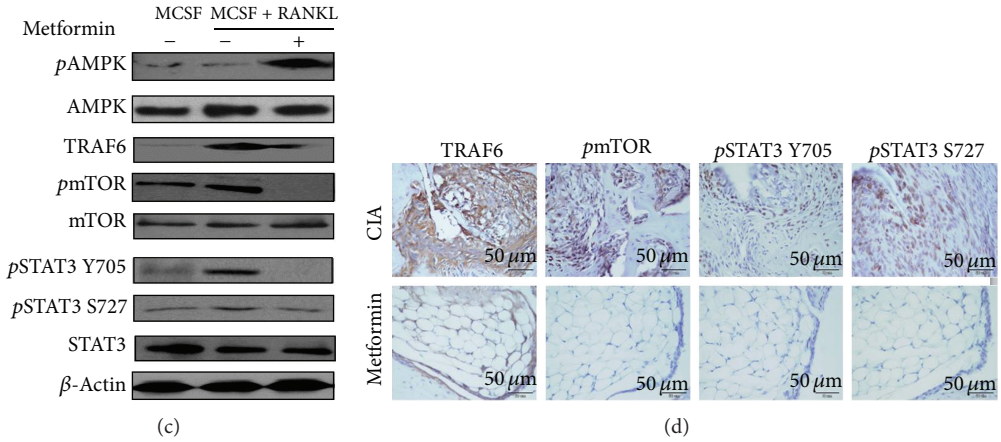

Figure 5: Metformin suppresses osteoclastogenesis *in vitro*. C57BL/6 mice preosteoclasts were cultured in the presence of 10 ng/mL M-CSF and/or 50 ng/mL RANKL in the presence or absence of metformin 1 mM. (a) Differentiated osteoclasts were stained for TRAP (scale bar = 200 μm) and The TRAP+ cells were indicated in graph (under). (b) The mRNA levels of TRAP, MMP-9, calcitonin receptor, carbonic anhydrase II, cathepsin K, integrin β3, HIF1-α, and AMPK were quantified by real-time PCR. (c) Protein levels of *p*AMPK, TRAF6, *p*mTOR, mTOR, *p*STAT3 Y705, *p*STAT3 S727, STAT3, and β-actin were analyzed using Western blot in osteoclasts. (d) Immunohistochemical detection of TRAF6, *p*mTOR *p*STAT3 Y705, and *p*STAT3 S727 were stained in the synovium of CIA and metformin-treated CIA. All tissues were counterstained with hematoxylin (original magnification, ×400) (scale bar = 50 μm). Data are presented as the mean ± SD of four independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$).

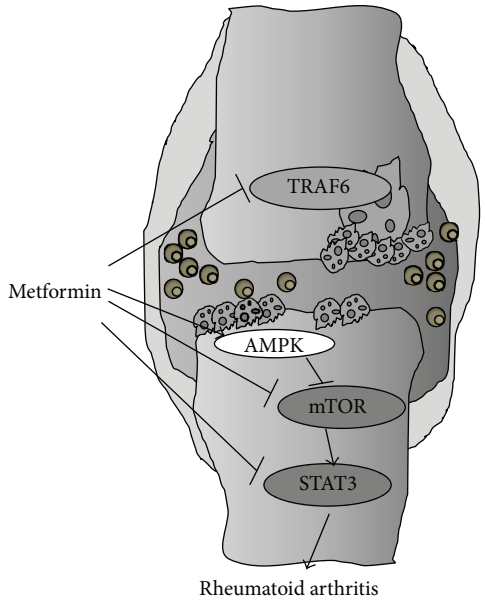

Figure 6: The signaling pathway that metformin uses to regulate T cell and osteoclasts in autoimmune arthritis.

Recently, metabolism has been raised as an important check point in regulating Th17/Treg balance [16]. Therefore, the role of AMPK, the master metabolic sensor, in T cell fate was investigated. Kang et al. reported that metformin-activated AMPK inhibited mTOR-STAT3 pathway contributing to a reduction in the population of Th17 cells [17]. Furthermore, our results revealed that metformin not only suppressed Th17 cells but also simultaneously enhanced Treg cells. This might be explained as the metabolic check point determines the differentiation of Th17 cell or Treg cell. Th17 cell and Treg cell are known to use different energy sources. For instance, Th17 cells are highly glycolytic while Treg cells have a high lipid oxidation rate [18]. The high glycolytic activity of Th17 cell is dependent on mTOR, which is inhibited by AMPK. mTOR enhances the glycolytic pathway promoting Th17 cell differentiation whereas AMPK inhibits mTOR and promotes mitochondrial oxidative metabolism to enhance Treg cell. Furthermore, the high glycolytic activity of Th17 cells is attributed to HIF-1α, a downstream molecule of mTOR pathway [19]. Th17 differentiation was impaired in HIF-1α deficient mice. In addition, HIF-1α promotes the nuclear translocation of RORγT and degrades Foxp3, resulting in a decrease in Treg cell [20]. Thus, AMPK regulate the balance between Th17 and Treg. AMPK has also been shown to negatively regulate the activation of STAT3 [21]. It is widely known that *p*STAT3 competes for the same binding locus as *p*STAT5 in IL-17 promoter to enhance IL-17 [22]. Therefore, activating AMPK and inhibiting mTOR are an efficient way to regulate Th17 and Treg concurrently. Our results revealed that metformin suppressed Th17 cell while increasing Treg cell in CIA mice. Though confocal microscopy showed an increase in STAT5 phosphorylation, it seems to result from reduced *p*STAT3 and consequently increase the number of Treg rather than the direct effect of metformin on STAT5 phosphorylation. Undeniably, metformin did not affect IL-2 induced STAT5 phosphorylation levels *in vitro* (data not shown).

It was reported that metformin ameliorated experimental autoimmune encephalitis [23] and collagen antibody induced arthritis [17], both involving Th17 cells. Our results confirmed that metformin-activated AMPK could suppress CIA via reciprocal regulation of Th17 cell and Treg cell. Therefore, future investigation of whether metformin can be used as a

therapeutic in Th17-driven chronic inflammatory diseases is promising.

Metformin also suppressed osteoclastogenesis both *in vivo* and *in vitro*. The number of TRAP+ multinucleated cells was lower in the joints of metformin-treated mice. This may partially result from decreased expression of inflammatory cytokines that promote osteoclastogenesis via the upregulation of RANKL in the arthritic joint. Metformin stimulates osteoprotegerin and reduces RANKL expression in osteoblasts [24], and AMPK inhibits RANK signaling [25], which might also contribute to reduced osteoclastogenesis. To investigate the direct effect on osteoclastogenesis, preosteoclasts were cultured with M-CSF and RANKL in the presence or absence of metformin, decreasing osteoclastogenesis in the metformin-treated cells. Our results also revealed that the suppression was associated with an increase in AMPK phosphorylation and subsequent decrease in mTOR and STAT3 phosphorylation. The decreased expression of HIF-1α in metformin-treated osteoclasts verifies the recent observation that mTOR-HIF-1α pathway was critical in osteoclastogenesis [10].

To date, there is no prevention in the development of rheumatoid arthritis. However, proper early treatment can prevent progressive damage of the joints. In this study, the effect of metformin on joint destruction and regulation of immune cells in occurrence of rheumatoid arthritis was evaluated. Additionally, the prevention of rheumatoid arthritis by metformin should be further investigated.

Collectively, these results suggest a new role of metformin. Metformin has been shown to ameliorate autoimmune arthritis by regulating the Th17/Treg balance and inhibiting osteoclastogenesis through AMPK. In cases where secondary diabetes mellitus develops due to excessive corticosteroid use in RA patients, metformin can be the optimal therapeutic option due to its glucose lowering and anti-inflammatory properties.

5. Conclusions

The present study demonstrated that metformin had an anti-inflammatory effect on CIA due to the inhibition of Th17 cell differentiation and the upregulation of Treg cell differentiation along with the suppression of osteoclast differentiation. Our data suggest that metformin might be a potential candidate for therapeutic modulation of experimental animal model with rheumatoid arthritis.

Conflict of Interests

The authors have no conflict of interests of any kind to declare regarding the materials or services referred to in this paper.

Acknowledgments

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Review Article

Evidence for the Gut Microbiota Short-Chain Fatty Acids as Key Pathophysiological Molecules Improving Diabetes

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In type 2 diabetes, hyperglycemia, insulin resistance, increased inflammation, and oxidative stress were shown to be associated with the progressive deterioration of beta-cell function and mass. Short-chain fatty acids (SCFAs) are organic fatty acids produced in the distal gut by bacterial fermentation of macrofibrous material that might improve type 2 diabetes features. Their main beneficial activities were identified in the decrease of serum levels of glucose, insulin resistance as well as inflammation, and increase in protective Glucagon-like peptide-1 (GLP-1) secretion. In this review, we updated evidence on the effects of SCFAs potentially improving metabolic control in type 2 diabetes.

1. Introduction

Type 2 diabetes (T2D) is a metabolic disorder characterized by hyperglycemia caused by the relative lack of insulin production, due to the exhaustion of pancreatic beta cell function after the establishment of insulin resistance [1]. The onset of inflammation in metabolic diseases (including obesity and diabetes) has been shown to be potentially related to alteration in the gut microbiota [2]. Since interindividual variations in the microbiome impact multiple human pathologies, understanding the factors that underlie changes in the composition of gut microbiota may be useful in designing more selective and effective therapies [3, 4]. Recently, a potential relationship between gut microbiota and T2D pathophysiology has been clearly suggested by two independent studies, which compared metagenomes from healthy and T2D subjects. For instance, an increase in Clostridium clostridioforme and a decrease in Roseburia_272 were demonstrated in T2D subjects from both Chinese and European populations [5, 6]. Accordingly, increased levels of Roseburia were associated with improved insulin sensitivity after gut microbiota transplantations from lean donors to recipients with metabolic syndrome [7]. These effects were potentially related to levels and activities of short-chain fatty acids (SCFAs). In particular, propionic and butyric acids were shown to reduce low-grade inflammation, to regulate cell proliferation and differentiation, and to induce hormone release [8–13]. Butyrate producing intestinal bacteria seems to play an important role in blood glucose regulation and lipid metabolism, as shown by fecal transplantation studies [7, 14]. Recently, Remely and coworkers demonstrated that distinct composition of gut microbiota producing different SCFAs may affect epigenetic gene regulation in obesity and T2D [15]. In addition, partial prevention of obesity through histone acetylation by SCFAs was reported by Duranton and colleagues more than ten years ago [16]. These beneficial

effects of SCFAs were not only related to their property as histone deacetylase inhibitors, but also related to their activation of the transmembrane cognate G protein-coupled receptors [17].

2. Short-Chain Fatty Acids (SCFAs)

SCFAs are organic fatty acids with 1 to 6 carbon atoms existing in straight- and branched-chain conformations [18, 19]. SCFAs are produced in the distal gut by bacterial fermentation of macro-fibrous material that escapes digestion in the upper gastrointestinal tract and enters the colon, including resistant starch, dietary fiber, simple sugars, sugar alcohols, unabsorbed or undigested proteins, and endogenous substrates, such as sloughed off epithelial cells, mucus, intestinal enzymes, and other secretions [8, 20, 21]. Although common SCFAs include formic, acetic, propionic, butyric, isobutyric, valeric, isovaleric, and caproic acids [22], 90–95% of the SCFAs present in the colon are constituted by acetate, propionate, and butyrate [8], with intraluminal concentrations of about 60% acetate (C2), 25% propionate (C3), and 15% butyrate (C4) [23]. Butyrate is considered as a major energy source for the colonic epithelium. Indeed in the colon, butyrate oxidation occurs with an increased rate as compared to acetate and propionate [24]. Propionate, entering the portal circle, is primarily utilized in gluconeogenesis in the liver, whereas significant amount of acetate enters systemic circulation and reaches peripheral tissues. Consequently plasma levels of SCFAs are dominated by acetate [25, 26]. Absorption of SCFAs is rapid and the colon absorbs more than 95% of SCFAs [23], mainly through specific apical solute carriers such as the monocarboxylate transporter 1 (MCT1) and the sodium-coupled monocarboxylate transporter 1 (SMCT1) present in colonic epithelial cells [27]; consequently absorption of SCFAs contributes to maintain acid-base equilibrium and promotes the absorption of Na+. By providing a significant contribution to the total caloric intake, metabolized SCFAs concur to maintain energy homeostasis [28]. Interestingly, SCFAs of colon origin contribute approximately 5–10% towards human energy requirements [29]. Positive metabolic health effects (such as satiety increase, blood glucose, and cholesterol lower levels) have been shown after ingestion of resistant starch and have been associated with increased fecal SCFAs concentrations, particularly propionic and butyric acids [23, 30]. For instance, it has been shown that butyrate and propionate reduce food intake. In addition, butyrate plays important roles both at the intestinal level, by regulating transepithelial fluid transport, ameliorating mucosal inflammation and oxidative status, reinforcing the epithelial defense barrier, and preventing colorectal cancer, and at the extraintestinal level, by exerting ameliorating effects on many pathologies, including hemoglobinopathies, metabolic diseases, hypercholesterolemia, insulin resistance, and ischemic stroke [31].

Several studies demonstrated that differences in the rate and ratio of SCFA production depend primarily on the type of substrate [8, 32]. Ingestion of fermentable dietary fibers increased SCFA concentration, whereas the high-fat diet (HFD) reduced formation of SCFAs [23, 33]. In 2010, Freeland and colleagues observed that in hyperinsulinemic subjects receiving either a high-wheat fiber cereal or a low-fiber cereal daily for 1 year, concentrations of the acetate and butyrate were higher in the subjects on the high-fiber than the control cereal diet [34]. Furthermore, this effect was associated with improved metabolic control, suggesting the onset of protection from the deleterious effects of high fat diet-induced obesity and diabetes. Recently, the group of Jakobsdottir observed changed formation of SCFAs in rats administered with low-fat or high-fat diets supplemented with fermentable dietary fibers, showing that high-fat diet reduced the formation of butyrate, but increased succinate and cholesterol, as well as inflammation and liver fat in rats, whereas dietary fiber counteracts these effects [33]. In most of these studies the increased concentration of butyrate is associated with the improvement in metabolic health. This evidence was a start for many works investigating the potential clinical relevance for butyrate. Administration of butyrate in a rat model of insulin resistance and steatosis induced by HFD reduced liver steatosis and inflammation, normalized transaminases, insulin resistance, and glucose tolerance [35]. Interestingly treatment of diabetic rat with butyrate decreased plasma glucose, and HbA1c improved the beta-cell proliferation and plasma insulin levels [36].

3. SCFA Receptors

Besides acting as a local nutrient source, SCFAs can also trigger cell-specific signaling cascades by receptor activation. Cognate receptors for SCFAs were formally described through the process of "deorphanization" of known protein with unknown function. Receptors for SCFAs are two G-protein coupled receptors (GPCRs): FFAR2 (free fatty acid receptor2, previously known as GPR43) and FFAR3 (or GPR41) [37–40]. Although they share about 40% similarity [37, 38, 41], they differ in their specificity for ligands, with ligand potency affected by specie heterogeneity [37, 38], and for G protein coupling: FFAR3 couples exclusively through the pertussis toxin sensitive $G_{i/o}$ family, whereas FFAR2 couples either the $G_{i/o}$ and pertussis toxin-insensitive G_q families [38]. The intracellular signaling cascade triggers inositol 1,4,5-trisphosphate formation, intracellular [Ca^{2+}] mobilization, activation of extracellular signal-regulated kinase 1/2, and inhibition of intracellular cAMP accumulation [38]. Both receptors are expressed in a variety of cells, including colonic enteroendocrine L cells [42–44], mucosal mast cells [43], adipose tissue [37, 38], neutrophils, and monocytes [45]. Activation of the receptors affects distinct function depending on their tissue distribution, for instance FFAR3 is involved in SCFAs-stimulated leptin production by adipocytes and lipid profiles regulation, whereas FFAR2 is involved in modulation of inflammation and Glucagon-like peptide-1 (GLP-1) secretion [36, 40, 46]. Kimura and coworkers demonstrated that FFAR2 is involved in controlling body energy utilization. Indeed FFAR2-deficient mice are obese on a normal diet, whereas mice overexpressing FFAR2 specifically in adipose tissue remain lean even when fed with a high-fat diet [47].

Interestingly, it has been recently reported that expression of FFARs may be affected by different compositions of gut microbiota through epigenetic regulation of gene expression [15]. In particular the promoter region of FFAR3 showed a significantly higher methylation in the lean control group compared to type 2 diabetics and to obese subjects. These interactions between microbiota and epigenetic regulation may cause changes in expression and signaling of FFARs and influence the onset of metabolic disease.

Deficiency of FFAR2 results in increasing or maintaining inflammation in models of colitis, arthritis, and asthma, related to increased production of inflammatory mediators and increased immune cell recruitment [48]. In FFAR2-deficient mice it has also been reported impaired GLP-1 secretion in responses to SCFAs and reduced basal levels of active GLP-1 when compared with controls [49]. Although deficiency of FFAR3 in mice has been reported to be associated with a lower expression of peptide YY (PYY) and an attenuated microbiota-induced secretion of PYY [50], FFAR3 seems to play a minor role in stimulation of GLP-1 secretion [49, 51] and is not required for butyrate- and propionate-dependent induction of Glucose-dependent insulinotropic peptide secretion from K cells [51].

4. SCFAs and GLP-1

GLP-1 is an incretin hormone that participates to glucose homeostasis, mainly by lowering plasma glucose concentration, improving insulin secretion and resistance, and preserving pancreatic beta-cell function [52, 53]. GLP-1 is secreted by the intestinal L cells, an open-type intestinal epithelial endocrine cells [54], in response to a variety of nutrients [55, 56].

SCFAs have been linked to increased GLP-1 secretion in both animal and human models [57–60]. The effect of SCFAs on GLP-1 release may be affected by different fiber feeding compositions and experimental setting [58, 61–63]. Moreover, Freeland and Wolever reported that in hyperinsulinemic subjects a long-term diet rich in SCFAs is needed to increase GLP-1 concentration. Interestingly rectal, but not intravenous, infusion of SCFAs was effective in acute increment of GLP-1 secretion [60]. Since GLP-1 secreting L cells are mainly located in the distal ileum and colon, and the primary site of SCFAs production is the colon, ability of SCFAs to induce GLP-1 secretion has been widely investigated. Expression of both FFAR2 and FFAR3 was reported in the colon, with particularly strong expression in GLP-1 producing L-cells [42–44, 64]. Evidence that SCFAs evoked the release of GLP-1 into plasma came more than 15 years ago from Dumoulin and coworkers [65]. However the intracellular mechanism linking SCFAs with GLP-1 release is not yet fully understood. Recently, Tolhurst and colleagues [49] demonstrated that in primary murine colonic cultures, expressing both mRNAs for FFAR2 and FFAR3, acetate and propionate stimulate GLP-1 secretion into the basolateral side through activation of FFAR2, via $G_{q/11}$ and subsequent increased of the intracellular calcium level. Furthermore they found that FFAR2-deficient mice had significantly reduced GLP-1 protein content and reduced basal levels of active GLP-1, suggesting that FFAR2 may be involved in maintaining L-cell function.

Among SCFAs butyrate seems to have a slower potency than acetate and propionate in stimulating GLP-1 secretion [49]. However butyrate treatment of the human L-cell line NCI-H716 cells resulted in enhanced secretion of GLP-1 and increased expression of genes involved in GLP-1 synthesis and secretion [57]. Furthermore the beneficial effects of administration of the probiotic VSL#3 such as reduced food intake, protection from body weight gain and insulin resistance, and rise in GLP-1 secretion were associated with the increased levels of butyrate [57]. Mechanisms through which butyrate increases GLP-1 secretion are still matter of debate. The poor responsiveness to SCFAs of the GLP-1, secreting cell line GLUTag, has been associated with the very low expression of FFAR2 [49]. Conversely, Yadav and colleagues suggested that butyrate interaction on L-cells might be mediated via FFAR3, since rising in secretion of GLP-1 induced by butyrate was associated with increased expression of FFAR3 [57]. Moreover, butyrate-induced total GLP-1 secretion was attenuated in the FFAR3 knockout mice [51].

5. SCFAs and Insulin

Insulin is a peptide hormone produced by pancreatic beta cells, which regulates the level of blood glucose inducing cellular glucose uptake, especially by adipose and skeletal muscle cells, and inhibiting glycogen lysis in liver cells. Therefore, the balance between insulin secretion and insulin action maintains homeostasis of glucose. In some metabolic disorders, such as T2D, cells fail to respond to the normal actions of insulin, resulting in insulin resistance [1]. Evidence that SCFAs protect against diet-induced obesity and insulin resistance has been reported by many authors [51]. Changes in the microbiota, and consequently in SCFAs composition, have been hypothesized to be associated with the development of obesity, insulin resistance, and diabetes. Colonization of normal and germ-free mice with microbiota harvested from the caecum of obese mice results in increased body weight gain [66]. Oppositely, modulation of the gut microbiota composition leading to increased butyrate production results in suppression of body weight gain and insulin resistance in high fat diet-fed and obese mice [57]. Furthermore improved insulin sensitivity has been found after infusion of butyrate-producing intestinal microbiota from lean donors to male subjects with metabolic syndrome [7]. Interestingly activation of FFAR2 by SCFAs may suppress insulin signaling in adipocytes, inhibiting fat accumulation in adipose tissue, but promoting the metabolism of unincorporated lipids and glucose in other tissues [47].

Among SCFAs, butyrate seems to play an important role in the pathology of obesity and diabetes; therefore the attention of the most recently studies has been focused on it. Interestingly, consistent with pancreatic beta-cells stimulation by incretins, oral administration of sodium butyrate in mice significantly elevated plasma insulin [51]. Recently,

a metagenomic study on obese versus lean subjects showed that butyrate-producing bacterial abundance was substantially decreased in obese subjects [6], supporting the fact that butyrate may be responsible of healthy metabolism. It has been widely recognized that a fiber enriched diet protects against obesity and insulin resistance. Supplementation of butyrate to the high-fat diet led to an increase in insulin sensitivity and a reduction in obesity in C57BL/6 mice [67]. Administration of the probiotic VSL#3 to high fat diet-fed (HFD) mice was associated with increased butyrate production from the microbiota, suppressed body weight gain and insulin resistance, and reduced fed blood glucose levels [57].

Regulation of insulin levels seems to depend on FFAR2 expression. Indeed lower insulin levels have been reported in FFAR2 knockout mice compared with wild-type controls after a prolonged period on a high-fat diet [68]. Furthermore reduced insulin levels and impaired glucose tolerance have been observed in the FFAR2 knockout mice during an oral glucose tolerance text [49]. Interestingly the reduced insulin levels were not associated with differences in insulin tolerance indicating that the observed impaired glucose tolerance may reflect an impairment of insulin secretion, probably due to the reduced circulating GLP-1 concentrations. However, finding that FFAR2 is expressed in pancreatic beta-cells [69] suggested that FFAR2 may be involved in direct regulation of SCFAs on islet cell function. This hypothesis is supported by certain studies about effect of butyrate on pancreatic beta-cell differentiation and function. In 2006, Goicoa and coworkers reported that short exposure of embryonic stem cells to sodium butyrate activates early pancreatic development genes and increases the beta-cell differentiation [70]. Furthermore it has been reported that nestin-EGFP-positive progenitor cells (NPPCs) cultured in the presence of GLP-1 and sodium butyrate increased levels of transcripts encoding pancreatic developmental factors and insulin, leading to differentiation of NPPCs into insulin-producing cells [71]. These promising results may be related to the activity of sodium butyrate as histone deacetylases (HDAC) inhibitor; indeed it is well known that HDAC inhibitors promote beta-cell development, proliferation, differentiation, and function [72]. Furthermore, in a rat model of juvenile diabetic treatment with sodium butyrate improved glucose homeostasis as well as beta-cell proliferation and function and reduced beta-cell apoptosis [36].

6. SCFA and Inflammation Associated with Diabetes

TDM2 is characterized by low-grade inflammation with increased levels of cytokines, such as interleukin (IL)-6, IL-1, or tumor necrosis factor-alpha (TNF-α). These inflammatory molecules are upregulated in insulin-target tissues, including the liver, adipose tissue, and muscles [73], thus contributing to insulin resistance [74, 75].

It has been reported that alterations in the intestinal microbiota composition promote a proinflammatory state of the adipose tissue that is associated with obesity and subsequent insulin resistance [76]. The composition of the gut microbiota, and consequently of its metabolic products, is mainly affected by dietary changes [77–79]; an appropriate intake of dietary fibers is often associated with a SCFA profile that might increase anti-inflammatory response in the body, whereas a high-fat diet was shown to be associated with reduction of SCFAs and increase in lipopolysaccharide (LPS) levels [80], suggesting a colonization of Gram-negative bacteria. LPS was shown to induce the release of proinflammatory molecules [81], which contributed to the establishment of increased permeability and inflammation in the intestinal epithelium. This condition is known as "metabolic endotoxemia." On the contrary, incubation of a human colonic epithelial cell line with butyrate was shown to increase transepithelial resistance by promoting the assembly of tight junction [82, 83]. The compromised epithelial integrity allows movement of bacteria and/or dissemination of their products from the gut lumen to tissues, thus resulting in the increase of systemic inflammation. Therefore, LPS released by gut microbiota may pathophysiologically affect the function of other organs, further increasing insulin resistance. LPS receptors were also identified to mediate critical activities potentially underlying insulin resistance. For instance, activation of Toll-like receptor 4 (TLR4) in pancreatic islets was shown to increase proinflammatory cytokine production (both in activated macrophages and in beta-cells) that impaired function and decreased viability of beta-cells [84–86]. Indeed, TLR4 activation directly resulted in a decreased mRNA expression of pancreas-duodenum homebox-1 (PDX-1), insulin gene expression, reduced insulin content, and diminished insulin-induced glucose secretion. In addition, LPS upregulated the expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activated mitogen-activated protein kinase (MAPK)-mediated pathways in adipocytes [87]. The upregulation of TLR4 mRNA levels induced by HFD in the liver was shown to be counteracted by butyrate [35]. High-fat diet was associated with the upregulation of TNF-α and phosphorylation of NF-κB in the ileum [88], confirming that fat intake might increase mediators of intestinal permeability and inflammation. The dietary fibers can partly counteract these harmful effects, probably through production of SCFAs, particularly propionic and butyric acids, that could have anti-inflammatory effects in the body [33, 89]. The anti-inflammatory effects of SCFAs are probably due to a balance between suppression of proinflammatory mediators, such as TNF-α and IL-6, and induction of anti-inflammatory cytokines. For instance, administration of butyrate to HFD fed animals significantly reduced hepatic expression of TNF-α, IL-1β, and IL-6, therefore reducing liver steatosis and inflammation [35]. On the other hand, butyrate was also shown to abrogate secretion of the proinflammatory cytokines IL-12 and TNF-α and increase the release of the anti-inflammatory cytokine IL-10 by monocytes exposed to bacteria [90]. Finally, propionate and butyrate reduced expression of the proinflammatory cytokines TNF-α and IL-6 in human adipose tissue [89]. The ability of SCFAs to reduce low-grade inflammation is related to their capacity in modulating leukocyte and adipocyte function, thus reducing expression and production of inflammatory cytokines and

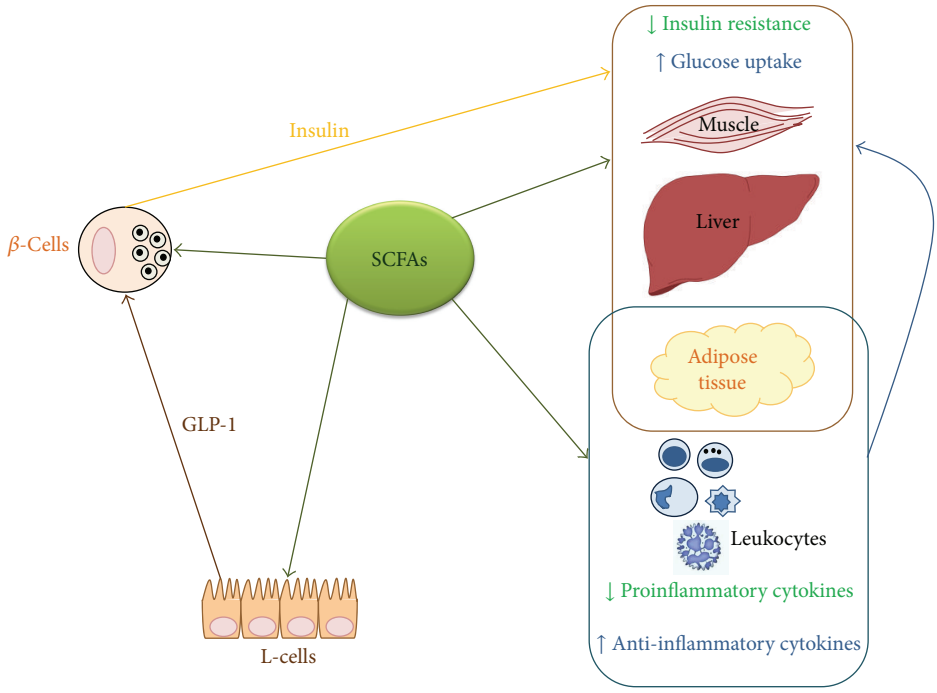

Figure 1: SCFAs improve metabolic functions in T2D. SCFAs were shown to affect pancreatic beta-cell function by directly acting as HDAC inhibitors (promoting β-cell development, proliferation, and differentiation) or by indirectly increasing GLP-1 secretion from enteroendocrine L-cells (leading to insulin release). Furthermore, SCFAs reduce the release of proinflammatory cytokines by adipose tissue and weaken leukocyte activation. These anti-inflammatory effects improve insulin resistance, tissue glucose uptake, and blood glucose levels.

chemokines [35, 48, 89, 91, 92]. Tedelind demonstrated that SCFAs decreased neutrophil release of TNF-α induced by LPS [93]. Accordingly, the exposure of adipocytes to propionic acid significantly downregulated several inflammatory cytokines and chemokines (such as TNF-α, resistin, and CCL5) [13, 94].

The molecular mechanisms through which SCFAs exert their anti-inflammatory effects may be related to the activation of their cognate G protein-coupled receptors FFAR2 and FFAR3, in addition to their function as HDAC inhibitors [95]. It has been reported that SCFAs might affect leukocyte subsets, including polymorphonuclear (PMN) cell activation [96–98]. FFAR2 seems to be a critical molecule regulating these effects. Firstly, FFAR2 mRNA is predominantly expressed in immune cells, particularly in PMN cells [37–39]. Moreover it has been reported that FFAR2 is induced during the differentiation of leukocyte progenitor cells to monocytes or neutrophils, suggesting that it could have an important function in the differentiation and/or activation of leukocytes [45]. Furthermore, Maslowski and colleagues demonstrated that inflammatory responses are regulated by interaction between SCFAs and FFAR2 [48], showing that immune cells from FFAR2-deficient mice have increased production of inflammatory mediators in comparison with wild-type mice. SCFA-mediated activation of FFAR2 was shown to trigger recruitment of circulating leukocytes to the inflammatory site via activation of intracellular signaling pathways including MAPK, Protein Kinase C (PKC), and Phospholipase C (PLC) [95]. On the other hand, by acting as HDAC inhibitors, SCFAs modulated the transcription of several target genes leading to downregulation of the expression of CXC chemokine receptor 2 (CXCR2) and inhibiting migration of neutrophils [48]. Finally, butyrate was shown to inhibit macrophage migration in response to LPS [99].

Another anti-inflammatory mechanism mediated by SCFAs was represented by adhesion molecule downregulation on endothelial cells, finally resulting in inhibition of leukocyte migration to inflammatory sites [78, 82]. By preventing inflammatory cell adhesion and chemotaxis, SCFAs were able to reduce immune cell infiltration to adipose tissue [100]. In addition, butyrate was shown to reduce TNF-α-induced VCAM-1 expression on umbilical vascular endothelial cells (HUVECs). Accordingly, preincubation with propionate and butyrate reduced both surface expression and mRNA levels of VCAM-1 in TNF-α- and IL-1β-stimulated HUVECs in a dose-dependent manner [100].

7. Conclusion

SCFAs have been associated with improvement of metabolic functions in T2D (including the control of blood glucose levels, insulin resistance, and GLP-1 secretion). These effects result from to the different tissues expressing SCFA receptors and, thus, are capable of responding to the beneficial effects induced by these molecules. Evidence reviewed in our paper indicates that regulation of blood glucose concentrations may involve several positive effects exerted by SCFAs occurring at different levels (Figure 1): (i) the decreased inflammatory

state that reduces insulin resistance, (ii) the contemporarily increased GLP-1 secretion that stimulates insulin release, and (iii) the improved beta-cell function that contribute to amelioration of glucose homeostasis.

Conflict of Interests

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

Authors' Contribution

Fabrizio Montecucco and Giorgio Luciano Viviani equally contributed as last authors to this work.

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Research Article

Serum Levels of LL-37 and Inflammatory Cytokines in Plaque and Guttate Psoriasis

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Psoriasis is a chronic inflammatory skin disease. It is assumed that the plaque phenotype of psoriasis is associated with T helper (Th) 1 immune response activation, while the guttate phenotype is associated with the Th17 immune response. Previous investigations of differences in the serum levels of cytokines relative to the clinical psoriatic phenotype have yielded conflicting results. This study compared the levels of circulating inflammatory cytokines and LL-37 relative to the morphological phenotype in patients with psoriasis. Seventy-four age-matched patients with psoriasis (32 with guttate psoriasis and 42 with plaque psoriasis) and 12 healthy controls were included. A multiplex cytokine assay and enzyme-linked immunosorbent assay were used to measure levels of Th1- and Th17-derived cytokines and LL-37, respectively. Circulating levels of interferon- (IFN)-γ, interleukin- (IL)-1RA, IL-2, and IL-23, and LL-37 were significantly higher in patients with psoriasis than in healthy controls. However, the serum levels of inflammatory cytokines (IL-7, IL-22, and IL-23) and LL-37 did not differ significantly between the guttate and plaque phenotypes of psoriasis. There was a positive correlation between serum inflammatory cytokine levels and the Psoriasis Area and Severity Index score. The findings of this study suggest that the serum levels of inflammatory cytokines reflect the disease activity rather than determine the morphological phenotype.

1. Introduction

Psoriasis is a chronic inflammatory skin disease with characteristic histological changes, including abnormal epidermal proliferation and a cellular infiltrate composed of neutrophils and T cells [1]. Psoriasis was originally classified as a T helper (Th) 1 disease because cytokines involved in the Th1 pathway, such as interferon- (IFN-) γ, interleukin- (IL-) 2, and IL-12, are elevated in lesional skin and peripheral blood [2–4], and the success of classical treatments is related to the result of redirecting a Th1 response towards a Th2 response [5]. Recent evidence suggests that a newly recognized subset of Th cells (Th17 cells, characterized by IL-17-producing CD4+ effector T cells) plays an important role in the pathogenesis of psoriasis [6]. Th17 cells differentiate from naïve CD4+ T cells when stimulated by IL-1β, IL-6, and IL-23 and can produce IL-17, IL-21, and IL-22 [7].

Recent studies also suggest that dendritic cells (DCs) and altered expression of antimicrobial peptides play a role in the pathogenesis of psoriasis. Plasmacytoid DCs are activated through a cathelicidin LL-37 and DNA complex in a toll-like-receptor-dependent manner [8]. This activation induces increased production of type I IFN, leading to myeloid DC activation and consequently leading to Th1/Th17 differentiation and keratinocyte activation [9]. This subsequently induces the expression of various cytokines [10, 11]. Both expression on lesional skin and systemic levels of LL-37 increase as a result and a correlation was found between LL-37 expression and proinflammatory cytokines in patients with psoriasis [12]. However, there has been a relative dearth of data regarding the circulating levels of LL-37 and the correlations with specific serum cytokines.

Psoriasis is classified into 5 morphological subtypes [13], although it is also thought that the phenotypes may transform into other clinical forms of the disease [14]. It has recently been suggested that IL-12/IFN-γ or IL-23/IL-17 signaling can influence the determination of clinical phenotypes.

Table 1: Demographics of healthy controls and patients with 1 of 2 types of psoriasis.

	Healthy controls	Psoriasis patient	Guttate psoriasis	Plaque psoriasis
Number	12	74	32	42
Age, years	32.6 ± 10.0	36.5 ± 14.2 (15~77)	37.7 ± 14.8	35.6 ± 13.9
Duration of psoriasis, years	N/A	6.2 ± 7.3	6.1 ± 7.5	6.2 ± 7.3
PASI score	N/A	7.9 ± 3.9	7.2 ± 3.4	9.1 ± 4.1

PASI: Psoriasis Area and Severity Index; N/A: not applicable.
Except where indicated otherwise, data are mean ± SD values.

For example, the plaque and guttate types of psoriasis are thought to be related to Th1 cytokines and Th17 immune responses, respectively. However, few studies have attempted to determine the correlations of biochemical markers with clinical phenotypes.

The present study compared differences in the serum levels of circulating Th1 and Th17 cytokines between plaque and guttate psoriasis and investigated the correlation between disease activity and serum levels of inflammatory cytokines. In addition, serum LL-37 levels in patients with psoriasis were compared with those in healthy controls, and the correlations between serum levels of LL-37 and inflammatory cytokines were analyzed.

2. Methods

2.1. Participants. Seventy-four patients with psoriasis (44 males and 30 females) and 12 healthy controls without psoriasis and without any family history of psoriasis were included in this study (Table 1). The controls were age- and sex-matched; accordingly, the age and sex distributions did not differ between the patient and control groups ($P > 0.05$). Psoriasis was diagnosed clinically and histopathologically and the following major inclusion criteria were implemented: no local or systemic treatment for at least 4 weeks prior to entering the study, no significant infection or immune suppression, and no history of specific medical diagnoses with renal, hepatic, cardiovascular, pulmonary, rheumatic, or endocrine involvement. Patients with erythrodermic, pustular, or palmoplantar-specific forms of psoriasis were excluded, as were those with psoriatic arthritis. The severity of the disease was evaluated by using the Psoriasis Area and Severity Index (PASI) score. The patients were divided into either the guttate or the plaque group according to their morphological psoriasis phenotype at the time of admission. Guttate psoriasis was defined as acute onset or reactivation of scattered, small plaque lesions of <1 cm in diameter, while patients with plaque psoriasis exhibited nummular and large plaques (≥1 lesion with a long-axis diameter of >5 cm). A total of 74 patients were assigned to either the guttate ($n = 32$) or plaque ($n = 42$) psoriasis group. The mean duration of disease and age did not differ between the 2 psoriasis groups ($P > 0.05$) as they had been matched for age and disease duration.

This study was conducted in accordance with the guidelines from the Helsinki Conference (52nd World Medical Association General Assembly, Edinburgh, United Kingdom, October 2000) and Korean Good Clinical Practice, with the participants' rights and safety taking precedence. Approval from the institutional review board was obtained. All participants were provided with detailed information about the study's purpose, methods, and expected results, after which the patients' informed consent to participate was obtained prior to screening.

2.2. Collection and Preparation of Blood Samples. Venous blood samples (5–10 mL) were collected into vacuum tubes under sterile conditions from both the patient and control groups. Serum was obtained by spinning the samples in a centrifuge and then immediately frozen at −70°C and stored until required for analysis.

2.3. Assays. Multiple cytokine analysis was performed by using xMAP technology (Luminex 200, Luminex, Austin, TX, USA) to measure serum IFN-γ, IL-1 receptor antagonist (IL-1RA), IL-2, IL-12(p40), IL-17A, IL-22, and IL-23. The Milliplex MAP multiplex assay was conducted in a 96-well microplate format according to the manufacturer's recommendations (Millipore, Billerica, MA, USA). Briefly, each of the bead solutions was transferred into a mixing vial and adjusted to a volume of 3 mL with bead diluents. Internal controls and standards, ranging from 0 to 10 000 pg/mL for each cytokine, were included with every assay. Following the addition of sera and beads, the resulting mixture was incubated overnight at 4°C. Detection antibodies and streptavidin-phycoerythrin were then added sequentially at room temperature for 30 minutes, and the plate was analyzed with the Luminex 200 system.

Serum cathelicidin LL-37 levels were measured by performing an enzyme-linked immunosorbent assay with commercially available kits (Hycult Biotech, Plymouth Meeting, PA, USA). The minimum detectable concentration of serum LL-37 when using this assay was 0.15 ng/mL.

2.4. Statistical Analysis. All data were analyzed by using the Statistical Package for the Social Sciences (SPSS) 17.0 software for Windows (SPSS, Chicago, IL, USA). The Mann-Whitney U test was used to compare mean values between groups and correlation analysis was performed by using the Pearson correlation test. Differences were considered to be statistically significant when $P < 0.05$.

3. Results

3.1. Comparison of Serum Levels of Cytokines and LL-37. Circulating levels of cytokines and LL-37 were analyzed in both the patient and control groups (Table 2). Serum levels of IL-1RA, IL-2, IL-23, IFN-γ, and LL-37 were elevated in

Table 2: Statistical analysis results of serum levels of cytokines and LL-37 in patients with psoriasis and healthy controls.

	Patients ($n = 74$) Median (IQR), mean	Healthy controls ($n = 12$) Median (IQR), mean	P^a
IL-1RA (pg/mL)	2.26 (0.00–7.18), 4.40	0.00 (0.00–1.62), 1.47	0.020*
IL-2 (pg/mL)	0.81 (0.59–1.25), 1.05	0.00 (0.00–0.92), 0.84	0.007*
IL-12 (pg/mL)	2.71 (0.00–4.80), 3.23	0.00 (0.00–1.36), 1.33	0.071
IL-17A (pg/mL)	0.31 (0.00–1.37), 1.59	0.00 (0.00–2.75), 1.52	0.936
IL-22 (pg/mL)	0.11 (0.09–0.18), 0.15	0.10 (0.10–0.13), 0.11	0.625
IL-23 (pg/mL)	1.57 (1.44–2.00), 1.99	0.95 (0.45–1.24), 1.02	<0.001*
IFN-γ (pg/mL)	2.94 (2.46–5.18), 4.53	0.50 (0.00–2.06), 1.34	<0.001*
LL-37 (ng/mL)	11.78 (9.75–16.90), 13.46	8.73 (7.73–11.60), 9.43	0.011*

IL: interleukin; IL-1RA: IL-1 receptor antagonist; IFN: interferon; IQR: interquartile range.
[a]Mann-Whitney U test between healthy controls and patients with psoriasis.
*denotes statistically significant difference at $P < 0.05$.

Table 3: Statistical analysis results of levels of specific serum cytokines and LL-37 in patients with guttate and plaque psoriasis.

	Guttate psoriasis ($n = 32$) median (IQR), mean	Plaque psoriasis ($n = 42$) median (IQR), mean	P^a
IL-1RA (pg/mL)	2.75 (1.12–7.18), 4.84	1.20 (0.00–5.21), 4.06	0.229
IL-2 (pg/mL)	0.81 (0.70–1.31), 0.11	0.84 (0.59–1.14), 1.00	0.558
IL-12 (pg/mL)	0.00 (0.00–4.80), 2.75	2.71 (0.00–5.91), 3.61	0.320
IL-17A (pg/mL)	0.00 (0.00–1.23), 1.31	0.68 (0.00–1.54), 1.80	0.580
IL-22 (pg/mL)	0.10 (0.09–0.14), 0.14	0.11 (0.09–0.20), 0.15	0.443
IL-23 (pg/mL)	1.57 (1.44–1.86), 2.01	1.58 (1.44–2.03), 1.97	0.756
IFN-γ (pg/mL)	2.90 (2.62–5.00), 4.64	3.02 (2.46–5.18), 4.45	0.768
LL-37 (ng/mL)	10.95 (8.96–16.94), 13.20	12.56 (10.60–16.90), 13.65	0.204

[a] Mann-Whitney U test between patients with guttate psoriasis and plaque psoriasis.

Table 4: Correlations between disease severity (PASI score) and serum levels of cytokines and LL-37 in all patients with psoriasis.

	Statistical parameters	IL-1RA	IL-2	IL-12	IL-17A	IL-22	IL-23	IFN-γ	LL-37
PASI	r	0.21	0.16	0.30	0.21	0.26	0.10	0.23	0.08
	P	0.07	0.16	0.01*	0.07	0.02*	0.42	0.048*	0.50

r: Pearson correlation coefficient.
*denotes statistically significant difference at $P < 0.05$.

patients with psoriasis compared with the controls. Although the serum IL-12(p40), IL-17A, and IL-22 levels also appeared to be elevated compared with the healthy control group, the difference was not statistically significant in the experimental setting of our study. Furthermore, the serum levels of all the tested cytokines and LL-37 did not differ significantly between the guttate and plaque psoriasis groups (Table 3).

3.2. Correlations between Serum Levels of Cytokines and LL-37 and PASI. The serum levels of 7 inflammatory cytokines and LL-37 in the 74 patients with psoriasis were compared with disease severity (as defined by PASI score) in order to establish the presence of any correlations. IL-12(p40), IL-22, and IFN-γ were significantly and positively correlated with PASI in all patients with psoriasis (Table 4, Figure 1). Although not statistically significant, there was a tendency for both IL-1RA and IL-17A to be positively correlated with PASI (Figure 1).

3.3. Correlations between Serum Levels of Individual Cytokines and LL-37. The correlations between the serum levels of LL-37 and individual cytokines are presented in Table 5. Serum LL-37 levels were significantly correlated with serum IL-22 and IFN-γ levels in patients with psoriasis. Although the correlations between serum IL-17A, IL-23, IL-1RA, and LL-37 levels were statistically nonsignificant, there appeared to be a trend towards an overall positive correlation ($P = 0.09$, $P = 0.15$, and $P = 0.18$, resp.). These findings suggest the presence of interplay between LL-37 in innate immunity and the cytokines involved in the Th1 and Th17 immune responses in psoriasis.

4. Discussion

Psoriasis is a chronic inflammatory skin disease involving the induction of Th1 and Th17 cell responses and the aberrant expression of proinflammatory cytokines [15]. The findings

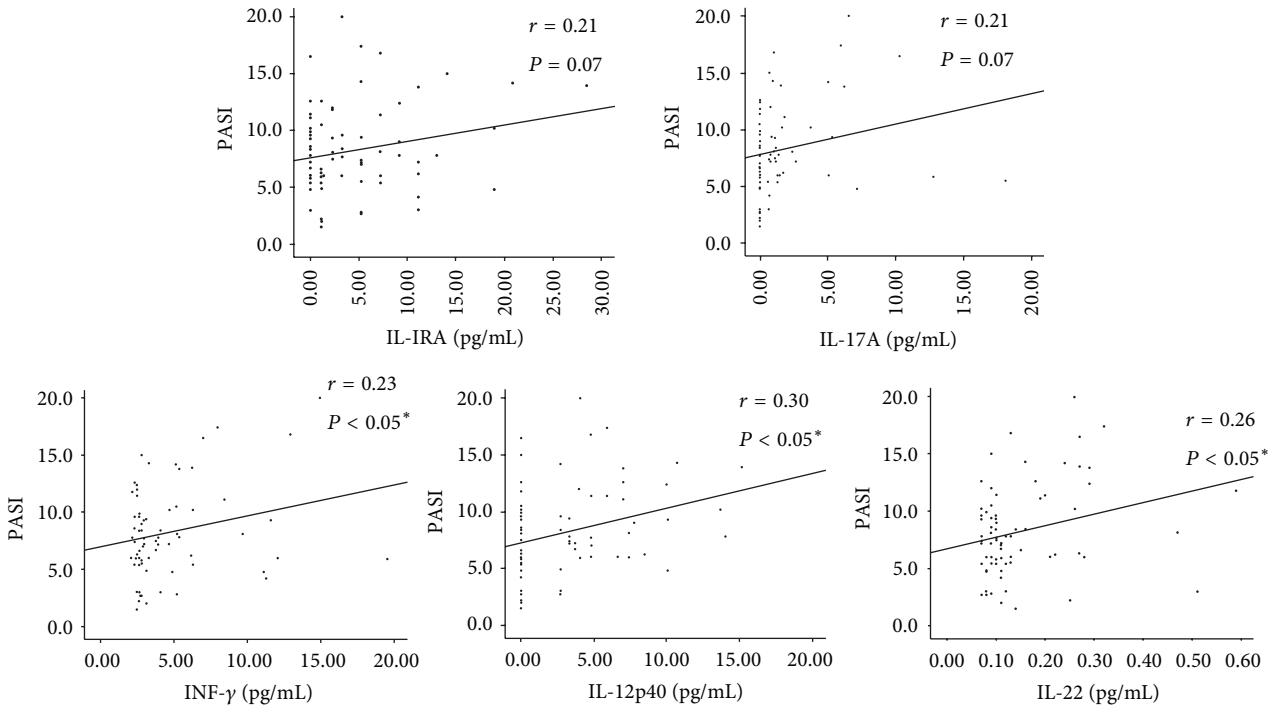

Figure 1: Correlations between serum levels of interleukin- (IL-) 1 receptor antagonist (RA), IL-12 (p40), IL-17A, IL-22, and interferon (IFN)-γ and Psoriasis Area and Severity Index (PASI) scores in patients with psoriasis. The Pearson correlation test and linear regression were used to calculate the linear regression lines.

Table 5: Correlations between serum levels of LL-37 and cytokines in patients with psoriasis.

LL-37	r	P
IL-1RA	0.16	0.18
IL-2	0.04	0.75
IL-12	0.03	0.81
IL-17A	0.20	0.09
IL-22	0.24	<0.05*
IL-23	0.17	0.15
IFN-γ	0.31	<0.01*

r: Pearson correlation coefficient.
*denotes statistically significant difference at $P < 0.05$.

of the present study confirm previous reports [16, 17] that the serum levels of most of the Th1- and Th17-related cytokines are elevated in the serum of patients with psoriasis.

The patients included in the present study were divided into the following 2 morphological phenotypes of psoriasis: guttate and plaque. Comparing the levels of serum inflammatory cytokines between these psoriasis groups revealed no statistically significant differences. These results suggest that morphological phenotype is not determined by specific activities of either the Th1 or Th17 pathway. A few studies have compared the serum cytokine levels among psoriasis subgroups, but their results were conflicting and did not definitively determine whether there is a significant relationship between the specific cytokines and phenotypes [18–20]. The present study is the first to compare the serum levels of inflammatory cytokines in different morphological phenotypes of psoriasis with similar disease duration and may provide a partial explanation for the conflicting results regarding cytokine levels previously reported. Thus, it seems that both Th1 and Th17 pathways are associated with the pathogenesis of psoriasis.

This study found significant correlations between the serum levels of IFN-γ, IL-12(p40), and IL-22 and patients' PASI scores. Combined with results of previous studies [3, 21, 22], the correlations between PASI score and the serum levels of some cytokines suggest that serum cytokine levels could be used as an objective parameter to reflect the disease activity of psoriasis.

The cathelicidin LL-37 is overexpressed in skin lesions [10, 23] and has recently been identified as a critical factor for the activation of an autoinflammatory cascade in psoriasis [8]. In the present study, the serum LL-37 levels were elevated in patients with psoriasis compared with healthy controls but the levels were not correlated with the PASI score. This finding is consistent with previous reports that serum LL-37 is not related to psoriasis severity [12, 24]. One possible explanation for the lack of an association between PASI and serum LL-37 is that, unlike the LL-37 found in lesional skin, the serum type represents the sum total produced by LL-37-producing cells in the intestine, airways, lymph nodes, and bone marrow. Thus, the serum LL-37 level could be affected by multiple organs' immune activities as well as many other factors such as vitamin D levels or ultraviolet and microbial exposure in the environment. In addition, LL-37 can play both a regulatory and a provocative role in immune responses

[11, 12, 25]. It may also be a modulator acting to balance the levels of pro- and anti-inflammatory cytokines [26], and hence it may not be positively correlated with disease activity.

As with the cytokines, the serum level of LL-37 did not differ significantly between the guttate and plaque morphologic types, which suggests that serum LL-37 is not involved in the establishment of a particular psoriasis phenotype.

This study found that serum LL-37 levels were significantly correlated with serum IFN-γ and IL-22 levels. The circulating LL-37 appeared to affect both INF-γ and IL-22, both of which are key cytokines in the Th1 and Th17 pathways involved in psoriasis. Sophisticated interactions between the cathelicidin and these 2 cytokines might exist [26–29], but no clear mechanism has yet been verified. Further investigation is necessary to determine the clinical significance and possible mechanism of this putative interaction.

In summary, the circulating levels of inflammatory cytokines and LL-37 do not appear to differ significantly between the guttate and plaque types of psoriasis. Serum levels of inflammatory cytokines appear to reflect disease activity rather than the morphological phenotype. Serum LL-37 is elevated in psoriasis but is not associated with either the morphological phenotype or the disease activity of psoriasis. Further studies are still needed to elucidate the relationship between serum LL-37 and cytokines in the pathogenesis of psoriasis and its clinical implications.

Conflict of Interests

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

Authors' Contribution

Young Ji Hwang and Ho Jung Jung had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design were done by Young Ji Hwang and Yong Beom Choe. Acquisition, analysis, and interpretation of data were done by Jae Wook Jung, Young Ji Hwang, Min Jung Kim, Nam Kyung Roh, and Yong Beom Choe. Drafting of the paper was done by Young Ji Hwang, Ho Jung Jung, and Yong Beom Choe. Critical revision of the paper for important intellectual content was done by Yang Won Lee, Yong Beom Choe, and Kyu Joong Ahn. Statistical analysis was done by Young Ji Hwang and Jae Wook Jung.

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Review Article

Ionotropic and Metabotropic Proton-Sensing Receptors Involved in Airway Inflammation in Allergic Asthma

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An acidic microenvironment has been shown to evoke a variety of airway responses, including cough, bronchoconstriction, airway hyperresponsiveness (AHR), infiltration of inflammatory cells in the lung, and stimulation of mucus hyperproduction. Except for the participation of transient receptor potential vanilloid-1 (TRPV1) and acid-sensing ion channels (ASICs) in severe acidic pH (of less than 6.0)-induced cough and bronchoconstriction through sensory neurons, the molecular mechanisms underlying extracellular acidic pH-induced actions in the airways have not been fully understood. Recent studies have revealed that ovarian cancer G protein-coupled receptor 1 (OGR1)-family G protein-coupled receptors, which sense pH of more than 6.0, are expressed in structural cells, such as airway smooth muscle cells and epithelial cells, and in inflammatory and immune cells, such as eosinophils and dendritic cells. They function in a variety of airway responses related to the pathophysiology of inflammatory diseases, including allergic asthma. In the present review, we discuss the roles of ionotropic TRPV1 and ASICs and metabotropic OGR1-family G protein-coupled receptors in the airway inflammation and AHR in asthma and respiratory diseases.

1. Introduction

Airway acidification has been shown to be attained by either the exogenous way, that is, the microaspiration of acid contents into the airway during gastroesophageal reflux and inhalation of low pH pollutant aerosol, or the endogenous way, that is, ischemia and inflammation of the airways in inflammatory diseases, such as asthma, cystic fibrosis, and chronic obstructive pulmonary disease (COPD) [1–3]. In ischemic and inflammatory situations, the stimulation of anaerobic glycolysis causes lactate production. In patients with asthma, it has been reported that airway pH reaches 5.2 to 7.1, depending on the severity: pH is normalized with corticosteroid therapy [1]. Although alteration of airway pH may serve an innate host defense capacity, that is, inhibiting the survival of bacteria in an acidic environment, it is also implicated in the pathophysiology of obstructive airway diseases. Thus, exposure to acids evokes a cough, bronchoconstriction, airway hyperreactivity (AHR), and microvascular leakage and stimulates mucus production [2]. However, molecular mechanisms underlying the extracellular acidic pH-induced actions in the airways have not been fully understood. In the present review, we discuss the proton-sensing mechanisms, focusing on proton-sensing ionotropic receptors, such as transient receptor potential vanilloid-1 (TRPV1) and acid-sensing ion channels (ASICs), and metabotropic ovarian cancer G protein-coupled receptor 1 (OGR1)-family G protein-coupled receptors (GPCRs), in the airway inflammation and AHR in asthma and respiratory diseases.

2. General Information Regarding Proton-Sensing Channels and OGR1-Family GPCRs

The mammalian transient receptor potential (TRP) superfamily of nonselective cation channels encompasses 28 isotypes and is divided into six subfamilies, that is, TRPC, TRPV, TRPM, TRPA, TRPP, and TRPML. These channels are expressed in neurons and a wide range of cell types in many biological systems [4–6]. TRP channels have six putative transmembrane domains and a pore-forming loop between the fifth and sixth segments. They are thought to be composed of homo- or heterotetramers [5]. Among them,

capsaicin-sensitive TRPV1 is activated by a diverse range of chemical and noxious stimuli, including protons [5–7]. TRPV1 senses relatively strong acidic pH of 4 to 5 through glutamic acid in the extracellular domain of the channel [7] (Figure 1). TRP channels other than TRPV1, including TRPA1, TRPV4, and TRPM8, are expressed in the respiratory system and involved in the regulation of airway functions [8–10]; however, whether protons practically trigger their channel activation remains unknown.

Another important family of proton-sensing channel is ASIC. ASICs are proposed to assemble as tetramers with homomeric or heteromeric subunits; each subunit consists of two transmembrane domains [11] (Figure 1). Six ASIC subunit proteins, encoded by four genes, have been identified: ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4. ASICs are voltage-independent channels that mainly conduct Na^+ [12]. Recent studies have demonstrated that ASICs activated by acidic pH play an important role in a wide range of physiological and pathophysiological processes such as nociception, mechanosensation, synaptic plasticity, and acidosis-mediated neuronal injury [11]. Histidine, glutamic acid, and aspartic acid may determine a broad range of optical pH of 4 to 7 for activation, depending on the subtypes [4, 13, 14]. Some forms of ASIC mRNAs have been detected in pulmonary sensory neurons [6].

In addition to TRPV1 and ASICs, there is increasing evidence that further acid-sensitive ion channels are involved in monitoring acidosis. These include TRP family ion channels other than TRPV1, including TRPV4, TRPC4, TRPC5, and TRPP2 (PKD2L1); two-pore domain K^+ (K_{2p}) channels; ionotropic purinoceptors (P2X); inward rectifier K^+ channels; voltage-activated K^+ channels; L-type Ca^{2+} channels; hyperpolarization-activated cyclic nucleotide gated channels; gap junction channels; and Cl^- channels [4]. Although most of these channels seem to be expressed and play important roles in the respiratory system, their extracellular proton-sensitivity has not, to our knowledge, been demonstrated for specific airway responses [10, 15–17].

Recent studies suggest that OGR1-family GPCRs, including OGR1 (GPR68), GPR4, and T cell-death associated gene 8 (TDAG8 or GPR65), also sense extracellular protons and, thereby, stimulates a variety of cellular activities through several types of G proteins [18–21] (Figure 1). This receptor family is expressed not only in neurons but also in nonneuronal cells. OGR1-family GPCRs were previously described as GPCRs for lysolipids, such as sphingosylphosphorylcholine [19–21]. Ludwig et al. [18], however, first discovered that OGR1 and GPR4 sense extracellular protons and are coupled to G_q and G_s, leading to activation of the phospholipase C/Ca^{2+} signaling pathway and the adenylyl cyclase/cAMP signaling pathway, respectively [18]. Later it was found that TDAG8 similarly senses extracellular protons, leading to the activation of the cAMP signaling pathway [22, 23]. OGR1-family GPCRs sense weak acidic to weak alkaline pH of 6 to 8 through histidine residues [18, 19, 24, 25]. G2A is also classified in this receptor family and is expressed by a broad range of immunoregulatory cell types, including macrophages, dendritic cells, neutrophils, mast cells, and T and B cells, and it is suggested to play an important role in innate and adaptive immunity [26]. However, the proton sensitivity of the receptor is very small and its role as a proton sensor has been questioned [27]. In the present review, therefore, we do not focus further on G2A. OGR1-family GPCRs are expressed in many cell types localized in the airways, and the cases in which their roles are demonstrated are summarized in Figure 2.

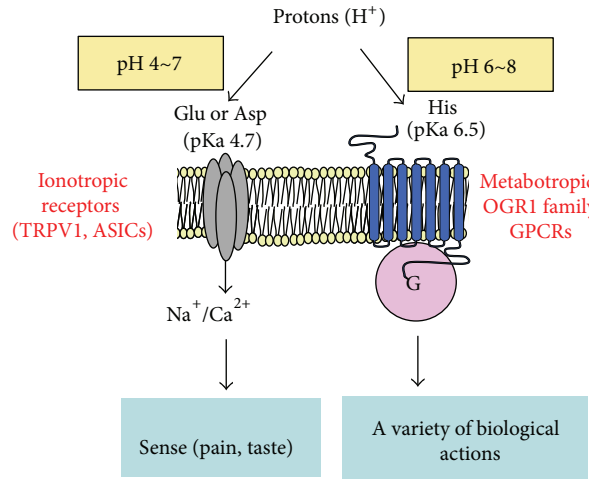

Figure 1: Ionotropic and metabotropic proton-sensing receptors. Extracellular acidification evokes a variety of airway responses. Ionotropic TRPV1 channel and ASICs mainly mediate severe acidic pH-induced cough, pain, and bronchoconstriction through sensory neurons, while OGR1-family GPCRs sense mild alkaline and mild acidic pH and exert a wide range of cellular actions in many types of structural and inflammatory cells.

3. Role of Proton-Sensing Channels and GPCRs in the Airways

3.1. Bronchoconstriction and AHR

3.1.1. Sensory Neurons. The proton-sensing TRPV1 channel and/or ASICs in sensory nerves have first been proposed to be involved in acidic pH-induced airway responses [2, 28, 29]. It has been established that TRPV1 on capsaicin-sensitive primary sensory neurons plays an important role in nociception and transmission of pain as a sensor of noxious stimuli [2, 4, 30]. The activation of TRPV1 on the sensory neurons by irritant compounds, including capsaicin and citric acid, generates reflex responses that, in turn, stimulate the release of tachykinins from the terminals of the sensory nerves, causing cough and bronchoconstriction [2, 5, 29]. The possibility that the afferent signals and neuropeptide release are coupled at the same nerve endings has also been proposed [31]. The neurogenic role of TRPV1 was based mainly upon experiments with selective agonists and antagonists. For example, capsazepine, a TRPV1-selective antagonist, and SR48968, a selective NK2 receptor antagonist, blocked citric acid inhalation-induced bronchoconstriction in guinea pig airways *in vivo* [32]. The role of TRPV1 in

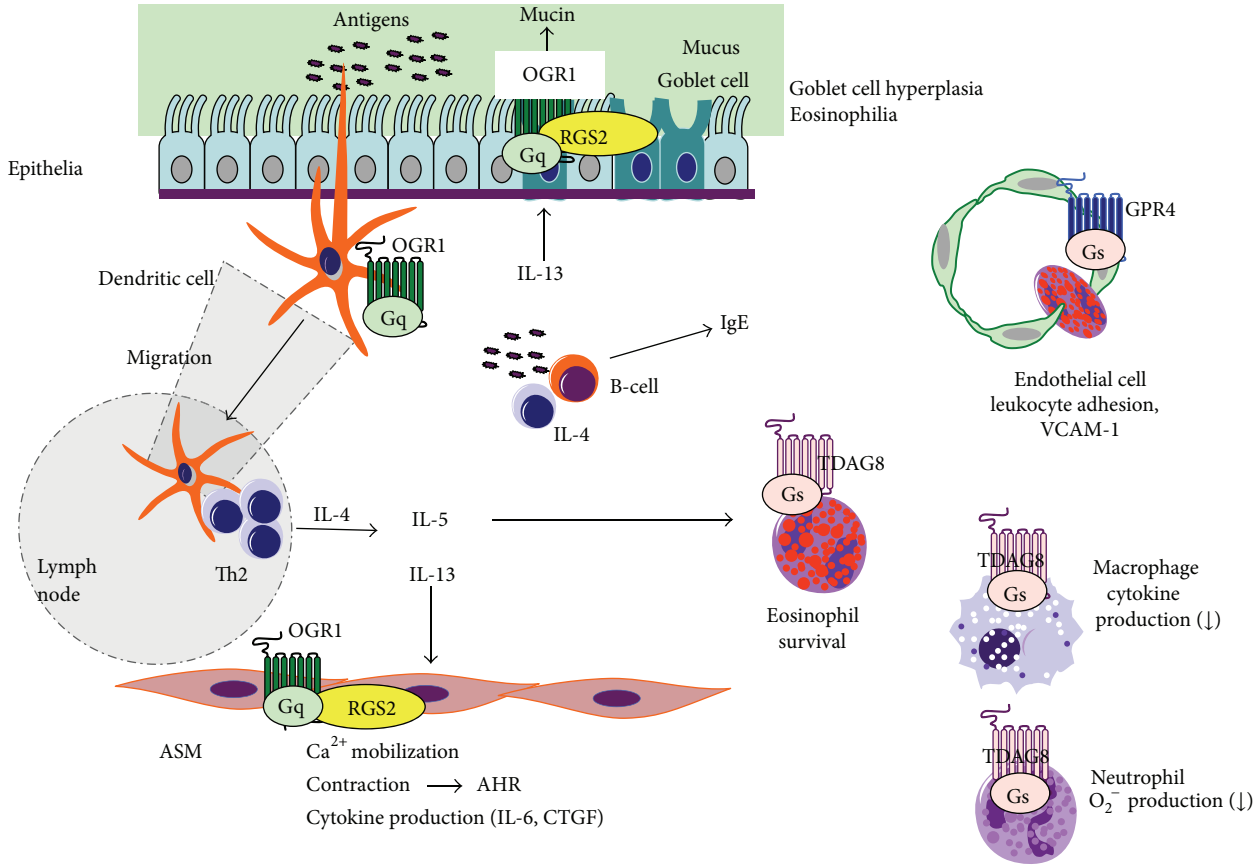

Figure 2: Role of OGR1-family GPCRs in Th2 polarization and subsequent airway inflammation and AHR. See the text in detail.

AHR has recently been demonstrated by the finding that oral administration of TRPV1-specific antagonists significantly attenuated AHR in the asthma model of OVA-sensitized guinea pigs [33]. In addition to TRPV1, involvement of ASICs in acidic pH-induced AHR was suggested by using selective ASIC inhibitors in the guinea pig tracheal rings *ex vivo* [28]. These results suggest that bronchoconstriction and AHR under acidic environments are indirectly mediated by sensory neurons through proton-sensing channels, such as TRPV1 and ASICs [6, 30]. In fact, no report has shown that TRPV1 and ASICs are expressed and are directly functioning in ASM cells, although vascular smooth muscle cells seem to express TRPV1 [8] and ASICs [34].

3.1.2. Airway Smooth Muscle (ASM) Cells. The previous studies, however, did not rule out the direct actions of acidic pH on ASM cells. Ichimonji et al. [35] showed that extracellular acidification stimulates mRNA expression and protein production of IL-6, a proinflammatory cytokine, in association with the phosphorylation of extracellular signal-regulated kinase (ERK) and p38MAPK, in human ASM cells. They also showed that extracellular acidification induced an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [35], which was accompanied by ASM cell contraction [36]. Acidification also induced expression of connective tissue growth factor (CTGF), a critical factor involved in the formation of extracellular matrix proteins and, hence, airway remodeling. TGF-β-induced CTGF expression was also enhanced by acidic pH [37]. In ASM cells, OGR1 is expressed at by far the highest levels among proton-sensing GPCRs [35]. The knockdown of OGR1 and G_q with their specific small interfering RNAs and an inhibition of G_q with YM-254890 attenuated the acidification-induced actions [35, 37]. These results suggest that extracellular acidification stimulates Ca^{2+} mobilization, inflammatory cytokine IL-6 and CTGF production, and contraction through OGR1/G_q in human ASM cells (Figure 2).

The role of OGR1 in AHR is supported by recent findings with RGS2- [38] and RGS5-deficient mice [39]. RGS2 and RGS5 are GTPase-activating proteins that inactivate G_q stimulated by G_q-coupled receptors, including OGR1. Xie et al. [38] showed that RGS2 deficiency caused spontaneous AHR in response to methacholine without any prior antigen sensitization/challenge. They also showed that the loss of RGS2 augmented Ca^{2+} mobilization and the contraction of ASM cells, increased ASM mass, and stimulated ASM cell growth via ERK and phosphatidylinositol 3-kinase (PI3K) pathways. These results are highly consistent with those of *in vitro* studies with human ASM cells [35, 36]. Interestingly, asthma patients display lower expression of RGS2 in epithelial cells and ASM cells in lung and circulating monocytes [38]. RGS5-deficient mice also showed spontaneous AHR, even without antigen sensitization in association with enhanced Ca^{2+} mobilization, ERK activation, and ASM cell contraction

[39]. In both cases, however, appreciable infiltration of inflammatory cells in the lung and production of cytokines, such as IL-4 and IL-5, was not observed [38, 39]. These results suggest that T-cell activation is not always required for GPCR-mediated asthma pathophysiology.

3.2. Mucus Hypersecretion in Epithelial Cells. Mucus hypersecretion is a common pathological feature of inflammatory airway diseases, including asthma. Liu et al. [40] have recently shown that an acidic pH of 6.4 stimulates mucin5AC (MUC5AC) secretion in the human bronchial epithelial cell line (16HBE) through the OGR1/G_q/phospholipase C pathway (Figure 2). Knockdown of OGR1 and G_q expression with small interfering RNAs inhibited acidification-induced increases in $[Ca^{2+}]_i$ and mucin production. Similarly, overexpression of RGS2 protein attenuated acidic pH-induced cellular responses, whereas knockdown of RGS2 slightly but significantly enhanced these responses. These results suggest that airway acidification induces mucin production through the OGR1 and G_q, of which activation can be regulated by RGS2. The same group has also shown that TRPV1 is expressed and citric acid (pH 5.0)-induced mucin secretion is mediated by Ca^{2+} influx via TRPV1 in the same cells [41]. These results suggest that epithelial cells utilize different proton-sensing machineries depending on the acidity of microenvironments; OGR1-family GPCRs sense mild alkaline to acidic pH of 8 to 6, whereas TRPV1 senses more acidic pH of 6 to 4, as described above.

3.3. Leukocyte Adhesion to and Leakage through Endothelial Cells. GPR4 has been shown to be expressed in endothelial cells and to be activated by extracellular acidification, leading to accumulation of cAMP, which may be relevant to normal blood vessel formation [42]. Chen et al. have recently shown that activation of GPR4 by acidosis increases endothelial cell adhesion of monocytes in association with the expression of adhesion molecules, such as VCAM-1 and ICAM-1, through the cAMP/Epac pathway [43]. Since VCAM-1 on the endothelium has been shown to mediate eosinophil adhesion and transmigration [44], this suggests that GPR4 is involved in the perivascular accumulation of eosinophils in the lung with inflammatory asthma (Figure 2). However, it remains unknown whether GPR4 deficiency affects eosinophilia in the airways with asthma.

3.4. Airway Eosinophilia due to Enhanced Survival of Eosinophils. Kottyan et al. [45] have reported that acidic pH increased eosinophil viability due to an inhibition of apoptosis, which was associated with an increase in cAMP accumulation. The acidic pH-induced increase in cell viability was attenuated by the adenylyl cyclase inhibitor and, in contrast, the phosphodiesterase inhibitor, the cell-permeable cAMP analog, and forskolin mimicked the acidic pH effect. The acidification-induced increase in eosinophil viability and cAMP accumulation was completely lost in the cells isolated from the TDAG8 knockout mice. These results suggest that eosinophil viability is increased in acidic microenvironments through the TDAG8/cAMP pathway (Figure 2). However, the mechanism by which cAMP inhibits eosinophil apoptosis remains unknown [45].

3.5. Cytokine and O_2^- Production in Macrophages and Neutrophils. In macrophages, TDAG8 and OGR1 are expressed. Extracellular acidification inhibited lipopolysaccharide (LPS)-induced TNF-α and IL-6 protein and mRNA production in mouse peritoneal macrophages [46]. The inhibitory action on cytokine production by acidic pH was significantly attenuated in macrophages from TDAG8-deficient mice but not in those from OGR1-deficient mice. Further characterization revealed that acidic pH inhibited proinflammatory cytokine production through the TDAG8/G_s/cAMP/PKA signaling pathway in mouse macrophages. The expression of TDAG8 was increased by glucocorticoid in the macrophage, which was associated with the enhancement of acidification-induced inhibition of TNF-α production [47]. Thus, TDAG8 seems to be indirectly involved in glucocorticoid-induced anti-inflammatory actions.

Extracellular acidification has been shown to induce human neutrophil activation, inducing an increase in $[Ca^{2+}]_i$, a shape-changing response, upregulation of the expression of CD18, an inhibition of apoptosis, and enhancement of agonist-induced H_2O_2 production [48]. These acidic pH-induced responses are accompanied by the activation of Akt and ERK. On the other hand, acidic pH has also been shown to inhibit some neutrophil functions. Extracellular acidic pH inhibits migration [49] and O_2^- production in human neutrophils [50]. Although intracellular acidification was supposed to, in part, explain the acidic pH-induced actions [48, 50], the precise proton-sensing mechanisms remained largely unknown. Murata et al. [51] have shown that acidic pH inhibited fMLP- and C5a-induced superoxide anion production. The acidic pH effect was mimicked by cAMP increasing agents and attenuated by a PKA inhibitor. Moreover, acidic pH increased cAMP accumulation. TDAG8 is coupled to cAMP signaling pathways and is abundantly expressed in neutrophils. These data suggest that TDAG8 may mediate extracellular acidification-induced inhibition of O_2^- production through cAMP [51].

Thus, TDAG8 is coupled to anti-inflammatory cAMP signaling pathways in macrophages and neutrophils. Consistently with an anti-inflammatory role, TDAG8-deficient mice showed exacerbation of anti-type II collagen antibody-induced arthritis, in which macrophages and neutrophils have been shown to play a critical role [52]. However, it remains uncharacterized whether the anti-inflammatory actions of TDAG8 in macrophages and neutrophils are involved in the pathophysiology of airway inflammation of asthma.

3.6. T-Cell Priming and Polarization by Dendritic Cells. Geffner and his colleagues have shown that extracellular acidification (pH 6.5) stimulates internalization of antigens; upregulated the expression of cell surface proteins, including CD11c, MHC class II, CD40, and CD86, involved in antigen presentation; and promoted an efficient MHC class

I-restricted presentation of antigen peptides in dendritic cells (DCs) of C57BL/6 mice [53]. Antigen-pulsed DCs under acidic pH showed an improved efficacy for inducing both specific $CD8^+$ cytotoxic T lymphocytes and specific antibody responses *in vivo* [53]. They further characterized acidic pH effects using human DCs and found that the transient exposure of human DCs to pH of 6.5 markedly increases several costimulatory proteins and improves the T-cell priming ability of DCs, which was associated with a dramatic increase in p38MAK-dependent IL-12 production. DC maturation by acidic pH stimulated the production of IFN-γ, but not of IL-4, by antigen-specific $CD4^+$ T cells. These results suggest that extracellular acidification may contribute to the initiation of adaptive immune responses by DCs, favoring the development of the Th1 phenotype in humans [54].

Basu and Srivastava suggested that TRPV1 is expressed in mouse DCs and involved in their maturation [55]. However, the role of TRPV1 is controversial. TRPV1 expression was not confirmed and either capsaicin or acidic pH failed to elicit a change in $[Ca^{2+}]_i$ or the membrane current in mouse DCs [56]. Recent studies have shown that TRPV1 is expressed in human DCs; however, in this case, the channel seems to inactivate rather than activate their maturation [57]. The role of ASICs has also been suggested [58]. ASIC1, ASIC2, and ASIC3 are expressed in mouse DCs, and selective inhibitors for ASICs, such as amiloride and nonsteroidal anti-inflammatory drugs (NSAIDs), inhibited acidic pH 6.5-induced expression of cell-surface molecules CD11c, MHC class II, CD80, and CD86.

We have recently examined the role of proton-sensing GPCRs in DC functions [59]. Mouse DCs express OGR1-family receptors, including OGR1; the functional expression of OGR1 was confirmed by the extracellular acidic pH- and OGR1-dependent increase in $[Ca^{2+}]_i$. OVA-sensitized DCs from OGR1-deficient mice showed the reduction in the expression of CCR7, a chemokine receptor for mature DCs, and the migration responses to CCL19 and CCL21, ligands for CCR7, as compared with those from wild-type mice [59]. Thus, OGR1 seems to be functioning in the migratory process of DCs to draining lymph nodes (see the next section).

4. Role of Proton-Sensing Channels and OGR1-Family GPCRs in Asthma Models

Although the contribution of TRPV1 to acidic pH-induced cough and bronchoconstriction is well demonstrated as discussed, it remains to be proven whether TRPV1-mediated neurogenic inflammation plays a central role in asthma and other respiratory diseases. The inarticulate conclusion is based on the lack of obvious effects by gene targeting experiments; TRPV1 deficiency did not attenuate or rather enhanced the airway inflammation and AHR as induced by LPS [60], antigens [61, 62], and cigarette smoke [63] in TRPV1 knockout mice. The anti-inflammatory role of TRPV1 might be, in part, explained by the release of anti-inflammatory somatostatin from the sensory nerve terminals in response to TRPV1 stimulation [60]. Regardless, the role of TRPV1 in the airway inflammation and AHR remains to be established in mice. However, uncertain results with mice may be partly explained by the strain difference: the previous studies [60–63] used C57BL/6. Rehman et al. [64] have recently shown that TRPV1 knockdown with siRNA attenuates IL-13- and antigen-induced asthmatic features including airway inflammation and AHR in Balb/c mice.

In addition to proton-sensing channels, proton-sensing GPCRs also play an important role in the pathophysiology of asthma. As described above, Kottyan et al. have shown that TDAG8 deficiency causes the stimulation of eosinophil apoptosis and, thereby, reduces airway eosinophilia in OVA- and *Aspergillus fumigatus*-sensitization models of mice *in vivo* [45]. They have also shown increased expression of TDAG8 in lungs from OVA-sensitized mice and in nasal brushing samples from pediatric asthma patients. Unfortunately, whether AHR and airway inflammation as cardinal features of asthma are modulated by TDAG8 *in vivo* has not been examined; however, it is noted that IL-13 production, which plays a role in these processes, was not affected by TDAG8 deficiency [45].

The findings of the expression of proton-sensing OGR1 in DC, a critical cell for antigen recognition and its presentation to T cells, and the involvement of OGR1 in the DC migration process [59] suggest participation of OGR1 in the pathophysiology of allergic asthma. Indeed, OGR1-deficient mice are resistant to the cardinal features of asthma, including airway eosinophilia, AHR, and goblet cell metaplasia, in association with a remarkable inhibition of the production of Th2 cytokines, including IL-4, IL-5, and IL-13, and OVA-specific IgE in an OVA-induced asthma model [59]. Intratracheal transfer to wild-type mice of OVA-primed bone marrow-derived DCs from OGR1-deficient mice developed lower AHR and eosinophilia as compared with the transfer of those from wild-type mice, which was associated with lower migratory activity to the peribronchial lymph nodes in OGR1-deficient DCs than in wild-type DCs. These results suggest that stimulation of OGR1 on DCs is critical for the early processes, that is, migration to lymph nodes and initiation of Th2 polarization, and, thereby, induces eosinophilia, airway inflammation, and AHR [59]. Since OGR1 is expressed in structural cells, including ASM cells and epithelial cells, and functions in a variety of cell-specific responses, the reduction of AHR and goblet hyperplasia in OGR1-deficient mice may be partly attributed to the reduction of acidification-induced constriction of ASM cells [35, 36] and mucin production in epithelial cells [40].

Finally, it should be noted that acidic pH modulates DCs leading to Th1 polarization in humans as described [51], which contrasts with the role of OGR1 in Th2 polarization in mice. The reason for the difference in the fate of T cells, that is, Th1 or Th2, is currently unknown. DCs express proton-sensing GPCRs other than OGR1, and OGR1-family GPCRs are expressed in neutrophils and macrophages as well. Therefore, differences in experimental conditions, for example, species, stimulants, antigens, and pH, may modify the state of DC activation and DC-T cell interaction, making naïve T cells polarize to Th1, Th2, Th17, or other phenotypes.

How change in the pH microenvironment modulates DC function and T cell polarization warrants further study.

5. Conclusions

Proton-sensing channels, such as TRPV1 and ASICs, and OGR1-family GPCRs are expressed in structural cells, including ASM cells and epithelial cells, and inflammatory and immune cells, including eosinophils and DCs, and play a variety of roles in airway responses, depending on the optimum pH of proton-sensing channels and GPCRs. Cough and bronchoconstriction are activated by severe acidic pH of 4 to 5 and are mainly mediated by pH-sensing channels through sensory neurons, although pH-sensing OGR1 on ASM cells may also be involved in the cell constriction. At mild alkaline or mild acidic pH of more than 6, however, OGR1-family GPCRs may be the main receptors involved in the regulation of airway responses under pathophysiological situations, such as allergic asthma. Thus, ionotropic and metabotropic proton-sensing receptors may be therapeutic targets for inflammatory and ischemic diseases, such as asthma, for which drugs that are more specific and have fewer side effects are still required.

List of Abbreviations

AHR: Airway hyperresponsiveness
ASIC: Acid-sensing ion channel
ASM: Airway smooth muscle
$[Ca^{2+}]_i$: Intracellular Ca^{2+} concentration
DC: Dendritic cell
ERK: Extracellular signal-regulated kinase
GPCR: G protein-coupled receptor
G_q: G_q protein
G_s: G_s protein
LPS: Lipopolysaccharide
OGR1: Ovarian cancer G protein-coupled receptor 1
PI3K: Phosphatidylinositol 3-kinase
RGS: Regulator of G protein signaling
TDAG8: T cell-death associated gene 8
TNF-α: Tumor necrosis factor-α
TRPV1: Transient receptor potential vanilloid-1
PKA: Protein kinase A.

Conflict of Interests

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

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Research Article

Red Ginseng Extract Ameliorates Autoimmune Arthritis via Regulation of STAT3 Pathway, Th17/Treg Balance, and Osteoclastogenesis in Mice and Human

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Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic joint inflammation. Red ginseng is a steamed and dried *Panax ginseng* C.A. Meyer, which has been used as alternative medicine for thousands of years. This study was undertaken to investigate the effects of red ginseng extracts (RGE) on autoimmune arthritis in mice and humans and to delineate the underlying mechanism. RGE was orally administered three times a week to mice with arthritis. Oral administration of RGE markedly ameliorated clinical arthritis score and histologically assessed joint inflammation in mice with CIA. A significant reduction in STAT3 phosphorylation and a decrease in the number of Th17 cells were observed with RGE treatment. There was also a marked reduction in RANKL-induced osteoclastogenesis with treatment of RGE. The inhibitory effect of RGE on Th17 differentiation and osteoclastogenesis observed in mice was also confirmed in the subsequent experiments performed using human peripheral blood mononuclear cells. Our findings provide the first evidence that RGE can regulate Th17 and reciprocally promote Treg cells by inhibiting the phosphorylation of STAT3. Therefore, RGE can ameliorate arthritis in mice with CIA by targeting pathogenic Th17 and osteoclast differentiation, suggesting a novel therapy for treatment of RA.

1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic joint inflammation that can lead to joint destruction and disability. Although the exact molecular mechanism of the RA pathogenesis remains elusive, proinflammatory cytokines including tumor necrosis factor- (TNF-) α and interleukin- (IL-) 1β and autoreactive T cells are known to play central roles in the development and progression of RA [1]. Although RA was conventionally conceived as Th1-driven inflammatory disease, it is becoming clear that Th17, rather than Th1, is a major pathogenic cell that orchestrates the complex network of the sustained inflammation and disease progression [2–4]. Th17 produces IL-17, which is abundantly expressed in the arthritic joints and neutralization of IL-17 results in attenuation of arthritis [4]. IL-17 not only induces proinflammatory cytokines, but also directly enhances osteoclastogenesis by upregulating receptor activator nuclear kappa ligand (RANKL) on osteoblasts [5], contributing to bone erosion in RA. Recently, Th17 was reported to express RANKL itself as well as directly inducing mature osteoclasts via cell-to-cell contact, substantiating its role in inflammatory bone destruction [6].

The development of Th17 is largely dependent on cytokine milieu. Transforming growth factor- (TGF-) β and IL-6

primes initial differentiation and IL-23 promotes functional maturation of Th17 cells [7]. As signal transducer and activator of transcription (STAT) 3 phosphorylation directly regulates retinoic acid receptor-related orphan receptor (ROR) γt, which is the master molecule of Th17, the Janus kinase (JAK)2-STAT3 pathway initiated by IL-6 is essential in the Th17 development. With recent advances in understanding the central role of Th17 in the pathogenesis of RA, novel therapeutics targeting Th17 are being utilized or are still under development. The blockade of IL-6 (Tocilizumab) is currently available on the market, while the JAK2 inhibitor and monoclonal antibody against IL-17 are still under clinical trials among RA patients.

Ginseng, the root of *Panax ginseng* C.A Meyer (Araliaceae) is a perennial herb which has been used as herbal medicine in Eastern Asia for thousands of years [8, 9]. Ginseng has two types of preparation, one is air-dried white ginseng and the other is steamed and sun-dried red ginseng. It contains various active components including ginsenosides, polysaccharides, peptides, polyacetylenic alcohols, and fatty acids [10]. Ginsenosides, in particular, are also called saponin extracts and are known to exert the majority of the pharmacologic effects seen from ginseng. Red ginseng is made from a ginseng plant going through an intensive process of cleaning, steaming, and drying. Heat treatment of ginseng leads to chemical changes of ginsenosides giving them distinct physical characteristics. Major ginsenosides of red ginseng saponin extract were shown to include less polar red ginseng-unique saponins Rg3, Rk1, and Rg5 in a high-performance liquid chromatographic analysis [11]. As supplementation with red ginseng is believed to improve health, numerous studies have been conducted to validate its beneficial effects. They demonstrated that RGEs had anti-inflammatory [12, 13], antidiabetic [14, 15], and anticancer [16] effects. Anti-inflammatory property of RGE leads to the understanding that it may have antiarthritic effect via modulating inflammation. Indeed, accumulating evidence has suggested that ginseng extracts can ameliorate autoimmune arthritis. It was reported that treatment with ginsenoside Rb-1 significantly attenuated arthritis in mice with established CIA [17]. Orally administered RGEs including Rg3, Rk1, and Rg5 as major components also successfully suppressed CIA shown by decreased production of proinflammatory cytokines, matrix metalloproteinase (MMP), and nitrotyrosine in the arthritic joints [11]. More recently, compound K, an active ginsenoside metabolite with a high intestinal absorption rate, was shown to decrease TNF-α-induced MMP production in RA-fibroblast-like-synoviocyte (FLS) and suppress osteoclastogenesis by inhibiting the expression of nuclear factor of activated T cells (NFATc1) [18]. However, it has not been suggested whether or not RGE has a regulatory effect on Th17, which is a central pathogenic cell in RA. Given that ginseng was reported to repress STAT3 activation in cancer cells [19], it is plausible to assume that RGE can suppress Th17 by inhibiting STAT3 phosphorylation, enhancing enhanced Treg cells by reciprocal regulation [20].

In the present study, we verified that oral administration of RGE could suppress arthritis in CIA model. To delineate the mechanism underlying the antiarthritic effect in terms of Th17, the effect of RGE on pathogenic Th17 cell differentiation both *in vivo* and *in vitro* was investigated. In addition, the effect of RGE osteoclast formation, which is implicated in bone erosion in RA was examined.

2. Methods and Materials

2.1. Animals. Six week old male DBA/1J mice were purchased from SLC, Inc., (Shizouka, Japan) and IL-10 knockout (KO) mice in the DBA/1J background were kindly provided by Linda K. Myers (University of Tennessee). Mice were maintained under specific pathogen-free conditions at the institute of Medical Science at the Catholic University of Korea and were provided standard mouse chow (Ralston Purina, St. Louis, MO, USA) and water *ad libitum*. All experimental procedures were examined and approved by the Animal Research Ethics Committee of the Catholic University of Korea (permit number: CUMC:20), which conforms to all National Institutes of Health of the USA guidelines.

2.2. Preparation of Red Ginseng Extract. Red ginseng extract (RGE) was kindly provided by the Korea Ginseng Cooperation Daejeon Cooperation (Daejeon, Republic of Korea). RGE yields 4.37% saponins; the main components of ginsenosides were Rb1 (12.6%), Rb2 (6.2%), Rc (6.9%), Rd (3.4%), Re (6.4%), Rf (2.1%), Rg1 (15.8%), and Rg3 (1.4%). The identified constituents are well standardized and qualified by the Korea Ginseng Cooperation. Other constituents in RGE are starch, sugars, fat, fiber, proteins, vitamins, minerals, and so forth.

2.3. Induction of CIA and Treatment with Red Ginseng. To induce CIA in mice, 100 μL of an emulsion containing 100 μg bovine type II collagen (CII) and complete Freund's adjuvant (Chondrex, Redmond, WA, USA) was injected intradermally into the base of the tail as the primary immunization. Two weeks later, 100 μg CII, dissolved and emulsified 1 : 1 with incomplete Freund's adjuvant (Difco, Detroit, MI, USA) was administered into the footpad as a booster injection. To assess the influence of RGE on symptom severity in the CIA model, mice were orally treated with 10 mg/kg RGE in saline or with vehicle alone three times a week after booster immunization over the course of 6 weeks. The arthritis index in these mice was scored twice weekly and expressed as the sum of the scores of four limbs.

2.4. Measurement of CII-Specific IgG and IgG. Serum levels of CII-specific IgG (Total IgG, IgG1, IgG2a) and IgG (Total IgG, IgG1, IgG2) measurement antibodies were measured using a commercially available ELISA kit (Beathyl Laboratories, Montgomery, TX, USA).

2.5. Immunohistochemistry. Mouse joint tissue was fixed in 4% paraformaldehyde, decalcification EDTA bone decalcifier and embedded in paraffin. The section (7 μm) was stained with hematoxylin and eosin, Safranin O, and toluidine blue to detect proteoglycans.

Immunohistochemistry was performed using the Vectastatin ABC kit (Vector Laboratories, Burlingame, CA, USA). Tissue was first incubated with primary antibodies to IL-17, IL-6, IL-1b, TNF-α, Nitrotyrosine, NRF2 (Santa

Cruz Biotechnology, Santa Cruz, CA, USA), and HO-1, Inos (Abcam, Cambridge Science Park, Cambridge, UK) overnight at 4°C. The sections were counterstained with hematoxylin. Samples were photographed with an Olympus photomicroscope (Tokyo, Japan).

2.6. Mouse In Vitro Osteoclastogenesis. Isolation of bone-marrow-derived monocyte/macrophage (BMM) cells and differentiation of osteoclast precursor cells (preosteoclasts) was performed as described [21]. Three days later, the nonadherent cells were washed out and preosteoclasts were cultured further in the presence of 10 ng/mL M-CSF, 100 ng/mL RANKL (Peprotech, London, UK), and various concentrations of RGE for four days to generate osteoclasts. On day 2, the medium was replaced with fresh medium containing M-CSF, RANKL, and RGE.

2.7. Human in CD4 T Cell Isolation and Differentiation. CD4 T cells were isolated from peripheral blood mononuclear cells (PBMCs) using a CD4 T cell isolation kit (Miltenyi Biotec) according to the manufacturer's instruction. To establish Th17 cell-polarizing conditions, the $CD4^+$ T cells were stimulated with plate-bound anti-CD3, anti-CD28, anti-IFN-r, anti-IL-4, IL-1β (20 ng/mL), and IL-6 (20 ng/mL) for 3 days. All cytokines were from R&D Systems, with the exception of TGF-β.

2.8. Intracellular Staining and Flow Cytometry. The following antibodies were used for mouse cells; Th17 cells: PerCP-Cy5.5-conjugated anti-CD4 (eBioscience) and FITC conjugated anti-IL-17A (eBioscience) was used for intracellular staining. The following antibodies were used for human cells: Th17 and Treg cells were from PE-Cy7-conjugated anti-CD4, APC- conjugated anti-CD25 (both from BD Pharmingen), and FITC-conjugated anti-Foxp3, PE-conjugated anti-IL-17 (both from eBioscience).

2.9. Enzyme-Linked Immunosorbent Assay (ELISA). The amount of IL-17, IL-21, IL-22, and IL-10 were measured using a sandwich ELISA (R&D Systems). Absorbance at 405 nm was measured using an ELISA microplate reader (Molecular Devices).

2.10. Human In Vitro Osteoclastogenesis. The generation of human preosteoclasts was performed as described [21]. After three days, these preosteoclasts were cultured further in the presence of 25 ng/mL M-CSF, 30 ng/mL RANKL, and various concentrations of RGE for nine days to generate osteoclasts. On day 3, the medium was replaced with fresh medium containing M-CSF, RANKL, and RGE. TRAP stain was performed as described [21]. All the subjects gave informed consent before the study. The study received the approval of the institutional review board of Seoul St. Mary's Hospital from all healthy volunteers.

2.11. TRAP Staining. A commercial TRAP kit (Sigma, St Louis, ML, USA) was used according to the manufacturer's instructions; however, counterstaining with hematoxylin was omitted. TRAP-positive multinuclear cells (MNCs) containing three or more nuclei were counted as osteoclasts.

2.12. Gene Expression Analysis Using Real-Time PCR. PCR amplification and analysis were performed on a Light Cycler 2.0 instrument (Roche Diagnostic, Mannheim, Germany) with software version 4.0. All reactions were performed using LightCycler FastStart DNA master SYBR green I (Takara, Shiga, Japan), according to the manufacturer's instruction. The following primers for mouse samples were used: IL-17, 5′-CCT CAA AGC TCA GCG TGT CC-3′(sense) and 5′-GAG CTC ACT TTT GCG CCA AG-3′(anti-sense); RORrt, 5′-TGT CCT GGG CTA CCC TAC TG-3′(sense) and 5′-GTC CAG GAG TAG GCC ACA TT-3′(antisense); CCR6, 5′-CCA TGA CTG ACG TCT ACC TGT TGA ACA-3′(sense) and 5′-GAA CAG CTC CAG TCC CAT ACC CAG CAG-3′(antisense); Foxp3, 5′-GGC CCTT CTC CAG GAC AGA-3′(sense) and 5′-GCT GAT CAT GGC TGG GTT GT-3′(antisense); SOCS3, 5′-CCT TTG ACA AGC GGA CTC TC-3′(sense) and 5′-GCC AGC ATA AAA ACC CTT CA-3′(antisense); RANK, 5′-TGT ACT TTC GAG CGC AGA TG-3′(sense) and 5′-CCA CAA TGT GTT GCA GTT CC-3′(antisense); MMP9, 5′-CTG TCC AGA CCA AGG GTA CAG CCT-3′(sense) and 5′-GAG GTA TAG TGG GAC ACA TAG TGG-3′(antisense); cathepsin K, 5′-CAG CAG AGG TGT GTA CTA TG-3′(sense) and 5′-GCG TTG TTC TTA CGA GC-3′(antisense); TRAP, 5′-TCC TGG CTC AAA AAG CAG TT-3′(sense) and 5′-ACA TAG CCC ACA CCG TTC TC-3′(antisense). The following primers for human samples were used: RANK, 5′-GCT CTA ACA AAT GTG AAC CAG GA-3′(sense) and 5′-GCC TTG CCT GTA TCA CAA ACT-3′(antisense); MMP9, 5′-CGC AGA CAT CGT CAT CCA GT-3′(sense) and 5′-GGA TTG GCC TTG GAA GAT GA-3′(antisense); cathepsin K, 5′-TGA GGC TTC TCT TGG TGT CCA TAC-3′(sense) and 5′-AAA GGG TGT CAT TAC TGC GGG-3′(antisense); CTR, 5′-TGG TGC CAA CCA CTA TCC CTG A-3′(sense) and 5′-CAC AAG TGC CGC CAT GAC AG-3′(antisense). The level of mRNA expression was normalized to that of β-actin.

2.13. Immunoblot Analysis. PBMC were cultured with anti-CD3 and anti-CD28 in the presence or absence of RDE for 72 h. Mice splenocytes were cultured with the Th17 condition in the presence or absence of RDE for 72 h. Both cells were then harvested and lysed with lysis buffer. Protein concentration was measured using the Bradford method (Bio-Rad, Herculed, CA, USA). Protein samples were separated using 12% SDS-PAGE and transferred onto nitrocellulose membranes (AmersharmPharmacia Biotech, Piscataway, NJ, USA). For Western blot hybridization, the membrane was preincubated with blocking buffer for 2 h and then incubated with primary antibodies against Total IkBa, p-IkBa, Total ERK, p-ERK, Total STAT5, pSTAT5, Total STAT3, pSTAT3(727), pSTAT3(705) (all from Cell Signaling, Danvers, ma), and β-actin for 1 h. After washing, horseradish peroxidase-conjugated secondary antibodies were added, and the membranes were incubated for 1 h at room temperature. After washing, the hybridized bands were detected using an ECL detection kit (Pierce, Rockford, IL, USA) and Hyperfilm (Agfa, Belgium).

2.14. Statistical Analysis. Experimental values are presented as mean ± SD of triplicate cultures and representative of experiments performed on three occasions. Statistical significance was determined by Mann-Whitney U test or ANOVA with Bonferroni's post-hoc test using the Graphpad Prism (v.5.01). Values of $P < 0.05$ were considered statistically significant, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

3. Results

3.1. Red Ginseng Extract Suppresses Collagen-Induced Arthritis. Results showed that administration of oral RGE three times a week (10 mg/kg) reduced the arthritic score and arthritis incidence almost completely compared to the oral administration of the vehicle (Figure 1(a)). When CIA was induced among the IL-10 KO mice, there were higher rates of clinical signs and more severe knee and paw injury when compared to the wild type mice. As shown in Figure 1(b), RGE successfully suppressed the arthritic score and arthritis incidence in the CIA mice of the IL-10 knockout background. Histological examination of the joints demonstrated that the paws and ankles of the red ginseng extract-treated mice had a lower degree of inflammation and cartilage damage compared with those of the vehicle-treated mice, as determined on day 49 after immunization (Figure 1(c)). In addition, the red ginseng extract-treated mice expressed markedly lower levels of not only proinflammatory cytokines such as TNF-α, IL-1b, IL-6, and IL-17, but also oxidative stress markers such as nitrotyrosine and iNOS as demonstrated by immunohistochemical analysis (Figure 1(d)). The results implicated that antioxidant activity of RGE might contribute to attenuating oxidative stresses in CIA mice. We next examined whether treatment with red ginseng extract would modulate humoral immune responses by assessing Ab production in CIA mice. Figure 1(e) illustrates that treatment with RGE efficiently attenuated the production of total IgG and IgG2a, the Th1-type Ab, in the sera of CIA mice. The effect of red ginseng extract on the Ag-specific humoral immune responses *in vivo* was also assessed. The serum levels of the CII-specific IgG2a and total IgG were significantly lower in the mice treated with red ginseng extract (Figure 1(e)). Interestingly, the CII-specific IgG1a, the Th2-type Ab, was considerably increased among the red ginseng extract-treated CIA mice (Figure 1(e)).

3.2. Red Ginseng Extract Reciprocally Modulates Populations of Regulatory T Cells and Th17 Cells in CIA Mice. mRNA from splenocytes of either red ginseng extract-treated CIA mice or vehicle-treated CIA mice was isolated and the expression of Th17 cell- and Treg cell-related markers by RT-PCR was then analyzed. The results showed that the mRNA levels of Th17 cell-related molecules such as IL-17, RORC, and CCR6 were downregulated whereas the mRNA levels of Treg cell-related molecules such as Foxp3 and SOCS3 were upregulated in the red ginseng-treated CIA mice (Figure 2(a)). Furthermore, the red ginseng treatment reduced the number of IL-17-producing $CD4^+$ T cells in the splenocytes of CIA mice as analyzed by flow cytometry (Figure 2(b)). Additionally, we measured the numbers of $CD4^+CD25^+Foxp3^+$ regulatory T cells and $CD4^+IL\text{-}17^+$ T cells (Th17 cells) in tissues of spleens and drain lymph nodes by immunofluorescence confocal microscopy. The spleen tissues from the mice treated with red ginseng extract showed an increased number of $Foxp3^+$ regulatory T cells and a decreased number of Th17 cells compared with those of the vehicle-treated mice (Figure 2(c)). The number of Th17 cells was also significantly decreased in the tissues of the drain lymph nodes (data not shown), although the number of Treg cells remained unchanged (Figure 2(c)).

3.3. Red Ginseng Extract Reduces STAT3 Phosphorylation in the $CD4^+$ T Cells in Mice. The number of pSTAT3-expressing $CD4^+$ T cells was indeed decreased in the red ginseng-treated CIA mice compared with those of the vehicle-treated CIA mice (Figure 3(a)). To evaluate whether RGE promotes phosphorylation of STAT3 *in vitro*, we performed immunoblot analysis of protein extracts prepared from $CD4^+$ T cells isolated from spleens cultured in a Th17-polarizing condition with various concentrations of RGE. RGE dose-dependently decreased the amount of phosphorylated STAT3 at tyrosine 705 under the Th17-generating condition while the total amount of STAT3 remained the same (Figure 3(b)). Phosphorylation of IkB and ERK was also reduced with RGE treatment (Figure 3(b)). After stimulation under conditions favoring the development of Th17 cells, we found that phosphorylation of STAT3 at 705 and 727 was significantly decreased by the addition of red ginseng extract, although phosphorylation of STAT3 at 727 was reduced in a less degree (Figure 3(b)).

3.4. Red Ginseng Extract Inhibits Osteoclastogenesis in CIA Mice. The number of TRAP positive cells was markedly reduced in the joint tissues of RGE-treated mice compared with those of vehicle-treated mice (Figure 4(a)). We next investigated whether RGE would directly inhibit osteoclast formation *in vitro*. The BMM cells were prepared from WT mice and stimulated with M-CSF and/or RANKL to induce osteoclast differentiation. The addition of various concentrations of RGE during the induction of osteoclastogenesis significantly inhibited osteoclast formation in a dose-dependent manner (Figure 4(b)). Transcripts of various osteoclastogenic markers such as RANK, MMP9, cathepsin K, and TRAP were also considerably decreased by the addition of RGE (Figure 4(c)).

3.5. Red Ginseng Extract Increased Foxp3-Expressing Regulatory T Cells and Decreased IL-17-Expressing Th17 Cells in Human PBMCs. Concentrations of RGE used in these *in vitro* experiments did not affect cell viability as demonstrated by the MTT assay (Figure 5(a)). Transcripts of Treg-related molecules such as Foxp3, Socs3, and IL-10 significantly increased whereas mRNAs of IL-17 and IL-6 were markedly reduced by red ginseng extract in $CD4^+$ T cells of human PBMCs (Figure 5(c)). Additionally, Th17-associated cytokines like IL-26 and IL-21 were also decreased although IL-22 did not change significantly (Figure 5(c)) at the mRNA level. Subsequent flow cytometry analysis confirmed that the

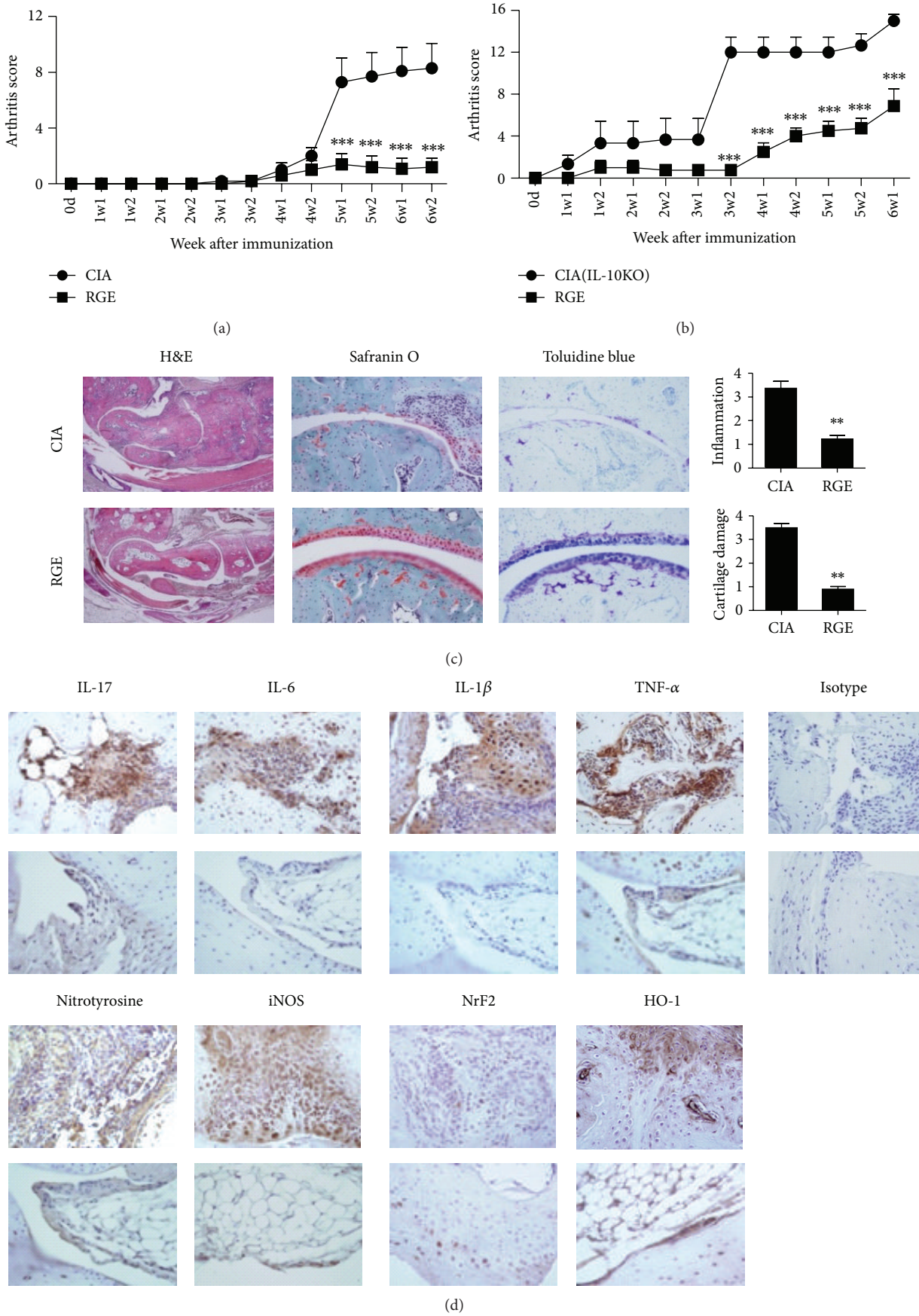
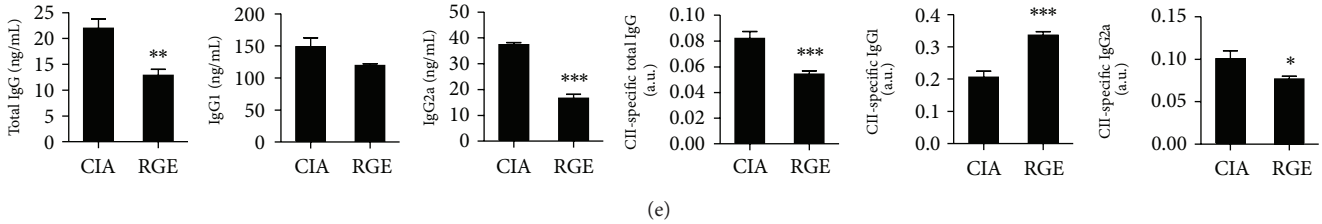


Figure 1: Continued.



(e)

Figure 1: Red ginseng extract suppresses collagen-induced arthritis. Collagen-induced arthritis (CIA) was induced. Mice were orally treated with 10 mg/mL of red ginseng extract (RGE) or vehicle only. (a) Clinical scores and incidence of arthritis in CIA-induced DBA/1J mice. (b) Clinical scores and incidence of arthritis in IL-10 KO mice. (c) Representative histological features of the joint of RGE or vehicle-treated mice with CIA. H&E, safranin O, and toluidine blue staining are shown. (d) Representative immunohistochemical staining of the joint of RGE or vehicle-treated mice with CIA (e) Concentrations of CII-specific IgG and IgG2a in sera of RGE or vehicle-treated mice with CIA were determined by ELISA. Data are presented as the mean ± SD of three independent experiments $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to control mice.

populations of Treg and Th17 cells were indeed reciprocally regulated, in which Treg cells were increased and Th17 cells were decreased by RGE in a dose-dependent manner (Figure 5(b)).

$CD4^+$ T cells either when activated with anti-CD3/28 antibodies alone (data not shown) or when cultured in a Th17-polarizing condition produced considerably lower levels of IL-17, IL-21, and IL-22 after the treatment with RGE. Interestingly, Treg-associated cytokines, IL-10 was significantly increased by the addition of RGE (Figure 5(d)). Phosphorylation of STAT3 at tyrosine 705 and 727 was markedly reduced with the treatment of 1000 μg/mL red-ginseng extract (Figure 5(e)). However, the amount of pSTAT5 was not affected by RGE treatment.

3.6. Red Ginseng Extract Inhibits Osteoclastogenesis in Humans. Figure 6(a) displays that RGE treatment effectively prevented human monocytes from differentiating into mature osteoclasts, which was determined by TRAP staining. It also reduced the expressions of osteoclastogenic markers such as the calcitonin receptor (CTR), cathepsin K, and MMP-9 (Figure 6(b)).

4. Discussion

In this study, oral administration of RGE ameliorated the clinical arthritis score and the histological severity of joint inflammation in mice with CIA. RGE inhibited differentiation of Th17, which is a main pathogenic T cell in RA, by suppressing phosphorylation of STAT3 and reciprocally increased Treg population. Furthermore, treatment with RGE substantially suppressed osteoclastogenesis, which might contribute to less bone erosion in CIA mice treated with RGE.

A significant reduction in proinflammatory cytokines including IL-17, IL-6, TNF-α, and IL-1β was observed in the joints of RGE-treated CIA mice. As *in vitro* studies have demonstrated that IL-17 is a powerful inducer of IL-6, TNF-α, and IL-1β [3], a decrease in Th17 which in turn reduced IL-17 in the arthritic joints seemed to cause the decrease in production of those cytokines. Moreover, decreased production of IL-6 and IL-1b might have contribution to suppressed Th17-IL-17 activation.

As oxidative stress is known to play an important role in RA pathogenesis [22–24], oxidative stress represented by iNOS and nitrotyrosine expression was also measured, which diminished with RGE treatment in CIA. Nrf2 is a transcription factor, activating upon the exposure to ROS. Expression of Nrf2 and HO-1 suppressed regulation of oxidative stress [25]. Activated Nrf2 binds to antioxidant response element (ARE) located in the regulatory regions of the genes coding for antioxidative enzymes such as HO and enhances transcription of the antioxidative enzymes [26]. Thus, decreased expression of Nrf2 and HO in the joints of RGE-treated mice indicates that the oxidative stress was lower than the vehicle-treated mice. Our data corroborate the previously reported antioxidative effect of RGE [27, 28]. In addition to inhibitory effect on Th17, the antioxidative property of RGE seems to exert an additional effect in suppressing CIA.

The main focus of this study was to investigate whether the antiarthritic effect of RGE was mediated by the regulation of pathogenic Th17 cells. As expected, the number of Th17 cells was significantly reduced among the RGE-treated mice with CIA, indicating that RGE suppressed Th17 differentiation in arthritic condition. As mentioned, Development of Th17 cell was caused by activation of STAT3 signal pathway [7]. Therefore, we investigated whether RGE could suppress STAT3 phosphorylation, and discovered that phosphorylation of STAT3 was decreased in RGE-treated mice as demonstrated by immunofluorescence confocal microscopy and in RGE-treated $CD4^+$ T cells cultured in Th17 polarizing conditions measured by immunoblotting. These data suggest that RGE might block the signal transduction pathway initiated by binding of IL-6 to its receptor, which activates downstream kinases and subsequently allows the phosphorylation of STAT3. This was consistent with previous report that American ginseng, albeit different genus, dramatically suppressed JAK2-STAT3 activation in aortic smooth muscle cell in rats [29]. Diminished expression of IL-6 also seems to have contributed to the Th17 regulation. To elucidate whether RGE directly regulates IL-6 expression requires future research.

The effect of RGE on Treg as well as Th17 in pathologic inflammatory condition in mice with CIA or in $CD4^+$ T cells

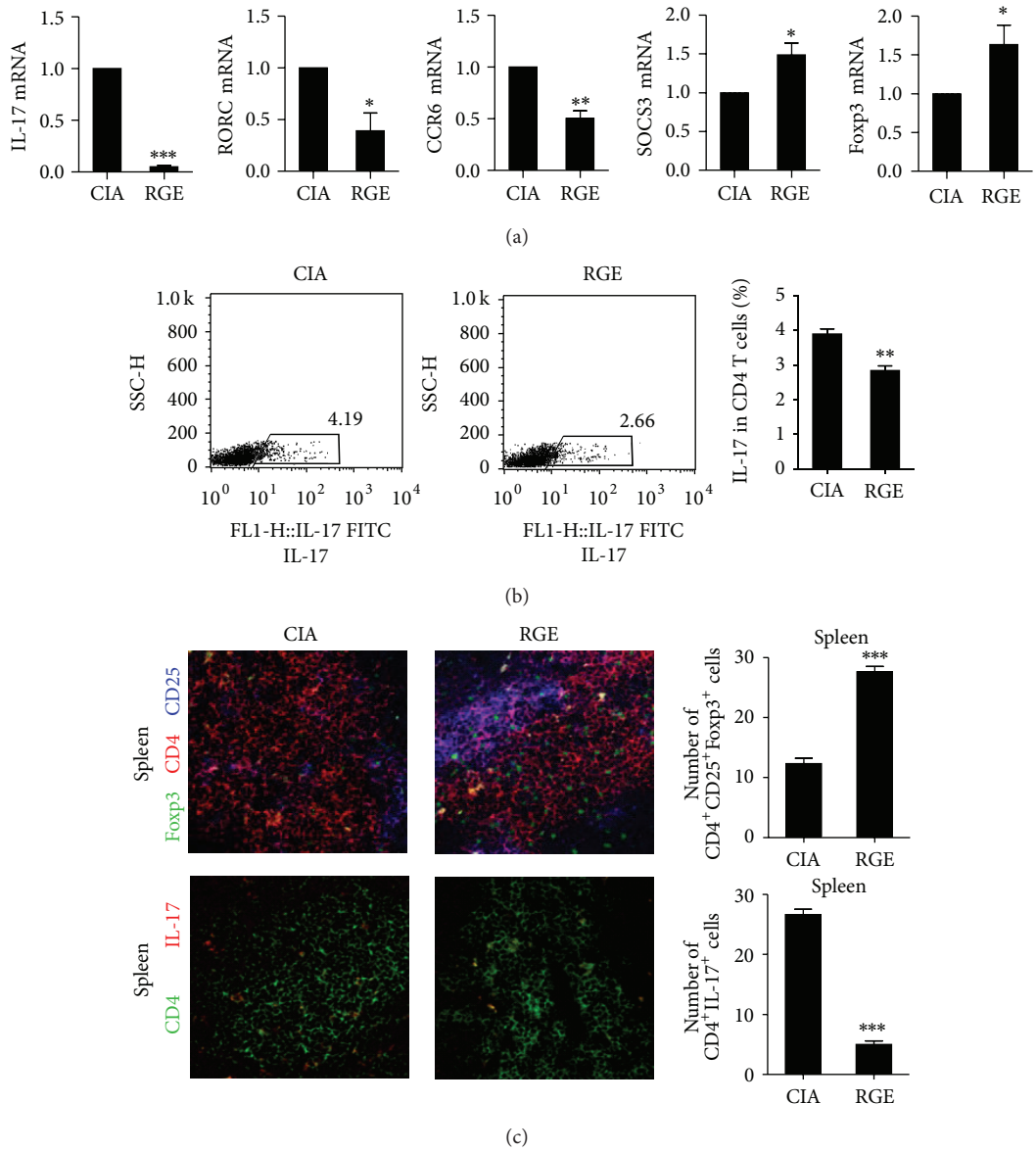


Figure 2: Red ginseng extract reciprocally modulates populations of regulatory T cells and Th17 cells in CIA mice. (a) Messenger RNA was isolated from splenocytes of either red ginseng extract (RGE) or vehicle-treated CIA mice. The mRNA expressions of Th17 cell-related molecules were measured by RT-PCR. (b) The proportion of IL-17-producing $CD4^+$ T cells in the splenocytes was analyzed by flow cytometry. (c) Spleens were subjected to immunostaining for $CD4^+CD25^+Foxp3^+$ or $CD4^+IL\text{-}17^+$ cells. The number of cells was counted in four independent quadrants. Data are presented as the mean ± SD of three independent experiments $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to control mice.

cultured in Th17 polarizing condition was investigated. Our data showed that the number of Treg cells increased with RGE treatment while Th17 differentiation was suppressed. This was in line with the previous finding that ginsenoside Rp-1 activated Treg cells [30]. However, this simultaneous regulation on Th17 and Treg by red ginseng has never been reported. The notion that Treg is reciprocally regulated with Th17 originates from the report that advocated the plasticity of T cell subsets [31]. Th17 and Treg are thought to have common precursors cell before they are destined to certain effecter cells. As transcription of IL-17 is regulated by competitive binding of pSTAT3 and pSTAT5 [20], and pSTAT5 is a critical transcriptional factor for Foxp3, the master molecule of Treg, the ratio of pSTAT3 and pSTAT5 is expected to be one of determinants for final effector cell type. While RGE significantly reduced pSTAT3, the level of STAT5 phosphorylation was not increased with RGE treatment. Although the RGE failed to increase the amount of pSTAT5, the decreased ratio of pSTAT3/pSTAT5 seemed to contribute to the Th17 suppression and the reciprocal increase in Treg. Namely, the relative reduction in pSTAT3 may have resulted in pSTAT5 binding on promoter site of IL-17 and suppressed IL-17 expression. With the suppression of Th17 differentiation, a shift toward Treg differentiation seemed to

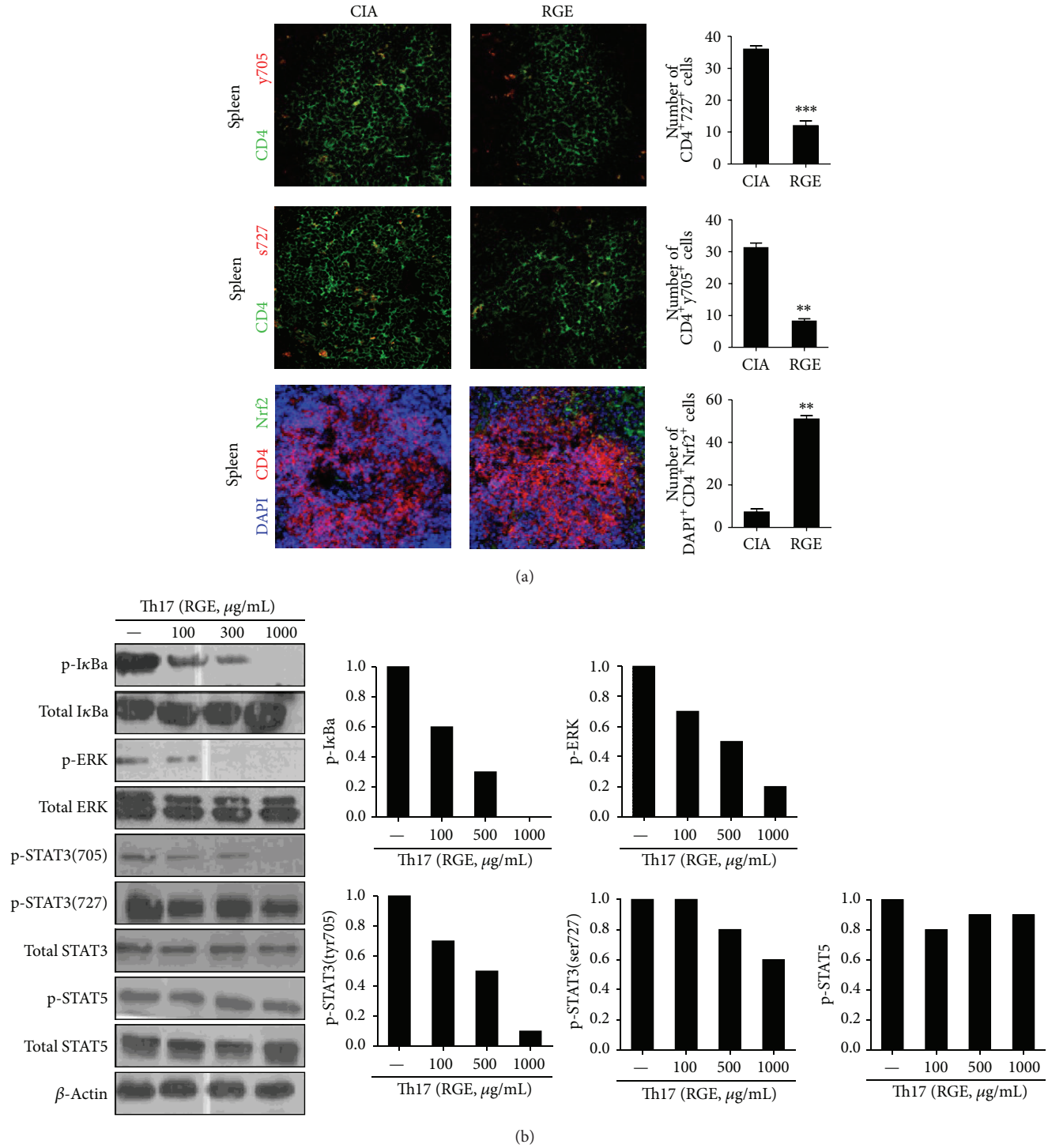


Figure 3: Red ginseng extract reduces STAT3 phosphorylation in the $CD4^+$ T cells in mice. (a) Spleens of red ginseng extract or vehicle-treated CIA mice were subjected to immunostaining for $CD4^+$pSTAT3y705$^+$ or $CD4^+$pSTAT3s727$^+$ cells. The number of cells was counted in four independent quadrants. (b) Isolated $CD4^+$ T cells were cultured in a Th17-polarizing condition for 3 days in the absence or presence of various concentrations of red ginseng extract (RGE). The expression of various signaling molecules was determined by western blotting. Data are presented as the mean ± SD of three independent experiments $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to control mice.

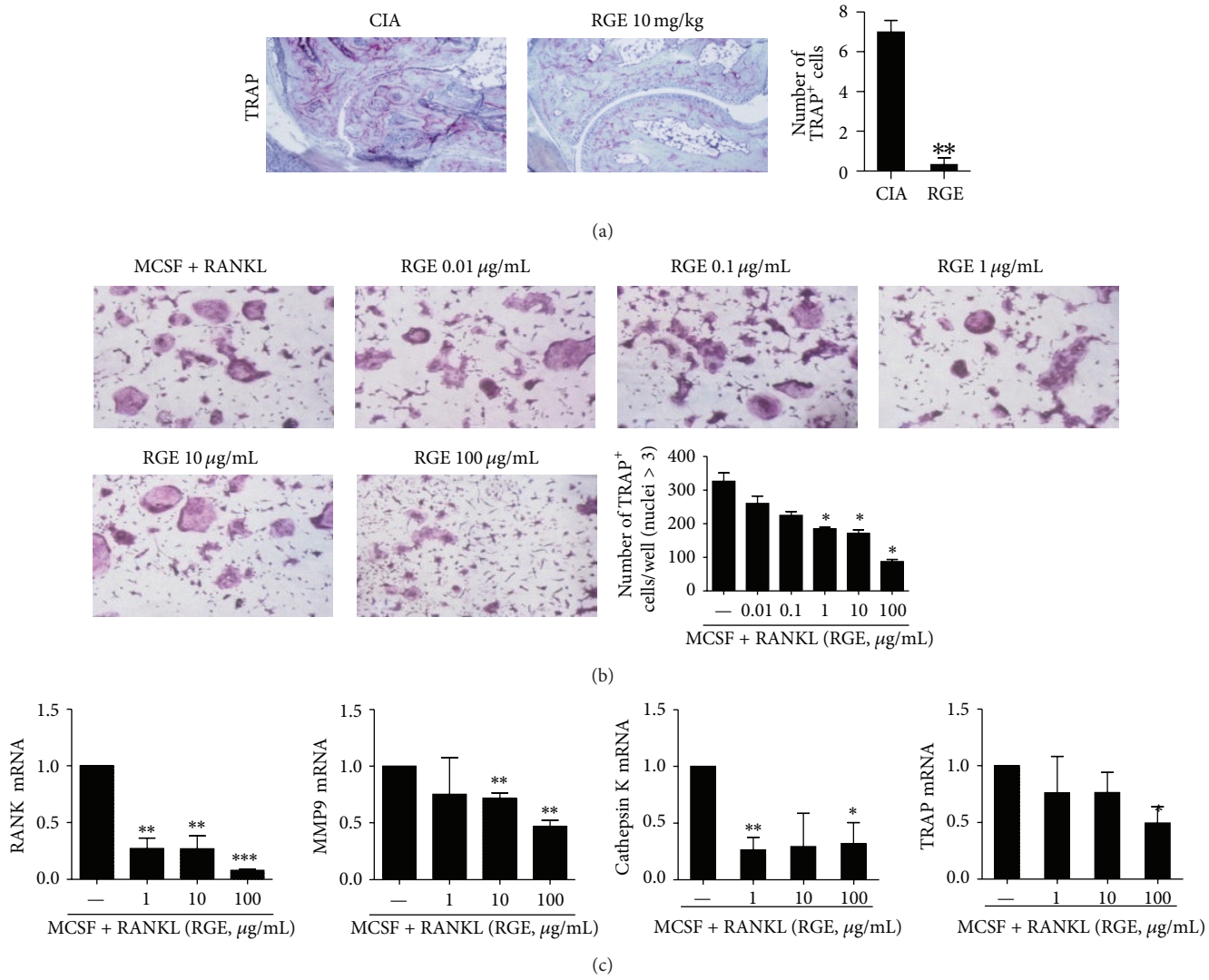


Figure 4: Red ginseng extract inhibits osteoclastogenesis in CIA mice. (a) The number of $TRAP^+$ cells in the arthritic joint of red ginseng extract (RGE) or CIA was counted using light microscopy. The BMM cells from the DBA/1J mice were cultured with M-CSF (10 ng/mL) and RANKL (50 ng/mL) in the presence or absence of various concentrations of RGE. (b) Cells were fixed and stained for TRAP (original magnification, ×100). The number of $TRAP^+$ cells was counted using light microscopy. The representative photographs from each group are shown. (b) The mRNA expressions of various osteoclastogenic markers such as RANK, MMP9. Cathepsin K and TRAP were analyzed using real time PCR. BMM: bone-marrow-derived monocyte/macrophage; MMP9: matrix metalloproteinase 9. Data are presented as the mean ± SD of three independent experiments $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to control mice.

be augmented. Although the differentiation into a specific T cell subset is mainly determined by master transcriptional regulator (e.g., RORγt, Foxp3) and phosphorylation of STATs, recent data indicate that other signaling pathways are also involved in this fine-tuning process. ERK signaling was suggested to be one of those pathways [32] where an inhibition of ERK decreases IL-6-induced RORγt expression and reciprocally enhances TGF-β-induced Foxp3 expression, resulting in decreased Th17/Treg ratio. Hence, the ability of RGE to suppress phosphorylation of ERK as well as STAT3 may be another efficient mechanism by which RGE regulated Th17/Treg balance.

The suppression of osteoclastogenesis by RGE was demonstrated by both *in vivo* and *in vitro*. In CIA joints, the decrease in number of Th17 cells, seems to have resulted in a reduction in TRAP(+) osteoclasts. The inhibitory effect of RGE on osteoclastogenesis *in vitro* was previously reported by He et al. [33]. They demonstrated that ginsenoside Rh2, a component of red ginseng, suppressed osteoclast differentiation by inhibiting RANKL-induced c-fos and NFATc1 expression. Ginsenoside Rh2 also inhibited phosphorylation of ERK and IκBα, thereby suppressing NF-κB, which was consistent with our findings observed in $CD4^+$ T cells. In addition, these results showed that RGE directly suppressed RANK expression during osteoclastogenesis *in vitro*, hampering RANKL-induced signaling.

The limitation of this study is that the exact chemical composition of RGE used was not analyzed. Therefore, it

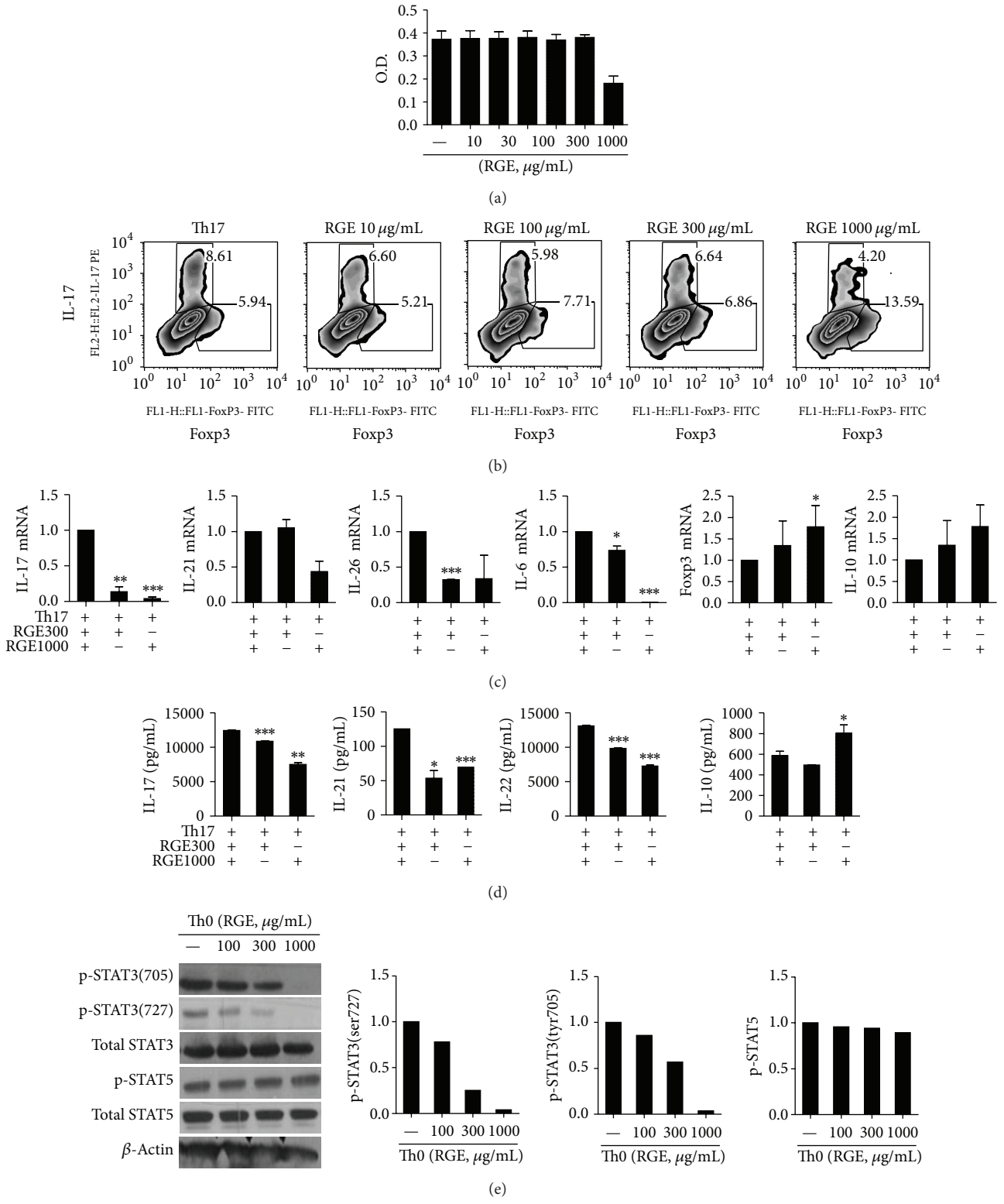


Figure 5: Red ginseng extract increased Foxp3-expressing regulatory T cells and decreased IL-17-expressing Th17 cells in human PBMCs. $CD4^+$ T cells were isolated from human peripheral blood and cultured with various concentrations of red ginseng extract (RGE) (a) MTT assay (b) The proportion of IL-17 positive cells and Foxp3 positive cells was analyzed by flow cytometry. (c) The mRNA expressions of Th17 - and Treg-associated molecules were measured by RT-PCR. (d) The concentrations of IL-17, IL-21, and IL-22 in the supernatant were measured by ELISA. (e) The level of total and phosphorylated STAT3 (at Y705 and S727, resp.) and STAT5 was assessed by western blotting. Data are presented as the mean ± SD of three independent experiments $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to control.

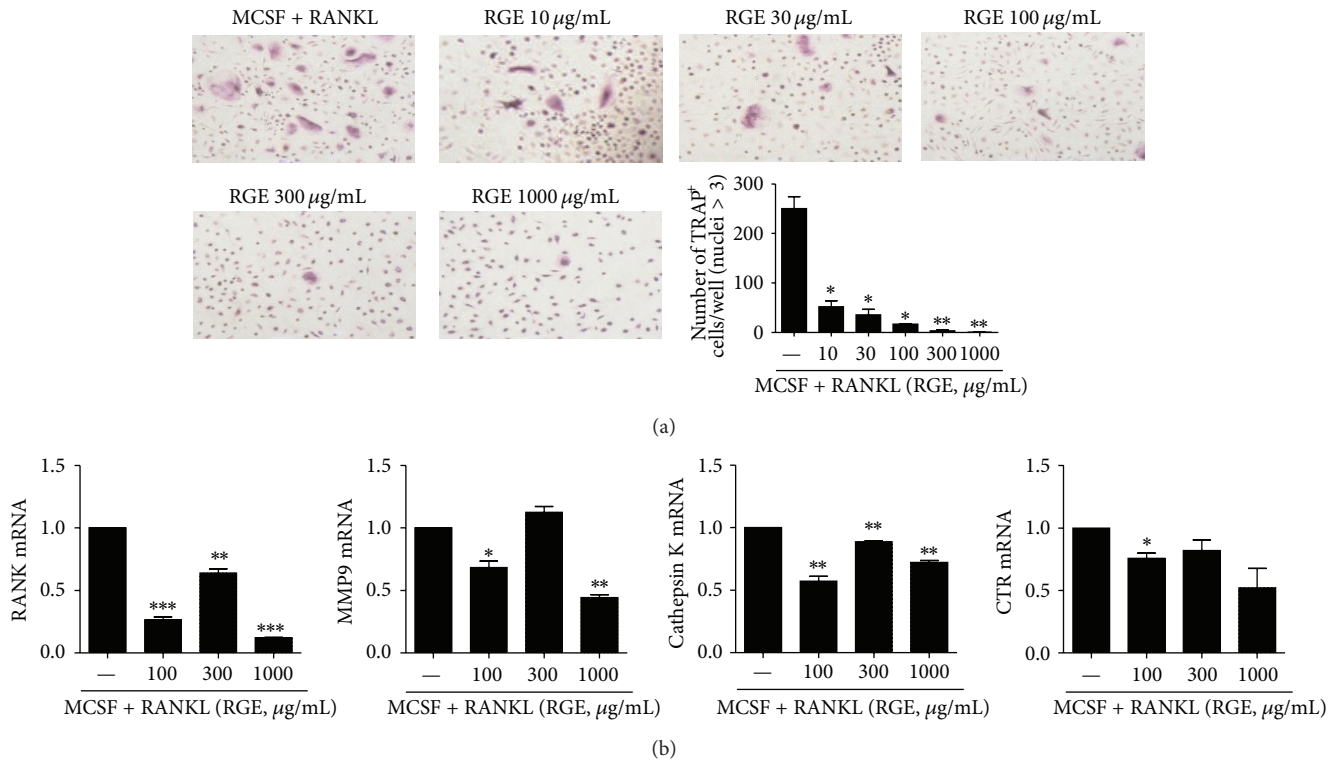


Figure 6: Red ginseng extract inhibits osteoclastogenesis in humans. Human peripheral blood mononuclear cells were cultured with M-CSF (10 ng/mL) and RANKL (50 ng/mL) in the presence or absence of various concentrations of RGE. (a) Cells were fixed and stained for TRAP (original magnification, ×100). The number of $TRAP^+$ cells was counted using light microscopy. The representative photographs from each group are shown. (b) The mRNA expressions of various osteoclastogenic markers such as RANK, MMP9. Cathepsin K and CTR were analyzed using real time PCR. BMM: bone-marrow-derived monocyte/macrophage; CTR: calcitonin receptor; MMP9: matrix metalloproteinase 9. Data are presented as the mean ± SD of three independent experiments $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to control.

is unclear which component of RGE is involved in specific molecular event. For instance, one ginsenoside may inhibit STAT3 phosphorylation in Th17 polarizing condition and another ginsenoside may suppress RANK expression during osteoclastogenesis or both effects can be mediated by one ginsenoside. Namely, the antiarthritic effect observed in the present study may be a constellate effect of several ginsenosides. However, red ginseng is generally ingested as a whole rather than any extracted form of specific ingredient. Hence, our data suggest red ginseng, as a whole, can be utilized as a novel therapeutic for RA.

The present study provides the first evidence that RGE can regulate Th17 differentiation. By inhibiting STAT3 phosphorylation, RGE concurrently suppresses Th17 and enhances Treg. This novel mechanism will provide the solid basis that red ginseng, conceiving as an alternative medicine, can be a promising therapeutic with scientific evidence. Furthermore, it can be expected that RGE may have therapeutic potential in other diseases where Th17 plays major role in pathogenesis such as inflammatory bowel disease or graft-versus-host disease. Future research on this topic will be promising.

5. Conclusion

In conclusion, our data demonstrate that RGE has a profound inhibitory effect on CIA development as well as its severity. The inhibitory effect appeared to be mediated through reciprocal regulation of Th17 and Treg by suppressive effect on STAT3 phosphorylation of RGE. RGE also directly inhibited osteoclastogenesis which is critical for bone erosion in RA. The regulatory effect was consistently found in experiments using human PBMCs, suggesting that antiarthritic effect of RGE could be applicable to human RA. In addition, RGE has been used widely in alternative medicine with relatively good safety profile. Therefore, RGE may be a novel therapeutic agent for the treatment of RA.

Conflict of Interests

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

Authors' Contribution

Jennifer Lee and Joo Yeon Jhun have equally contributed to this work. Sung Hwan Park and Mi-La Cho have equally contributed to this work.

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Review Article

Roles of Chronic Low-Grade Inflammation in the Development of Ectopic Fat Deposition

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Pattern of fat distribution is a major determinant for metabolic homeostasis. As a depot of energy, the storage of triglycerides in adipose tissue contributes to the normal fat distribution. Decreased capacity of fat storage in adipose tissue may result in ectopic fat deposition in nonadipose tissues such as liver, pancreas, and kidney. As a critical biomarker of metabolic complications, chronic low-grade inflammation may have the ability to affect the process of lipid accumulation and further lead to the disorder of fat distribution. In this review, we have collected the evidence linking inflammation with ectopic fat deposition to get a better understanding of the underlying mechanism, which may provide us with novel therapeutic strategies for metabolic disorders.

1. Introduction

Ectopic fat deposition refers to an excessive accumulation of lipids (mainly triglycerides) in nonadipose tissues, such as liver, muscle, and pancreas [1]. A large number of studies have shown that ectopic fat deposition is closely associated with insulin resistance (IR) and related metabolic diseases, including type 2 diabetes, atherosclerosis, and dyslipidemia [2]. However, its underlying mechanism has not yet been fully elucidated. In recent years, increasing evidence have shown that chronic low-grade inflammation is closely related to ectopic fat deposition and metabolic diseases; for example, elevated inflammatory factors are often observed in patients with ectopic fat deposition, such as fatty liver and fatty pancreas [3, 4]. Usually, this inflammatory condition is linked with overnutrition; however, a recent study reported elevated C-reactive protein (CRP) in nonobese or overweight subjects with nonalcoholic fatty liver disease, revealing that inflammation may play a critical and direct role, independent of excessive lipid from diet, in the development of ectopic fat accumulation [5]. Since ectopic fat accumulation involves both adipose tissue and nonadipose tissue, the purpose of the present review was to summarize the important evidence linking inflammation with ectopic lipid deposition in both adipose tissue and nonadipose tissue, in order to improve our understanding of the mechanism of ectopic lipid deposition.

2. Inflammation Causes Decreased Capacity of Fat Storage in Adipose Tissue

Adipose tissue is crucial for maintaining energy and metabolic homeostasis. One important function of adipocytes is to store TG, and the impairment of this function may have an effect on lipid handling in adipose tissue and, thereby, further contribute to excessive fat accumulation in nonadipose tissues. Many studies demonstrate that obese individuals often have enlarged adipocytes with overloaded lipid content and excess lipids "spill over" from the incompetent and dysfunctional adipose tissue, thereby exposing other tissues to an excessive influx of lipids, leading to ectopic fat deposition [6]. However, this "spill over" hypothesis cannot explain why some nonobese patients who are suffering metabolic disorders could also be accompanied by ectopic lipid accumulation. Besides, lipodystrophy, which is characterized by atrophic subcutaneous fat and IR, is often accompanied by ectopic fat accumulation in liver and/or in skeletal muscle. In mice models of lipodystrophy, transplantation of adipose tissue reversed IR and lipid content in both liver and skeletal

muscle [7]. Furthermore, the development of lipodystrophy is correlated with the mutations of several genes participating in adipose metabolism, such as peroxisome proliferator-activated receptor γ (PPARγ), 1-acylglycerol-3-phosphate-O-acyltransferase2 (AGPAT2), and Berardinelli-Seip congenital lipodystrophy (BSCL2), suggesting that this disease may be associated with adipocyte differentiation and lipid synthesis disorders in adipocytes [8]. Interestingly, lipodystrophic patients exhibited higher circulating concentrations of tumor necrosis factor-α (TNF-α) in adipose tissue, implying that inflammation may play an important role in the pathogenesis of the ectopic lipid deposition [9, 10].

Adipose tissue is composed of mature adipose cells and stroma-vascular fraction (SVF). In the SVF, mesenchymal stem cells as well as preadipocytes are able to differentiate into mature adipocytes for fat storage [11], and the whole process is controlled by some critical transcription factors and enzymes, including PPARγ, CCAAT enhancer binding protein (C/EBPα), sterol regulatory element-binding protein 1 (SREBP-1), fatty acid synthase (FAS), acetyl-CoA carboxylase enzymes (ACC), and stearyl coenzyme A desaturase 1 (SCD-1) [12]. Gustafson and Smith [13] have shown that both interleukin-6 (IL-6) and TNF-α prevented the normal development of preadipocytes to fully differentiated adipose cells and lipid accumulation with decreased expression of PPARγ2 and C/EBPα, which keep the cells undifferentiated. As an acute phase protein, serum amyloid A (SAA) is one of the most sensitive inflammatory markers, which is highly correlated with obesity, dyslipidemia, and insulin resistance. Remarkably depression of PPARγ, C/EBPβ, and C/EBPα was observed in preadipocytes treated with recombinant SAA (rSAA), leading to decreased intracellular lipid accumulation [14]. In Salles et al.'s study [15], after a 2-week high fat diet (HFD), TNF-α-knockout (TNF-α-KO) mice presented two-fold more adipose fat pad mass than control mice, while interestingly TNF-α-KO mice showed lower hepatic TG and ceramide accumulation in liver, with significantly declined adipose inflammatory markers, including resistin, monocyte chemotactic protein-1 (MCP-1), SAA3, and F4/80, implying decreased levels of inflammatory cytokines in adipose tissue might improve fat storage capacity of adipose tissue to prevent abnormal lipid deposition in nonadipose tissues.

Lipolysis of mature adipocytes is another important foundation for maintaining the balance of lipid metabolism in adipose tissue, which is conducted mainly by two rate-limiting enzymes, hormone-sensitive lipase (HSL) and fatty triglyceride lipase (ATGL) [16]. Except for impaired adipocyte differentiation, the inflammation-induced lipolysis may also be responsible for decreased lipid storage capacity of adipose tissue. In vitro, both IL-6 and TNF-α were proved to promote lipolysis in 3T3-L1 preadipocytes and increase free fatty acid in supernatant [13]. While in vivo, IL-6 injection also upregulated the level of fatty acid in serum. In morbidly obese patients, significantly elevated HSL and ATGL mRNA levels were observed, with enhanced serum CRP levels. Watt et al. [17] found that IL-6 administration promoted lipolysis with higher HSL mRNA and nonesterified fatty acid (NEFA) in serum. All evidence above supports that inflammation influences lipolysis by affecting the expression of HSL. However, some previous studies reported that TNF-α downregulated ATGL and HSL mRNA, without any changes in protein expression [18, 19]. Until recently, a study from Yang et al. [20] indicated that inflammation may stimulate basal lipolysis in adipocytes by regulating the function of ATGL. ATGL action in TNF-α-induced lipolysis was promoted by the depletion of G0/G1 switch protein 2 (G0S2) contents, which binds directly to ATGL and is capable of inhibiting its lipase activity. Yang found that TNF-α treatment inhibited the activity of ATGL by reducing both gene and protein expression of G0S2 to induce lipolysis in adipocytes, without any changes in expression of ATGL proteins. Remarkably, restoration of G0S2 protein levels by adenovirus-mediated ectopic expression was sufficient to prevent TNF-α-induced increase of glycerol release.

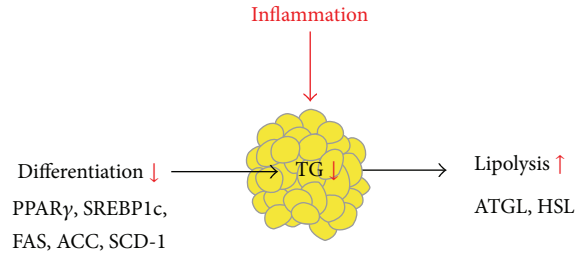

Figure 1: Inflammation decreases the lipid storage capacity of adipose tissue by inhibiting preadipocyte differentiation and increasing lipolysis.

Therefore, in terms of adipose tissue, inflammatory cytokines could decrease the lipid storage capacity by inhibiting preadipocytes differentiation and increasing lipolysis, which might further contribute to excessive fat accumulation in nonadipose tissues (Figure 1).

3. Effect of Inflammation on Nonadipose Tissue: Inflammation May Promote Fat Deposition in Nonadipose Tissue

3.1. Inflammation and Hepatic Fat Deposition. As a key metabolic organ, liver plays a crucial role in lipid metabolism. The balance of hepatic fat homeostasis depends on several pathways, influx of free fatty acids from adipose tissue due to lipolysis, de novo lipogenesis (DNL), fatty acid oxidation, and lipoprotein secretion [21]. Any abnormality of the processes above could contribute to lipid accumulation in liver.

Of the TG in liver, 59.0% derives from NEFAs, 26% from DNL, and 15% from the diet, which highlights the effect of fatty acid transportation on hepatic lipid content [22]. The transport of fatty acids into the liver is mediated via fatty acid transporters such as fatty acid transport protein (FATP), fatty acid translocase (FAT/CD36), fatty acid binding protein (FABP), and caveolin-1. Higher FAT/CD36 and FABP levels were observed in liver of nonalcoholic fatty liver disease (NAFLD) patients who are often accompanied with high levels of inflammatory factors, which were positively related to liver fat content [23, 24]. Furthermore, Margarita found that IL-6 induced higher FABP gene and protein expression

in both HepG2 cells and primary mouse hepatocytes, leading to increased intracellular lipid content [25]. Moreover, in Salles et al.'s study [15], after a 12-week HFD, TNF-α KO mice showed significantly lower liver ceramide and TG content with an obviously decreased CD36 mRNA compared to their wide type (WT) counterparts. All the evidence above gives us a clue that chronic inflammation may increase hepatic fat accumulation by enhancing fatty acid uptake with upregulated FA transporters.

Lipogenesis in hepatocytes is under control of a series of critical genes, such as SREBP-1c, FAS, ACC, and SCD-1. Above all, SREBP-1c plays the key role for regulating the expression of genes encoding rate-limiting enzymes responsible for de novo lipogenesis, of which FAS and ACC seem to be particularly important. Several studies have discussed the effects of inflammation on SREBP1c. In recent years, our group has focused on the effect of inflammation on lipid metabolism in liver. We induced a chronic systemic inflammation by subcutaneous injection of 10% casein in C57BL/6J mice. Significant increases of IL-6, TNF-α, and SAA were observed in casein-injected mice compared with the respective controls, suggesting that chronic systemic inflammation was successfully induced in vivo. Our results showed that chronic systemic inflammation induced by casein injection exacerbated lipid accumulation in the liver of mice fed with normal chow diet (NCD) and HFD, with upregulated mRNA and protein expression of SREBP-1, ACC, and FAS in liver, which indicated that chronic systemic inflammation increased lipogenesis in liver, resulting in hepatic lipid deposition [26]. In vitro, we also found that inflammatory factor TNF-α raised the expression of SREBP-1c, FAS, and ACC in HepG2 hepatocytes, leading to enhanced lipid accumulation (data unpublished). Similar results were observed in L02 hepatocytes [27]. Studies above were together extracted to a hypothesis that inflammation may aggravate hepatic steatosis by promoting lipid synthesis. Accordingly, inhibiting inflammation could improve lipid content in liver. In HFD treated TNF-α–/– mice, lipid content in liver was obviously reduced, accompanied with declined protein expression of SREBP1c and FAS [15]. Moreover, in recent years, sirtuin1 (SIRT1) has been shown to be involved in the process of anti-inflammation. The activation of SIRT1 is proved to exert anti-inflammatory effects by inhibiting production of TNF-α, MCP-1, and IL-8 via blockade of nuclear factor-κB (NF-κB). Some studies reported that administration of SIRT1 activator ameliorated fat accumulation in the liver of monosodium glutamate (MSG) mice which exhibited obesity and IR, and the expression of lipogenic genes, such as SREBP-1c, FAS, and ACC, was reduced by SIRT1 activator treatment, with declined expression of inflammatory cytokines, which also provided evidence for the critical role of inflammation in hepatocytes lipogenesis [28, 29].

Most fatty acids are metabolized through β-oxidation, which occurs mainly in mitochondria. The oxidation of intrahepatocellular fatty acid is regulated by a variety of key enzymes, including PPARα, PPARγ coactivator 1α (PGC-1α), and carnitine palmitoyltransferase 1 (CPT1). In patients with NAFLD, the expression of PPARα and CPT1 in liver was decreased, indicating impaired fatty acid oxidation [30]. Glosli et al. [31] found that in hTNF-α transgenic mice, hepatic triglycerides were enhanced and accompanied by reduced hepatic mRNA levels or activities of CPT-II, mitochondrial HMG-CoA synthase, peroxisome proliferator-activated receptor α (PPARα), and fatty acyl-CoA oxidase (FAO). In addition, as previously described, SIRT1 activators could attenuate liver steatosis with ameliorated inflammation. While in liver-specific SIRT1 knockout mice, lipid content in liver was obviously elevated, with decreased PPARα and PGC-1α and impaired fatty acid oxidation [32].

IL-6 is another critical inflammatory cytokine. However, the effect of IL-6 is still controversial. Long-term IL-6 injection ameliorates fatty liver of obese mice by stimulating hepatic fatty acid β-oxidation with increased hepatic PPARα [33]. Intriguingly, treatment of cultured hepatocytes with various concentrations of IL-6 downregulates the expression of PPARα [34, 35]. Matthews et al. [36] reported that mice with global deletion of IL-6 displayed hepatosteatosis, liver inflammation, and impaired whole-body insulin sensitivity when compared with control mice on a standard chow diet, revealing that IL-6 might be protective against hepatic steatosis and inflammation.

Hepatic TG could be delivered as VLDLs and secreted into circulation. The formation of VLDL involves the fusion of apoB with a TG droplet, which is mediated by microsomal triglyceride transfer protein (MTP). However, in patients with dyslipidemia, serum LDL-c and VLDL levels were observed to be positively associated with CRP and TNF-α [37]. Additionally, Pérez et al. reported that IL-6 treatment could upregulate hepatic apoB synthesis and secretion [38], implying a promoting effect of VLDL secretion of inflammation. This paradox might be explained since the lipid metabolism in liver is in a dynamic equilibrium and a fat deposition develops only when the inflammation induced fatty acid uptake or TG synthesis surpasses the inflammation-stimulated increase of hepatic lipid secretion.

It seems that the chronic low-grade inflammation, especially the proinflammatory cytokine TNF-α, might promote hepatic lipid accumulation through increased fatty acid uptake, enhanced TG synthesis, and reduced fatty acid oxidation in liver, and anti-inflammatory treatment may ameliorate this adverse effect. However, further studies are needed to investigate the effects of other different inflammatory cytokines on hepatic lipid accumulation (Figure 2).

3.2. Inflammation and Fat Deposition in Skeletal Muscle. Skeletal muscle is the major organ for fatty acid consumption, barely for lipid synthesis or storage. The increase of the lipid content in skeletal muscle which mainly results from increased fatty acid uptake and decreased β-oxidation can directly affect glucose and lipid metabolism and insulin sensitivity [39]. Several studies have indicated that inflammation may regulate fatty acid oxidation in skeletal muscles.

Increased lipid content as well as reduced β-oxidation of fatty acid and upregulated fatty acid uptake (a greater abundance of FAT/CD36) in muscle are observed in obese subjects who are characterized by inflammation state [40], indicating a negative role of inflammation on lipid accumulation in skeletal muscle. However, this view remains controversial,

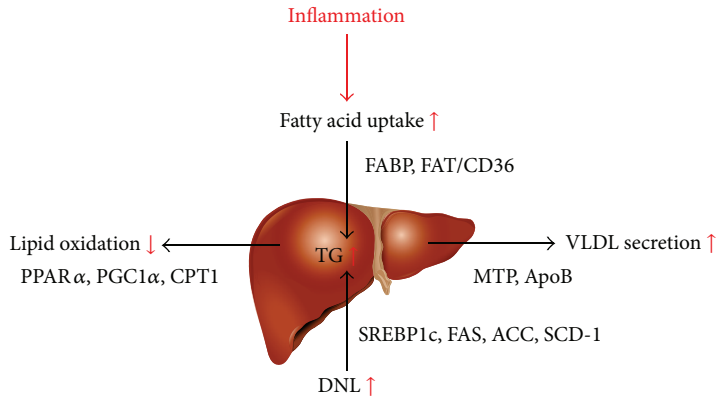

Figure 2: Inflammation promotes hepatic lipid accumulation through increased fatty acid uptake, enhanced TG synthesis, and reduced fatty acid oxidation.

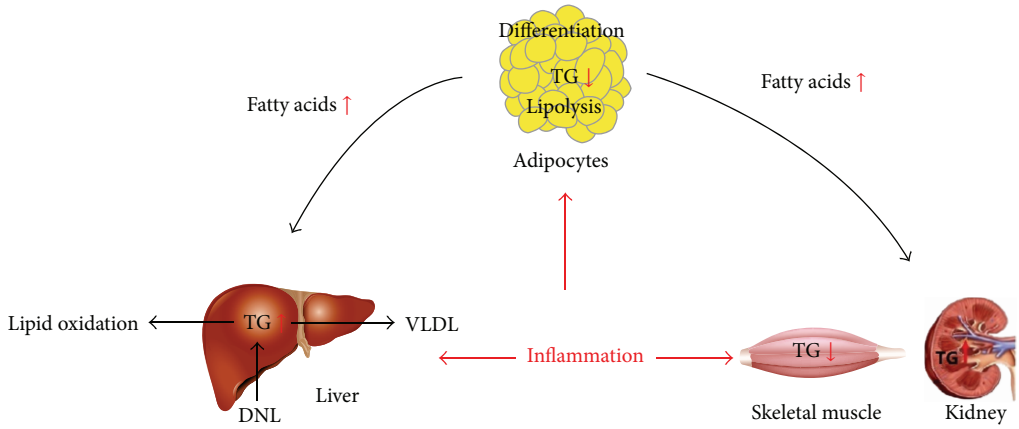

Figure 3: On one hand, chronic low-grade inflammation inhibits the differentiation of preadipocytes, increases lipolysis to upregulate the serum free fatty acid levels, and decreases the fat storage capacity of adipose tissue. On the other hand, inflammation directly influences lipid metabolism of liver, skeletal muscle, kidney, pancreas, and so forth, by different effects on process of ectopic fat deposition.

as in Salles et al.'s report [15] no significant difference of ceramide and TG concentrations was found in hind-limb skeletal muscles between TNF-α-KO and WT mice after a 2-week HFD. Furthermore, TNF-α-KO and WT mice showed similar levels of expression of genes involved in fatty acid oxidation, fatty acid uptake, or fatty acid synthesis, including PPARα, CPT1α, CD36, SREBP1, and FAS. In vitro, TNF-α treatment was reported having no effect on FA oxidation but increased FA incorporation into diacylglycerol. Besides, IL-6 was identified to raise lipid oxidation via upregulation of PPARα, PGC-1α, and PPARγ mRNA expression by activating AMP-activated protein kinase (AMPK) signal pathway in skeletal muscle and decreasing fat accumulation in human primary skeletal muscle cells [41–45]. Those results propose a different role of inflammation on lipid metabolism in skeletal muscle from the role in liver.

3.3. Inflammation and Fat Deposition in Other Tissues. As we discussed above, inflammation is closely correlated with fat content in liver and skeletal muscle; however, should inflammation be responsible for lipid deposition in other tissues? Wan et al. reported that inflammatory cytokines TNF-α and IL-6 upregulated the expression of FAT/CD36 at both mRNA and protein levels and exacerbated intracellular lipid accumulation in human mesangial cells (HMCs) and renal tubular epithelial HK-2 cells, showing the effect of inflammation on lipid metabolism in kidney [46]. In vivo, casein injection significantly increased lipid deposition in kidneys in C57BL/6J mice, which suggested that inflammatory stress increased lipid accumulation in kidneys [47]. Cardiac adiposity, characterized by an increase in intramyocardial triglyceride content and an enlargement of the volume of fat surrounding the heart and vessels, was reported to be positively associated with inflammatory markers [48]. Pioglitazone, which has been proved to reduce inflammatory cytokines, was shown to reduce intramyocardial triglyceride content in T2D patients [49]. Besides, Ma et al. reported that after injection of 10% casein for 8 weeks, the high-fat-fed apolipoprotein 3 (Apo3) KO mice represented significantly increased plasma SAA levels as well as elevated lipid accumulation in cardiac blood vessels, which indicated that inflammatory stress may markedly exacerbate lipid accumulation in cardiac blood vessels [50]. In another study, thirty C57BL/6J lean controls and 30 leptin-deficient obese female mice were

fed a 15% fat diet, and after 4 weeks, obese mice presented much higher TG content in pancreas compared with lean controls, with raised IL-1β and TNF-α concentrations. Those studies provide some clues that inflammation might promote lipid deposition in tissues such as kidney, heart, and pancreas; nevertheless, more evidence both in vivo and in vitro is still needed to further prove this effect [51].

4. Conclusion

In summary, chronic low-grade inflammation plays an important role in the development of ectopic fat deposition. On one hand, inflammatory cytokines decrease the lipid storage by inhibiting the differentiation of preadipocytes and increasing lipolysis, leading to upregulated free fatty acid level in serum and abnormal fat accumulation in other tissues. On the other hand, inflammatory cytokines directly interrupt the lipid metabolism in nonadipose tissues. In liver, increased lipid accumulation results from elevated import of fatty acids, lipid synthesis, and declined fatty acid oxidation induced by inflammation. Some evidence, but not enough, reveals that inflammatory cytokines might promote lipid deposition in other tissues such as kidney, heart, and pancreas. Further researches are required to investigate the effects and mechanisms of inflammation on nonadipose tissues (Figure 3).

Conflict of Interests

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

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Clinical Study

The Effect of a Community-Based, Primary Health Care Exercise Program on Inflammatory Biomarkers and Hormone Levels

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The aim of this study was to analyze the impact of a community-based exercise program in primary care on inflammatory biomarkers and hormone levels. The 1-year quasiexperimental study involved 13 women (mean age = 56.8 ± 11.4 years) and it was developed in two basic health care units in Rio Claro City, Brazil. The physical exercise intervention was comprised of two, 60-minute sessions/week. The inflammatory biomarkers were measured at baseline, 6 months, and 1 year. Repeated measures ANOVA analyses indicated that the intervention was effective in reducing CRP and TNFα after 1 year compared to baseline and 6 months ($P < 0.05$). There were no changes in IL10, IL6, and insulin after 1 year. However, leptin significantly increased at 1 year ($P = 0.016$). The major finding of this study is that a community-based exercise program can result in a decrease or maintenance of inflammatory biomarkers after 1 year, and thus has the potential to be a viable public health approach for chronic disease prevention.

1. Introduction

It is well established that chronic diseases are the leading cause of mortality in the world. According to the World Health Organization [1] 60% of all death is attributed to cardiovascular diseases, diabetes, cancers, and chronic respiratory diseases. The inflammatory process related to chronic diseases, characterized by dysregulation in the balance between pro- and anti-inflammatory processes, is linked with several complications such as insulin resistance, endothelial dysfunction, atherosclerosis, and vascular and metabolic disorders [2–5].

Regular physical exercise has been increasingly viewed as an effective therapeutic strategy for the management of chronic diseases [6]. It has long been known that regular physical activity induces multiple adaptations within skeletal muscles and the cardiorespiratory system, providing positive outcomes for the prevention and treatment of chronic diseases [7, 8]. Some studies have indicated that regular physical activity has anti-inflammatory effects and is associated with improvement in inflammatory biomarkers such as a reduction in levels of the proinflammatory cytokines [9–14]. According to Pedersen [8], the anti-inflammatory processes provided by physical exercise play important roles in the protection against diseases associated with low-grade inflammation such as cardiovascular diseases and type 2 diabetes.

Considering that physical inactivity is the fourth leading cause of death worldwide [15] and causes 6–10% of the major noncommunicable diseases [6], it is necessary to induce social, economic, and environmental changes and multiple strategies that promote public policies related to physical active life style. "Saúde Ativa Rio Claro" (SARC) is a community-based exercise intervention in primary care designed to promote and maintain physical activity levels of residents in Rio Claro City, Brazil. Since 2001, SARC operates in basic health care units and reaches approximately 400 low-income adults aged 35 years or older [16]. Evidence

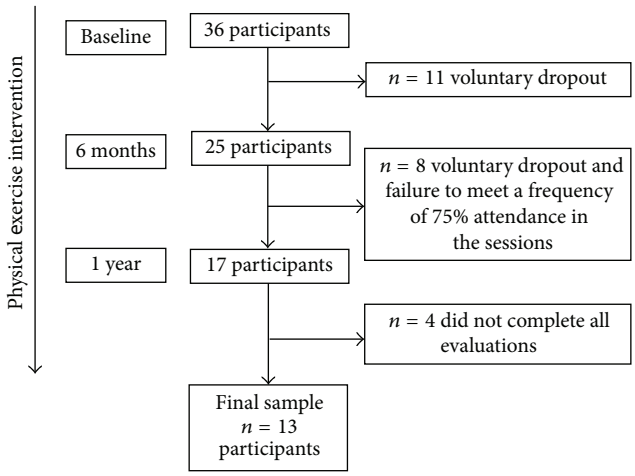

Figure 1: Recruitment of participants for the study. Evaluations were done at baseline, 6 months, and 1 year of SARC intervention.

suggests that this program improves blood cholesterol, LDL, HDL, and glucose profiles [17, 18]. However, it is unknown whether the SARC intervention can improve inflammatory biomarkers and thus potentially aid in the prevention of chronic disease and associated complications. Therefore, the aim of this study was to assess the impact of SARC on a range of common inflammatory biomarkers and hormone levels in adult women, including leptin, insulin, C-reactive protein (CRP), interleukin 6 (IL6), tumor necrosis factor alpha (TNFα), and interleukin 10 (IL10). It was hypothesized that there would be an increase in IL10 and a decrease in inflammatory markers (CRP, IL6, and TNFα) and hormone (leptin and insulin) levels after 1 year of SARC intervention.

2. Methods

2.1. Participants. This 1-year quasiexperimental study was developed in two basic health care units in Rio Claro City, Brazil. Adult females were recruited via flyers and newspaper advertisements. Participants were assigned to the intervention group based upon proximity from their residence. Thirty-six participants were recruited at the beginning of intervention. As a result of either voluntary dropout or failure to meet the inclusion criterion for the study (frequency of 75% attendance in the sessions), 25 participants remained in the intervention after 6 months. Although 17 participants completed the 1 year intervention, four participants did not complete all evaluations; thus the final sample size was 13 women (mean age = 56.8 ± 11.4 years, Figure 1). The study was approved by the Human Research Ethics Committee of Biosciences Institute, UNESP, protocol number: 2308.

2.2. Physical Exercise Intervention. SARC is a community-based exercise intervention comprised of two 60-minute sessions/week of physical exercises. The sessions were divided in warm-up activities (5 minutes), moderate intensity aerobic exercise (30 minutes), strength-training exercises (20 minutes), and cool-down activities (5 minutes). Furthermore, during each session, the participants received counseling designed to increase daily physical activity levels and encourage participation in physical exercise outside of the intervention.

The warm-up and cool-down activities included static stretching exercises and articular movements. Static stretching was maintained for a minimum period of 15 to 30 seconds, twice for each muscle group. The participants were advised to sustain a muscle stretch that did not cause pain [19, 20]. The aerobic exercises consisted of walking at moderate intensity (60–70% of peak heart rate). The target zone for exercise was calculated using the equation HRpeak = 206 − (0.88 × age), as suggested by Gulati et al. [21]. All participants were instructed to maintain a subjective effort between 13 and 15 [22] on the Borg scale [23] during walking. Four participants were randomly selected to measure the intensity of their activity twice a month using a cardiac rate monitor (Polar, FS1) and the subjective effort scale. The strength-training exercises were performed using free weights, exercise mats, and latex exercise bands. Exercises included all major muscle groups and were performed in 3 sets of 30 seconds, followed by one minute of recovery.

2.3. Inflammatory Biomarkers Measures. A 10 mL venous blood sample was collected at baseline, after 6 months, and after 1 year of intervention, in the morning after 12 hours of fasting. The blood sample was transported under refrigeration to the laboratory within 30 minutes, centrifuged for 10 minutes with the serum immediately separated following centrifugation. The inflammatory biomarkers were analyzed in duplicate using commercial kits. C-reactive protein (CRP) was analyzed using an enzyme-linked immunosorbent assay (ELISA). Interleukin 10 (IL10), interleukin 6 (IL6), tumor necrosis factor alpha (TNFα), leptin, and insulin were analyzed using Luminex technology assay (Luminex). Intra-assay coefficients were all <10%. To minimize analytical variations, the same technician tested all samples without changing reagent lots, standards, or control materials.

2.4. Statistical Analyses. Descriptive data are reported as means and standard deviations. The ratio of IL-10 to TNF-α (IL10/TNF-α) was calculated and compared in 1 year.

A repeated measures ANOVA was used to analyze the changes in anthropometric variables, inflammatory biomarkers, and hormones levels over time (baseline, 6 months, and 1 year). Significant differences were determined by Bonferroni post hoc tests. Statistical analyses were conducted using SPSS 20.0, with the alpha level set at $P < 0.05$.

3. Results

Table 1 shows the anthropometric characteristics of the participants ($n = 13$, mean age of 56.8 ± 11.4) at baseline, 6 months, and 1 year. No changes in weight, body mass index (BMI), or waist to hip ratio (WHR) were seen over time ($P > 0.05$). The prevalence of diseases was 7.7% ($n = 1$) for diabetes, 30.7% ($n = 4$) for obesity, and 38.5% for hypertension ($n = 5$).

Table 1: Anthropometric characteristics (mean, standard deviation) of participants at baseline, after 6 months, and after 1 year of exercise intervention.

	Baseline	6 months	1 year	*P* value BL versus 6 M	*P* value BL versus 1 Y	*P* value 6 M versus 1 Y
Weight (kg)	67.3 ± 11.5	66.8 ± 11.4	67.2 ± 10.9	0.541	0.631	1.000
BMI (kg/m^2)	27.5 ± 5.6	26.8 ± 6.0	27.9 ± 5.6	0.500	0.316	1.000
WHR	0.88 ± 0.8	0.86 ± 0.8	0.89 ± 0.7	0.863	0.326	1.000

BL: baseline; 6 M: 6 months; 1 Y: 1 year; BMI: body mass index; WHR: waist and hip ratio.

Table 2: Inflammatory biomarkers and hormone concentration levels (mean, standard deviation) at baseline, after 6 months, and after 1 year of exercise intervention.

Biomarker	BL	6 M	1 Y	*P* value BL versus 6 M	*P* value BL versus 1 Y	*P* value 6 M versus 1 Y
CRP (mg·L^{-1})	3.4 ± 1.2	3.0 ± 1.2	1.5 ± 1.0$^{*\alpha}$	0.999	0.001	0.003
IL10 (pg·mL^{-1})	4.8 ± 2.0	4.4 ± 2.3	4.2 ± 1.5	0.988	0.681	0.602
IL6 (pg·mL^{-1})	4.4 ± 1.1	4.2 ± 1.5	3.4 ± 0.7	0.999	0.236	0.163
TNFα (pg·mL^{-1})	10.6 ± 5.6	7.6 ± 4.0	5.6 ± 3.0$^{*\alpha}$	0.082	0.001	0.004
Leptin (ng/mL^{-1})	2.69 ± 2.25	2.30 ± 1.66	7.60 ± 4.89$^{*\alpha}$	0.999	0.016	0.003
Insulin (ng/mL^{-1})	1.09 ± 1.01	0.83 ± 0.41	0.67 ± 0.17	0.898	0.405	0.642
IL10/TNFα	0.59 ± 0.4	0.64 ± 0.2	0.85 ± 0.3	—	—	—

BL: baseline; 6 M: 6 months; 1 Y: 1 year; CRP: C-reactive protein; IL10: interleukin 10; IL6: interleukin 6; TNFα: tumor necrosis factor alpha.
*Statistically significant difference from baseline.
$^\alpha$Statistically significant difference after 6 months.

The prevalence of participants having at least 1 disease was 46.1% ($n = 6$).

Table 2 and Figure 2 illustrate the inflammatory biomarkers and hormone concentration levels and indicate the outcomes of statistical analyses between time at baseline, 6 months, and 1 year. CRP levels significantly decreased after 1 year of intervention (1.5 ± 1.0 mg·L^{-1}) compared to baseline (3.4 ± 1.2 mg·L^{-1}, $P = 0.001$) and 6 months (3.0 ± 1.2 mg·L^{-1}, $P = 0.003$). A significant decrease in TNFα levels was shown after 1 year of intervention (56.6 ± 3.0 pg·mL^{-1}) compared to baseline (10.6 ± 5.6 pg·mL^{-1}, $P = 0.001$) and 6 months (7.6 ± 4.0 pg·mL^{-1}, $P = 0.004$). IL10, IL6, and insulin did not change over 1 year ($P > 0.05$). Leptin levels were significantly increased after 1 year (7.6 ± 4.89 pg·mL^{-1}) of intervention compared to baseline (2.69 ± 2.25 pg·mL^{-1}, $P = 0.016$) and 6 months (2.3 ± 1.66 pg·mL^{-1}, $P = 0.003$). The IL10/TNFα ratio increased after 1 year of intervention (BL = 0.59 ± 0.4; 6 M = 0.64 ± 0.2; 1 Y = 0.85 ± 0.3).

4. Discussion

Chronic inflammation is an important pathophysiological factor in the development of several diseases and complications, through the effects of proinflammatory cytokines such as TNFα and IL6, among others [2–5]. On the contrary, anti-inflammatory cytokines, such as adiponectin and IL-10, seem to be protective against pathological conditions [24, 25].

Analyses indicate that the SACR intervention was effective in decreasing CRP and TNFα levels and maintaining IL10, IL6, and insulin levels over 1 year. However, leptin levels increased over 1 year. Several studies show that inflammatory biomarkers are reduced following longer term lifestyle modification involving reduced food intake and increased physical activity [9]. Thus, the effects of regular physical activity on basal levels of inflammatory markers have been used to recommend exercise as an anti-inflammatory therapy. According to Soares and de Souza [14] integrative interventions, including diet, moderate aerobic exercise (60% to 80% of HRmax or 50% to 60% of $VO_{2\,max}$) and circuit resistance training (8 to 10 exercises, 8 to 12 repetitions), health education, and counseling, used together, appeared to be effective strategies to improve inflammatory biomarkers in women.

Our results (Table 2 and Figure 2) indicated that SARC was effective in decreasing CRP levels after 1 year compared to baseline and 6 months. These findings are in agreement with other studies in the literature indicating that a physical lifestyle can reduce CRP levels [13, 26–29]. CRP has a long plasma half-life (>96 h), no variation of diurnal or seasonal, and no age or gender dependence [30, 31]. It plays a pivotal role in the innate immune response, is released in response to a variety of proinflammatory cytokines, and is triggered by many factors such as cardiovascular diseases, trauma, malignancy, and chronic arthritis [32]. In our study, the 56% decrease in CRP is clinically relevant because the value changed from a level considered "high risk" for cardiovascular disease at baseline (above 3.0 mg/L) to an "average risk" (1.0 to 3.0 mgL) after 1 year of the SARC intervention.

According to You et al. [33], findings about the relationship between physical exercise and inflammatory biomarkers

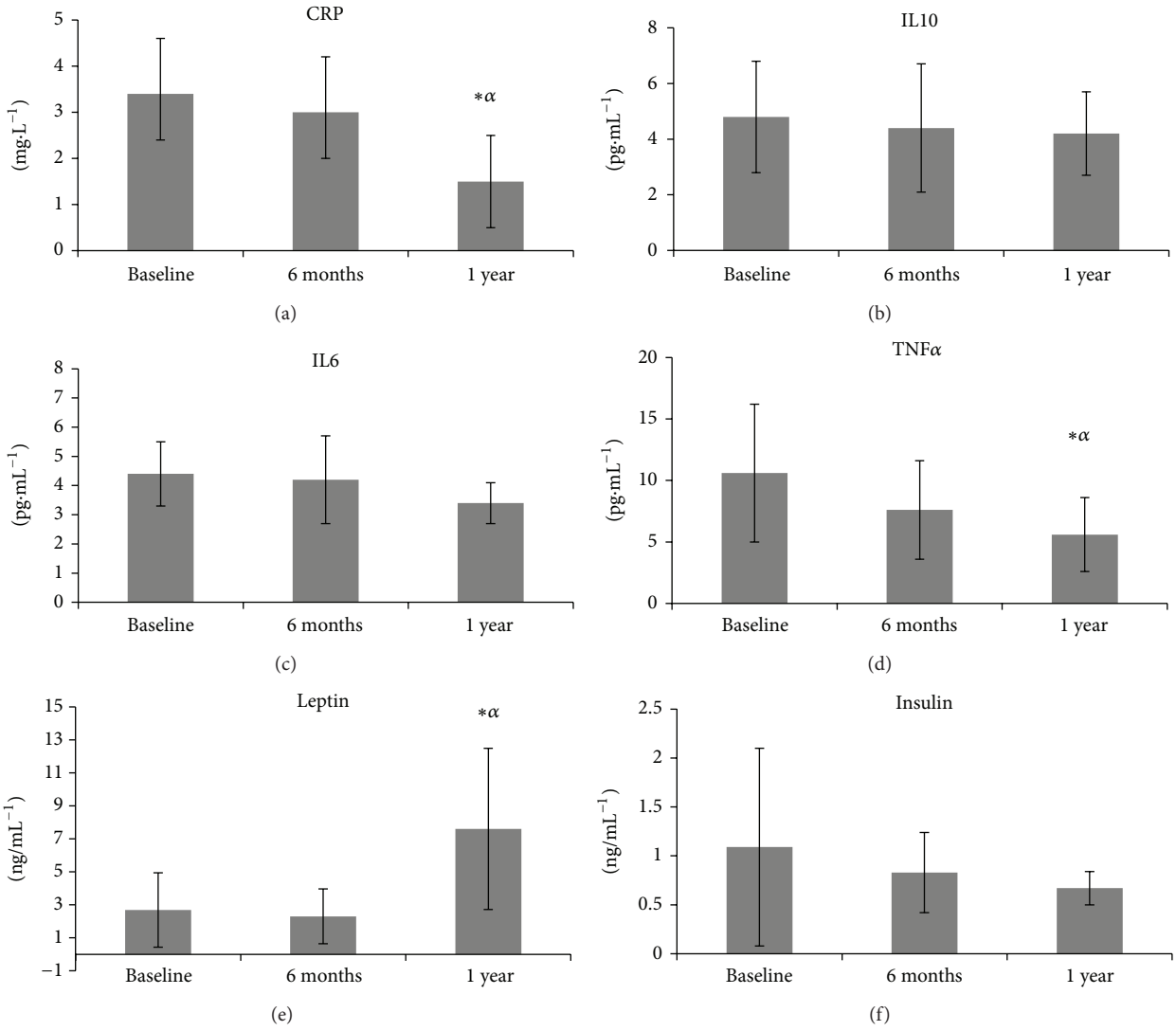

Figure 2: Levels of C-reactive protein (CRP), interleukin 10 (IL10), interleukin 6 (IL6), tumor necrosis factor alpha (TNFα), leptin, and insulin at baseline, after 6 months, and after 1 year of exercise intervention. *Statistically significant difference from baseline. $^{\alpha}$Statistically significant difference from 6 months.

are more consistent for CRP than for other biomarkers. However, the SARC intervention was effective in reducing TNFα (Table 2 and Figure 2) after 1 year compared to baseline and after 6 months. Studies have indicated that regular physical activity is associated with a reduction or no change in TNFα [27, 28, 34, 35]. TNF-α is a cytokine with a varied range of proinflammatory activities, such as influencing the atherosclerotic process both by causing metabolic perturbations and by increasing the expression of cellular adhesion molecules [36].

No changes were detected in IL10 following 1 year of intervention (Table 2). IL10 has multifaceted anti-inflammatory properties. It is able to reduce serum levels of TNFα and IL6 and plays a protective role against atherosclerosis [24, 25]. There is lack of consensus in the literature as to whether physical activity can improve IL10 levels. Kadoglou et al. [28] demonstrated in their study that a higher volume of aerobic exercise (four times/week, 45–60 min/session) was effective in increasing IL10 levels after 6 months. Similarly, Jankord and Jemiolo [37] compared groups performing different amounts of physical activity volume and concluded that the higher volume was associated with an increase of IL-10 [37]. Thus, it appears that 2 sessions per week of physical exercise delivered by SARC may be insufficient to improve IL10 levels. However, the IL10/TNFα ratio increased after 1 year of intervention. This result indicates that physical exercise was able to improve the proportion of anti- to proinflammatory cytokines after 1 year.

The SARC intervention did not change IL6 levels following 1 year of intervention (Table 2). Some studies have reported that physical exercise is correlated with lower IL6 levels [13, 29, 34, 37–40]. However, our results are in agreement with other studies. Olson et al. [41] found that an intervention consisting of at least two training sessions

per week was not effective in reducing IL6 levels after 1 year. Campbell et al. [42] and Donges et al. did not also find lower levels of IL6 following physical exercise interventions [43]. Different cells produce IL6 and this cytokine plays both "good" and "bad" roles depending on the circumstances. It has been suggested that an elevation in IL6 in response to physical exercise can exert an anti-inflammatory role. Myokine, the IL6 from muscle, can increase during physical exercise. It wields metabolic effects on liver and adipose tissues (activating glycogenolysis and lipolysis) and inhibits the production of TNFα [44, 45]. On the other hand, IL6 is also secreted by macrophages and lymphocytes in response to injury or infection [46] and has been associated with several pathological conditions as a marker of low-grade inflammation [47, 48]. Thus, the maintenance of IL6 levels during a 1-year intervention could be considered a positive outcome.

It is currently well accepted that regular physical exercise is an effective therapeutic intervention to reduce the risk of developing insulin resistance by improving glucose tolerance and insulin action in individuals predisposed to developing type 2 diabetes [7]. It has been hypothesized that insulin resistance increases with age due to increased adiposity, decreased lean muscle mass, changes in dietary habits, and reduced physical activity [49]. Although there was not a statistically significant change in insulin in the present study (Table 2), insulin levels decreased by 38.4% after 1 year of the intervention, suggesting that insulin sensitivity may have improved, although an insulin sensitivity test in participants would be needed to confirm this.

In the present study, leptin levels were maintained until 6 months and then increased significantly after 1 year of the intervention (Table 2 and Figure 2). Despite these changes, leptin levels remained in the normal range (2.5–21.8 ng/mL). According to Mota and Zanesco [50], the relationship between physical activity and plasma leptin is unclear, with some studies showing a reduction in their levels while others fail to find any change. Recently, Akbarpour [13] demonstrated that 12 weeks of physical exercise was able to reduce leptin levels, BMI, and IL6, and in contrast to our findings they did not find any changes in TNFα.

Plasma levels of leptin can increase as the result of obesity [51]; in the present study we saw no changes in body weight, BMI, or WHR after 1 year. In addition, TNF-α and CRP have been shown to be related to high levels in adipose tissue, and its level in the circulation indicates the production of these biomarkers in adipose tissue [51]. In the present study, although no decrease in BMI and weight was observed, the levels of TNFα and CRP were decreased, supporting the effect of exercise on these biomarkers independent of weight loss. Current evidence supports that exercise training reduces chronic inflammation and this effect is independent of the exercise induced weight loss [33].

The mechanisms related to physical exercise as a therapy in changing inflammatory biomarkers are not clear, despite studies showing positive outcomes. The discrepancy between the results from various studies in the literature can be attributed to the differences among the groups studied, training period, volume, intensity, duration, and type of training.

This study has a number of limitations that should be considered. The small sample size that resulted in the study has low statistical power and was a result of the difficulty in maintaining a 75% participation rate in the intervention sessions over the 1-year intervention period. We attempted to reduce dropout by assigning participants to an intervention groups geographically near their home. In addition, this study employed a quasiexperimental design, and thus we are not able to state with confidence that the changes in inflammatory markers are due to participation in the SARC intervention. We attempted to include a control group (doing no physical exercise over 1 year) to allow us to conduct a controlled trial, but the university ethics committee would not approve this study design.

Considering the fact that 46.1% of participants already had at least 1 disease related with the inflammation process, this study illustrates that a public health exercise intervention delivered in low-income communities has the potential to exert a beneficial effect and improve or maintain inflammatory biomarkers profiles, assisting in the prevention of chronic diseases. However, a larger randomized controlled trial needs to be conducted to confirm or refute these suggestive findings.

5. Conclusion

The major finding of the present study was that a public health exercise intervention was effective in decreasing CRP and TNFα levels and maintaining IL10, IL6, and insulin levels over 1 year. Developing and delivering a community-based, public health exercise intervention like SARC could be a viable initiative to promote health at the public health level.

Conflict of Interests

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

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Research Article

Study of the Correlations among Some Parameters of the Oxidative Status, Gelatinases, and Their Inhibitors in a Group of Subjects with Metabolic Syndrome

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Our aim was to examine some parameters of oxidative status, gelatinases, and their inhibitors and to evaluate their interrelationships in subjects with metabolic syndrome (MS). We enrolled 65 MS subjects, subdivided according to the presence or not of diabetes mellitus. We examined lipid peroxidation (expressed as thiobarbituric acid reacting substances, TBARS), protein oxidation (expressed as carbonyl groups), nitric oxide metabolites (NO_x), total antioxidant status (TAS), MMP-2, MMP-9, TIMP-1, and TIMP-2. We found that MS subjects, diabetics and nondiabetics, showed an increase in TBARS, PC, and NO_x. A significant decrease in TAS was observed only in nondiabetic MS subjects in comparison with diabetic MS subjects. We observed increased concentrations of MMP-2, MMP-9, TIMP-1, and TIMP-2, higher in diabetic subjects. Our data showed a positive correlation between TAS and MMP-2, TAS and MMP-9, and TAS and MMP-9/TIMP-1 and a negative correlation between TBARS and MMP-2 in diabetic MS subjects in the entire group. In MS subjects a prooxidant status and increased levels of gelatinases and their inhibitors are evident although the correlations between oxidative stress and MMPs or TIMPs are controversial and need further investigation.

1. Introduction

Today the metabolic syndrome (MS) is considered a public health problem [1]. As it is known, obesity and MS are associated with a low-grade of chronic systemic inflammation, reflected by an increase in circulating leukocytes and by elevated levels of proinflammatory cytokines [2, 3] that contributes to the development of insulin-resistance and atherosclerosis [4] via the activation of nuclear factor-kB pathway [5]. The alterations of the vascular wall start from the endothelial dysfunction and oxidative stress and also an altered matrix metalloproteinases (MMPs) expressions contribute to the consequent remodelling of the basal membrane.

In the last years, we have been interested in the evaluation of the redox balance [6–10] and of the MMPs profile [11, 12] in MS. MMPs are related to atherosclerotic disease and to cardiovascular morbidity and mortality [13–18]; these are endopeptidases responsible for the degradation of several extracellular matrix proteins, such as collagen, laminin, gelatin, and fibronectin [19] which are produced into the vascular wall and are denominated in relation to their target (collagenase, gelatinase, stromelysin, or matrilysin). Gelatinases A and B (MMP-2 and -9) are involved in the vascular remodelling that precedes the atherosclerosis development and also in its worse outcomes [20, 21]. MMP-9 has been discovered in older atherosclerotic lesions [22] and is responsible for fibrosis, matrix degradation, and angiogenesis resulting in plaque instability and rupture [22, 23], while MMP-2 has been correlated with a more stable plaque and with rare haemorrhages [24]. The regulation of MMPs production and activity is complex. Some MMPs (MMP-2) are constitutively expressed on cell surface, while others (MMP-9) are stored in secretory granules and are inducible by exogenous stimuli, such as cytokines, growth factors, and cell-matrix contacts [19, 20]. MMPs are synthesized as precursors (pro-MMP) and they must be activated, to expose the catalytic domain with the Zn^{2+}-binding site, by several proteases, such as plasmin, thrombin, chimase, and membrane-type MMP (MT-MMPs)

[19, 25], or by S-glutathiolation, S-nitrosylation, and phosphorylation reactions [23].

MMPs and oxidative stress seem to be strongly correlated in subjects with high cardiovascular risk [26–30] and this link has been demonstrated in several experimental models [31–34]. Peroxynitrite ($ONOO^-$) activates some MMPs via the S-glutathiolation [31, 34] but, at higher concentrations, can lead to the inactivation of MMP-2 [34]. Also hydrogen peroxide (H_2O_2) activates MMP-2 and promotes the expression of MMP-2 and MMP-9 in human venous endothelial cells [35]. Reactive oxygen species (ROS) can influence MMP transcription by means of the activation of the mitogen-activated protein kinase (MAPK), the inhibition of MAPK phosphatase, the inactivation of the histone deacetylase, and the recruitment of different chromatin remodelling factors [36]. MMPs activity is downregulated especially by the four tissue inhibitors of MMP (TIMPs): TIMP-1 inhibits MMP-1, MMP-3, MMP-7, and MMP-9, TIMP-2 inhibits especially MMP-2, and TIMP-3 can inhibit both the gelatinases, while TIMP-4 inhibits MT-1 MMP and MMP-2 activity [14].

In literature, there is no definite information regarding the effects of the oxidative stress on MMPs expression and activity in MS, even if classical cardiovascular risk factors, such as dyslipidemia and diabetes mellitus, increase the gelatinase levels via oxidative stress. In fact, the exposure of endothelial cells to oxidized LDL increases the levels of MT-1 MMP mRNA [37] and the treatment of monocytes-derived macrophages with oxidized HDL induces ROS production, release of TNF-α, and an overexpression of MMP-2 and MMP-9 [38]. Also the effects of hyperglycemia on MMP-9 and MMP-2 activity in cultured cells are mediated by ROS [39–41].

Previously, we have focused on the oxidative status [6, 7, 9] and the profile of gelatinases and tissue inhibitors [11, 12] in MS subjects. In this study, our aim was to evaluate, in a group of MS subjects, some parameters of the oxidative status, MMP-2, MMP-9, and their tissue inhibitors in order to investigate their statistical correlations.

2. Materials and Methods

We examined 65 subjects (41 men and 24 women; median age 51 yrs; interquartile range 12) selected from those referred to our observation from 2008 to 2011. MS was defined following the International Diabetes Federation (IDF) criteria [1]. The subjects were subsequently subdivided into diabetics (DMS) (22 men and 11 women; median age 59 yrs; IQR 7) and nondiabetics (NDMS) (19 men and 13 women; median age 46 yrs; IQR 9). The baseline characteristics of the two subgroups of DMS and NDMS subjects are described in Table 1. No subjects of both subgroups were taking antioxidants or practising exercise regularly. In the DMS subgroup, only 4 subjects were current smokers while 29 subjects were nonsmokers; in the NDMS subgroup, 11 subjects were current smokers while 21 were nonsmokers. Neither DMS nor NDMS subjects were heavy drinkers. In the subgroup of DMS subjects, diabetes had duration less than 5 years and was treated with diet and oral antidiabetic agents. In all participants, cholesterol and triglycerides were measured by standard enzymatic procedures, HDL-cholesterol after phosphotungstic acid/magnesium chloride precipitation and enzymatic determination of cholesterol, and LDL-cholesterol by the Friedewald formula.

Table 1: Medians (interquartile ranges) of the anthropometric profile, blood pressure values, and metabolic pattern in the whole group of MS subjects and in the two subgroups of MS subjects, respectively, with and without diabetes mellitus.

	All MS patients	Diabetic MS patients	Nondiabetic MS patients
Waist circumference (cm)	105 (14.5)	113.0 (16)	100.5 (8)§
BMI (Kg/m^2)	31.95 (4.71)	33.2 (4.4)	31.4 (4.2)
SBP (mmHg)	130 (20)	140 (27.5)	130 (15)$^{\#}$
DBP (mmHg)	80 (7.5)	80 (10)	80 (5)
Glycaemia (mg/dL)	101.5 (40.5)	130.5 (80)	91 (13)§
Total cholesterol (mg/dL)	207 (74)	186 (50)	227.5 (60)§
HDL-cholesterol (mg/dL)	40 (15)	41 (17)	38 (11.5)
LDL-cholesterol (mg/dL)	127.6 (53.6)	109 (47.8)	145 (55.5)§
Triglyceridemia (mg/dL)	180 (84)	159 (65)	191.5 (115)$^{\#}$
Triglyceridemia/HDL cholesterol	4.31 (3.31)	3.74 (2.41)	5.11 (2.66)$^{\#}$

$^{\#}P < 0.05$ and $^{\S}P < 0.001$ versus diabetic MS patients (Mann-Whitney test).

In this group of MS subjects we examined on fasting venous blood the following.

(i) *Lipid Peroxidation*. The oxidation of polyunsaturated fatty acids was evaluated in plasma by detection of the TBARS, generated by peroxidative processes, which include lipid peroxides and MDA. The evaluation of TBARS was made by fluorimetry, using 1,1,3,3-tetramethoxypropane as standard [42].

(ii) *Protein Oxidation*. The protein carbonyl (PC) content was measured by an enzyme-linked immunosorbent assay (ELISA) kit (BioCell PC test kit, Enzo Life Sciences AG, Switzerland), which uses the classic PC reagent 2,4-dinitrophenylhydrazine (DNP). In brief, plasma samples were incubated with DNP, and then plasma proteins were nonspecifically adsorbed on an ELISA plate. Unconjugated DNP and nonprotein constituents were washed away. The adsorbed proteins were probed with biotinylated anti-DNP antibody, followed by streptavidin-linked horseradish peroxidase. A chromatin reagent was added, and the reaction was stopped by adding an acid solution. Absorbance for each well was measured at 450 nm and related to a standard curve prepared for serum albumin containing increasing proportions of hypochlorous acid-oxidized protein, calibrated colourimetrically. Total protein concentration in plasma

Table 2: Medians (interquartile ranges) of gelatinase and inhibitor plasma concentrations in normal controls, in the whole group of MS patients, and in the two subgroups, respectively, with and without diabetes mellitus.

	Control subjects	All MS patients	Diabetic MS patients	Nondiabetic MS patients
TBARS (nmol/mL)	5.71 (2.28)	8.83 (0.70)‡	8.99 (0.71)‡	8.73 (0.665)‡
PC (nmol/mg prot.)	0.470 (0.125)	0.880 (0.240)‡	0.880 (0.240)‡	0.880 (0.220)‡
TAS (mmol/L)	0.910 (0.330)	0.880 (0.300)	0.910 (0.150)	0.720 (0.395)†§
NO_x (μmol/L)	28.05 (24.65)	74.80 (26.52)‡	77.10 (24.20)‡	67.70 (43.92)‡
MMP-2 (ng/mL)	29.19 (6.07)	44.50 (10.46)‡	47.84 (10.76)‡	39.69 (9.75)‡§
MMP-9 (ng/mL)	53.17 (6.57)	105.6 (36.15)‡	127.3 (28.7)‡	91.50 (11.61)‡§
TIMP-1 (ng/mL)	30.99 (2.63)	73.47 (10.31)‡	75.66 (8.65)‡	68.45 (9.35)‡§
TIMP-2 (ng/mL)	87.27 (6.28)	98.77 (14.38)‡	99.79 (12.86)‡	95.21 (15.32)$^{\dagger\#}$
MMP-2/TIMP-2	0.354 (0.080)	0.449 (0.130)‡	0.460 (0.104)‡	0.391 (0.141)$^{\dagger\#}$
MMP-9/TIMP-1	1.720 (0.150)	1.480 (0.345)	1.658 (0.339)	1.347 (0.298)†§

$^{*}P < 0.05$, $^{\dagger}P < 0.01$, and $^{\ddagger}P < 0.001$ versus control subjects (Mann-Whitney test).
$^{\#}P < 0.01$ and $^{\S}P < 0.001$ versus diabetic MS patients.

samples was evaluated by the method of Lowry et al. [43].

(iii) *Nitric Oxide Metabolites (NO_x).* The NO production was evaluated by a micromethod, which measures the concentration of the NO metabolites, nitrite and nitrate (NO_x). *In vivo* NO has a very short half-life (less than 0.1 sec) and it is converted, through different biochemical pathways, into nitrite, which has a half-life of a few minutes, and the more stable nitrate. Plasma concentrations of nitrate are 90–99% of the total NO metabolites concentration, indicated as NO_x. In the laboratory method adopted by us, nitrate was first converted into nitrite by a nitrate reductase; then nitrite was assessed by spectrophotometry after addition of the Griess reagent [44].

(iv) *Total Antioxidant Status (TAS).* TAS was obtained using an Assay kit (Calbiochem, La Jolla, CA, USA) which relies on the ability of plasma antioxidant substances to inhibit the oxidation of 2,2-azino-bis(3-ethylbenzthiazoline sulfonic acid) (ABTS) to the radical cation $ABTS^{\bullet+}$ by a peroxidase [45]. The radical concentration was measured by spectrophotometry.

(v) *Gelatinases and Their Inhibitors.* Plasma concentrations of gelatinases (MMP-2 and MMP-9) and their inhibitors (TIMP-1 and TIMP-2) were determined using, respectively, the Human MMP-2 ELISA and Human MMP-9 ELISA kit (Boster Biological Technology, Ltd.) and the Human TIMP-1 ELISA and Human TIMP-2 ELISA kit (Boster Biological Technology, Ltd.).

The same parameters have been examined in a group of 17 normal subjects (10 men and 7 women; median age 38 yrs; IQR 4), selected from the hospital staff. In this group of control subjects the basal glucose level was 89 (7) mg/dL, total cholesterol level was 200 (40) mg/dL, LDL-cholesterol was 142 (24) mg/dL, HDL-cholesterol was 46 (9) mg/dL, and triglycerides were 65 (36) mg/dL. The mean values of blood pressure in these subjects were 125 (10)/75 (5) mm/Hg; BMI was 26 (4); waist circumference was 98 (13) cm.

The Ethical Committee approved the study and each subject gave informed consent.

3. Statistical Analysis

The results were expressed as medians and interquartile ranges (IQR); the differences between MS subjects and normal controls as well as the differences between normal controls and subjects with MS subdivided in agreement with the presence or not of diabetes mellitus were estimated according to the Mann-Whitney test. The study of correlations was performed employing the Spearman rank correlation coefficient.

4. Results

Examining the baseline characteristics of subjects with MS we observed a significant decrease in waist circumference ($P < 0.001$), systolic blood pressure ($P < 0.05$), and basal glucose level ($P < 0.001$) and a significant increase in total cholesterol ($P < 0.001$), LDL-cholesterol ($P < 0.001$), triglycerides ($P < 0.05$), and triglycerides/HDL-cholesterol ($P < 0.05$) in NDMS subjects in comparison with DMS subjects (Table 1).

MS subjects showed an increase in lipid peroxidation, protein oxidation, and nitric oxide metabolites (NO_x) (Table 2). In the same group of MS subjects, we found an increase in MMP-2, MMP-9, TIMP-1, and TIMP-2 in comparison with normal subjects; we also observed an increase in MMP-2/TIMP-2 ratio, with no difference regarding MMP-9/TIMP-1 ratio (Table 2). Subdividing the MS group in the two subgroups, we found that the increase in lipid peroxidation, protein oxidation, and NO_x was similar in DMS and NDMS subjects, while the decrease in TAS was significantly evident only in NDMS subjects, in comparison with normal and DMS subjects (Table 2). We observed also that the plasma concentrations of MMP-2, MMP-9, TIMP-1, and TIMP-2

were significantly increased in the two subgroups in comparison with normal subjects, but in MS subjects with DM the values were higher than in nondiabetics (Table 2). The MMP-2/TIMP-2 ratio was significantly increased in the two subgroups in comparison with normal subjects although its value was lower in NDMS than in DMS subjects (Table 2). The MMP-9/TIMP-1 ratio instead was significantly decreased in MS subjects without DM, not only in comparison with normal subjects but also in comparison with DMS subjects (Table 2).

In normal controls, as well as in the two subgroups of subjects with MS, no statistical correlation was observed among age, parameters of oxidative status, gelatinases, and tissue inhibitors. Examining the linear regression among TBARS, gelatinases, and their inhibitors, we found a negative correlation between TBARS and MMP-2 in DMS subjects (Table 4). No correlation among carbonyl groups, gelatinases, and their inhibitors was evident (Tables 3, 4, and 5) and no relationship among NO_x, gelatinases, and their inhibitors was observed (Tables 3, 4, and 5). A positive correlation between TAS and MMP-2, TAS and MMP-9, and TAS and MMP-9/TIMP-1 ratio in the entire group of MS subjects was found (Table 3).

Table 3: Correlations between oxidative parameters and gelatinases in all MS subjects.

	Versus TBARS	Versus PC	Versus TAS	Versus NO_x
MMP-2	−0.162	−0.043	0.255[#]	−0.077
MMP-9	0.176	−0.025	0.315[#]	0.075
TIMP-1	0.094	−0.067	−0.052	−0.012
TIMP-2	0.126	−0.199	0.067	0.071
MMP-2/TIMP-2	−0.206	0.035	0.196	−0.100
MMP-9/TIMP-1	0.133	−0.017	0.320[§]	0.097

[#]$P < 0.05$; [§]$P < 0.01$ (Spearman's rank correlation).

Table 4: Correlations between oxidative parameters and gelatinases in diabetic MS subjects.

	Versus TBARS	Versus PC	Versus TAS	Versus NO_x
MMP-2	−0.363[#]	−0.240	−0.235	−0.144
MMP-9	0.180	−0.144	−0.057	0.295
TIMP-1	0.002	0.000	−0.287	0.079
TIMP-2	0.224	−0.266	0.148	0.287
MMP-2/TIMP-2	−0.283	−0.100	−0.263	−0.149
MMP-9/TIMP-1	0.167	−0.136	0.028	0.216

[#]$P < 0.05$ (Spearman's rank correlation).

Table 5: Correlations between oxidative parameters and gelatinases in nondiabetic MS subjects.

	Versus TBARS	Versus PC	Versus TAS	Versus NO_x
MMP-2	−0.346	0.097	0.207	−0.167
MMP-9	−0.041	−0.021	0.236	−0.163
TIMP-1	0.055	−0.174	−0.289	−0.167
TIMP-2	−0.067	−0.154	−0.193	−0.140
MMP-2/TIMP-2	−0.280	0.195	0.316	−0.077
MMP-9/TIMP-1	−0.104	0.084	0.317	−0.065

5. Discussion

The results of this research confirm all the data previously published by us [7, 9, 10, 12]: the parameters of the oxidative stress distinguish DMS from NDMS subjects and are altered even in middle-aged MS subjects [10]. Also the gelatinases and their inhibitors discriminate DMS from NDMS subjects and in fact their means were significantly higher in MS diabetic subjects.

Our goal was in particular the evaluation of the statistical correlations between the parameters of the oxidative status and the gelatinases and their tissue inhibitors in MS.

It has been observed that ox-LDLs upregulate MMP-9 expression and reduce TIMP-1 expression in monocyte-derived macrophages [46] and that MDA, which is included in TBARS, is correlated with the MMP-9 activity in subjects with acute coronary syndrome [27]. In this study however the TBARS that reflects lipid peroxidation was negatively correlated with MMP-2 only in DMS subjects.

No correlation was observed among protein oxidation, gelatinases, and their inhibitors, although in experimental models [35] a significant correlation between carbonyl groups and MMP-9 has been described.

We noted especially a positive correlation between TAS and MMP-2, between TAS and MMP-9, and between TAS and MMP-9/TIMP-1 ratio in the entire group of MS subjects. As it is known, TAS includes enzymatic antioxidants (superoxide dismutase, catalase, and glutathione peroxidase) and nonenzymatic antioxidants (uric acid, ascorbic acid, bilirubin, vitamin E, and carotenoids). The examination of the literature data regarding the correlations between antioxidants and MMPs profile shows controversial aspects. In fact, MMP-9 plasma levels are negatively associated with provitamin A carotenoids in a general population [47] while, in experimental models, the deficiency of vitamin A seems to be responsible for a gelatinase decrease without any variations of TIMPs [48]. In subjects with acute stroke the infusion of uric acid induced a decrease of total and active MMP-9 levels [49] and the treatment with antioxidants (polyethylene glycol-superoxide dismutase and N-acetyl-L-cysteine) reduces the MMP-9 activity in plasma and in aortic tissue homogenates of experimental models of diabetes mellitus [39] and the use of tempol (a ROS scavenger) reduces MMP-2 levels and its activity in aortic rings of animal models of renovascular hypertension [50]. Even in experimental models of oxidative stress (obtained with the depletion of glutathione), taurine inhibits MMP-2 activation in cardiac tissues [51]. Differently, the treatment with retinoic acid increases significantly MMP-9 but not MMP-2 [48] and lutein, a carotenoid, enhances MMP-9 synthesis in animal models [52]. Therefore, all these studies do not clarify how in subjects with MS the TAS could be positively related to the gelatinases and their tissue inhibitors and then all these data need further investigation.

With regard to the behaviour of NO_x, its increase in MS is related especially to a nitric oxide overproduction by macrophages, in which the NO synthase activation is caused by cytokines, such as TNF-α and IL-1β [53–55] that are able to induce also MMPs expression [56–58]. In this study, no statistical correlation was observed among NO_x, gelatinases and their inhibitors in the whole group and in the two subgroups of MS subjects. Keeping in mind that NO production and gelatinases expression are induced by the same cytokines that are increased in MS [59], the inflammatory state could be the link between oxidative stress and MMPs. In addition, it must be considered that, during an inflammatory response, leukocyte infiltration through basal membranes is only possible if these cells produce enzymes that can degrade the extracellular matrix so MMPs, as well as ROS, are crucial effector molecules of inflammatory cells, which play a sure role in atherosclerosis and other chronic inflammatory and metabolic diseases [60].

6. Conclusions

There are several data regarding the influence of the oxidative status on the gelatinases and their tissue inhibitors. In this preliminary study concerning a small group of MS subjects, we observed a significant alteration of all these parameters, although from the statistical analysis of the data it is difficult to clarify how the oxidative stress could influence the plasma levels of the gelatinases and their inhibitors. Further investigation seems to be necessary, considering the impact of MS on cardiovascular morbidity and mortality and especially the opportunity of specific therapeutic strategies.

Conflict of Interests

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

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Review Article

Guidance Cue Netrin-1 and the Regulation of Inflammation in Acute and Chronic Kidney Disease

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Acute kidney injury (AKI) is a common problem in the hospital setting and intensive care unit. Despite improved understanding, there are no effective therapies available to treat AKI. A large body of evidence strongly suggests that ischemia reperfusion injury is an inflammatory disease mediated by both adaptive and innate immune systems. Cell migration also plays an important role in embryonic development and inflammation, and this process is highly regulated to ensure tissue homeostasis. One such paradigm exists in the developing nervous system, where neuronal migration is mediated by a balance between chemoattractive and chemorepulsive signals. The ability of the guidance molecule netrin-1 to repulse or abolish attraction of neuronal cells expressing the UNC5B receptor makes it an attractive candidate for the regulation of inflammatory cell migration. Recent identification of netrin-1 as regulators of immune cell migration has led to a large number of studies looking into how netrin-1 controls inflammation and inflammatory cell migration. This review will focus on recent advances in understanding netrin-1 mediated regulation of inflammation during acute and chronic kidney disease and whether netrin-1 and its receptor activation can be used to treat acute and chronic kidney disease.

1. Introduction

Acute kidney injury (AKI) has now replaced the old terminology acute renal failure. Clinically AKI is defined as a rapid decline in kidney function resulting in failure to maintain fluid, electrolyte, and acid-base homoeostasis. The incidence of AKI is increasing which is further complicated by lack of effective therapies to reduce or prevent it from happening. AKI has a frequency of 1–9% in hospital inpatients and over 40% in critically ill patients in the intensive care units if sepsis is present [1–3]. Similarly, chronic kidney disease due to diabetes contributes to a significant amount of mortality and morbidity. In the United States, approximately 20 million people or 7% of the population are estimated to have diabetes and the incidence of diabetes is growing. Diabetes has become the primary cause of end-stage renal disease (ESRD). Approximately 44% of new patients entering dialysis in the USA are diabetics [4, 5]. Studies in animals and human suggested that acute and chronic kidney diseases are inflammatory disease and inflammatory mediators play a major role in tissue injury seen in both forms of kidney disease [6–14].

Inflammation is defined as a cellular response to injurious stimulus which is classified into two broad categories: (1) nonsterile inflammation and (2) sterile inflammation. Nonsterile inflammation usually occurs during infection whereas sterile inflammation usually occurs without infection but during tissue injury due to surgery, metal toxicity, ischemia, drugs, or chemicals. Similar to nonsterile inflammation in response to infection, sterile inflammation also exhibits a similar manifestation such as vasodilation, edema, leukocyte infiltration into the tissues, and cellular damage by apoptosis

and necrosis [15, 16]. However, the initial events that elicit and control the response can be very different between sterile and nonsterile inflammation. The dying and dead cell often release intracellular contents that are not usually exposed to immune systems such as ATP, uric acid, heat shock proteins, high mobility group of proteins, nucleic acid, and many others [17–21] which may act as ligands for pattern recognition receptors on the cell surface of innate immune system and adjacent cells causing activation of those cells. Activated innate immune cells and adaptive immune cells release cell damaging reactive oxygen and nitrogen species, proteases, and cytokines [15, 22]. Although these damaging molecules are beneficial during infection to clear pathogen and during tissue regeneration process or wound healing, uncontrolled release of these molecules during early stages of tissue injury often causes excessive damage to normal tissue which can lead to further reduction in organ function [7, 12–14, 23].

Cells have defensive protective mechanism often activated in parallel with the inflammatory response to counteract the damaging effects of innate immune cells. These cytoprotective molecules include anti-inflammatory cytokines (IL-10 and TGFβ1), neuronal guidance cues netrins, adenosine, heme oxygenase, and others. Inadequate response or downregulation of these counteracting pathways may exacerbate inflammatory response and tissue injury. But at the same time, complete suppression of the inflammatory response is detrimental to system survival as it may experience susceptibility to infection or reduced capacity to regenerate injured tissues.

2. Inflammation in Acute Kidney Injury

Animal and human studies suggest that AKI such as ischemic kidney disease and cisplatin induced kidney damage is inflammatory disease mediated by both innate and adaptive immune systems [12–14, 24, 25]. The role of immune cells in ischemia reperfusion injury of the kidney has been known for more than a decade. Using gene knockout, specific cell depletion, adoptive transfer of immune cells, and generation of bone marrow chimera model, the role of specific immune cell type in ischemia reperfusion injury was determined. These studies indicated the involvement of both the adaptive and innate immune systems in AKI [7, 12, 26–31]. Most components of innate (macrophage, neutrophils, and NKT) [26, 30, 32] and adoptive ($CD4^+$ T cells and B cells) [31, 33] immune systems have been shown to mediate ischemia reperfusion injury of the kidney. Depletion of macrophage [30, 34], neutrophils [35], CD4 T cells [31], B cells [33], or NKT cells (with NK1.1 mAb) [26] is protective against ischemia reperfusion injury of the kidney, whereas depletion of regulatory T cells (Treg) exacerbates AKI [36]. Recent studies suggest that the administration of Treg or adenosine treated dendritic cells before ischemia is also protective [36, 37]. In contrast to the above observations, opposing views on the role of adaptive immune systems in ischemia reperfusion injury have also been documented [38, 39]. For example, depletion of neutrophils in T and B cell deficient mice is not protective against ischemia reperfusion injury of the kidney [39]. Similarly, dendritic depletion is protective against ischemia reperfusion injury of the kidney [25], whereas dendritic cell depletion exacerbates cisplatin induced AKI [40]. Many of T cell, macrophage, and neutrophil secreted cytokines (IL-6, TNFα, IL-17, and IFNγ) [14, 26, 41, 42] and chemokines (MCP-1, KC, and MIP-2) [9, 43, 44] are also shown to mediate renal ischemia reperfusion injury and cisplatin induced AKI. In addition, toll-like receptors 2 and 4 are shown to be critical mediators of AKI [10, 29]. The conclusion drawn from these studies is that every cell type of immune system or their secreted components (cytokines and chemokines) play a critical role in mediating ischemia reperfusion injury of the kidney (Figure 1). The challenge is to translate these findings into useful therapies in human. Depletion of all cell types of immune systems for therapeutic purpose will be difficult and also may leave the patients with immune deficiency. Depletion of one specific cell type may not be protective or may offer only marginal protection. Moreover, in most of these studies in animal models of AKI, immune cell depletion was performed prior to reperfusion injury. However, in the hospital setting this is not possible as AKI development is difficult to predict but can be diagnosed early once it develops. Therefore, effectiveness of depletion after reperfusion injury is initiated has never been studied. Moreover, in the model system used for ischemia reperfusion injury such as bilateral renal pedicle clamping, injury happens so rapidly and depletion of immune cells after initiation of injury could be problematic. Recent animal study suggests that TLR4 ligand HMGB1, cytokines such as IL-1α and -β, IFNγ, KC, GM-CSF, MIP-1α, and VEGF peak as early as 1 minute after reperfusion [45]. In addition, recent studies also suggest that AKI is a systemic inflammatory disease that affects other organs as well. Therefore, therapy based on molecules which regulate inflammation by suppressing immune cell activation and migration into injured organs will be effective in treating ischemic kidney disease and other forms of AKI. Moreover, these molecules will suppress inflammation and leave immune system intact; thereby possible immunodeficiency will be avoided. However, knowledge on such molecules which can negatively regulate innate and adaptive immune systems is lacking. Understanding such molecule, like netrin-1, will provide new direction to treat inflammatory ischemic kidney disease. This review will focus on one such counteractive anti-inflammatory pathway netrin-1 and its receptor UNC5B in the regulation of inflammation during acute (ischemic, drug induced) and chronic (diabetes) kidney injury.

3. Netrins and Their Receptors

The name netrin-1 is derived from Sanskrit Netr, meaning "one who guides." Studies in the nematode *Caenorhabditis elegans* identified genes required for circumferential axon guidance [46, 47]. One of the genes identified, *unc*-6, encoded a secreted protein with sequence homology to laminins [48]. In 1994, using commissural axon outgrowth from explants of embryonic rat dorsal spinal cord as a functional assay,

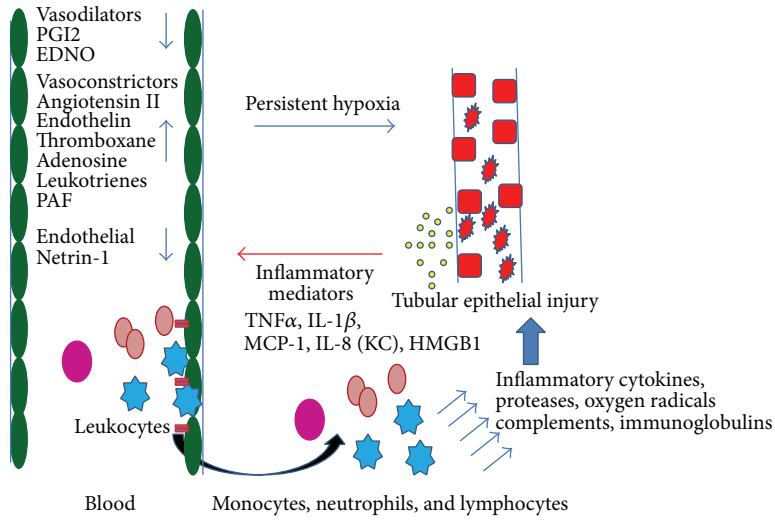

Figure 1: Inflammation in acute kidney injury. Ischemia reperfusion or nephrotoxin causes tubular epithelial stress or necrosis which releases inflammatory mediators. These inflammatory mediators activate endothelial cells and expression of adhesion molecules (II) such as ICAM-1 induces leukocyte migration into the interstitium and release of vasoconstrictor. At the same time inflammation downregulates anti-inflammatory molecules such as netrin-1 and endothelial nitric oxide. Infiltrated white blood cells such as neutrophils, monocyte, and T cells release cytokine, chemokines, proteases, and oxygen radicals cause further damage to the epithelium. All these events lead to vasoconstriction and persistent hypoxia which contributes to further tissue damage.

two proteins were purified from homogenates of embryonic chick brain and discovered to be homologous to UNC-6 [49]. They were named netrin-1 and netrin-2. Netrin-1 is a laminin-related molecule initially discovered as a diffusible molecule produced by a ventral structure in the developing spinal cord, the floor plate, which attracts commissural axons [49]. Netrin-1 was thus shown to act as a chemoattractive or chemorepulsive cue for many migrating axons and neurons during the development of the nervous system [50, 51].

Five netrins have been identified in vertebrates so far: netrin-1 has been identified in chicken [52], mouse [53], *Xenopus* [54], zebrafish [55, 56], and humans [57]; netrin-2 in chickens [52]; and netrin-3 in humans (NTN2L) [58] and mouse [59]; netrin-4 in mouse, human, rat, *Xenopus*, and chicken [60]. In addition, two glycosylphosphatidyl inositol- (GPI-) modified, membrane anchored netrin family members (netrin G1 and netrin G2) were discovered. The membrane-anchored netrin Gs are reported to have evolved independently of the secreted netrins [61, 62] and are found only in vertebrates. All netrins are composed of approximately 600 amino acids and have a molecular mass of approximately 70 kilodaltons. Netrins 1, 2, 3, and 4 are all structurally related to the short arms of laminin γ chains and contain a laminin VI domain and three EGF like repeats similar to the laminin V domain (V-1, V-2, and V-3); they also contain a positively charged heparin-binding COOH-terminal domain termed domain C [49, 63].

Netrin-1 effect is regulated by the interaction with its main receptors, deleted in colorectal cancer (DCC) [53, 63] and uncoordinated family member 5 (UNC5A-D or UNC 5H 1–4) [64, 65] (Figure 2). In addition, recently, four additional receptors have been identified which include Down syndrome cell adhesion molecule (DSCAM) [66], integrin $\alpha 3\beta 1$ and $\alpha 6\beta 3$ [67, 68], cerebellin 4 (CBLN4) [69] (Figure 2), and a controversial receptor adenosine receptor (A2bR) [70]. Structurally, netrin-1 resembles the extracellular matrix protein laminin and is thought to have derived from the laminin γ chain. Netrin-1 comprises a globular domain (VI) at the amino terminus which is followed by three epidermal growth factor (EGF) repeats, namely, V1, V2, and V3. Domains VI and V bind to the fibronectin type III domains of DCC and immunoglobulin domains of UNC-5 families of netrin-1 receptors [65], which are followed by the positively charged C terminal domain (C). Domain V is the most highly conserved across the netrins. Given the homology between DCC and neogenin, it is likely that netrin-1 also binds the fibronectin type III domains of neogenin. However, recent studies suggest that neogenin has specific ligand called repulsive guidance molecule (RGM) [71]. Netrin-4 is also known to bind neogenin [72]. Netrin-1 C-terminal domain contains the integrin recognition sites including the RGD motif recognized by $\alpha 3\beta 1$. The C domain also contains binding sites for membrane glycolipids and extracellular matrix components including heparan sulfate proteoglycans [73].

Netrin-1 signaling is complex and is not always attractive or stimulatory. Binding of netrin-1 to DCC induces axonal attraction [70], whereas binding to the UNC5 receptor family causes repulsion [64, 74]. Binding of netrin-1 to DCC and UNC5B induces activation of multiple pathways which include MAPKs, PKC, src, PI3 kinase, Rac and Rho kinase, focal adhesion kinase, and many others (Figure 2) [60, 65, 75, 76].

Netrin-1 receptors DCC and UNC5B are also called dependence receptors. They transmit signal even in the absence of ligand. When the ligand is available, these

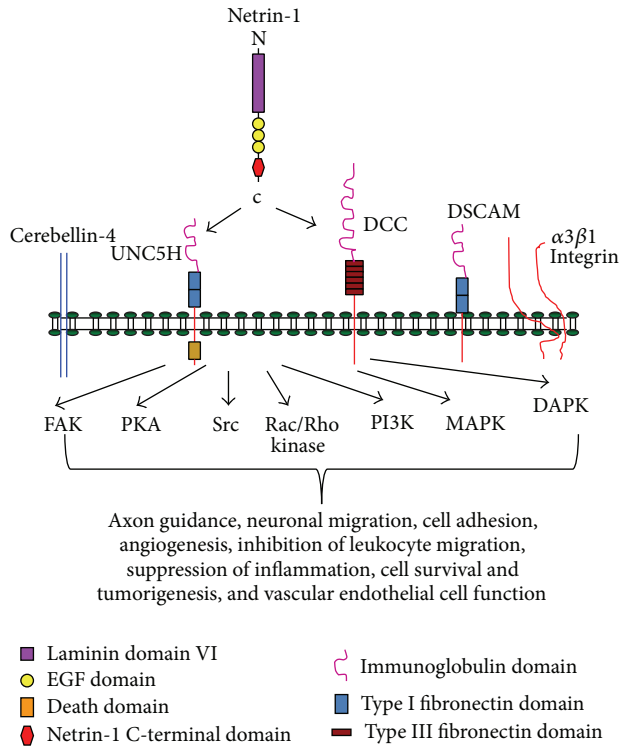

Figure 2: Netrin-1 receptor signaling and known functions. Netrin-1 is known to bind several receptors which include uncoordinated 5 H (UNC5H1-4), deleted in colorectal cancer (DCC), down syndrome cell adhesion molecule (DSCAM), integrins ($\alpha 3\beta 1$ & $\alpha 6\beta 3$), and cerebellin-4. Binding of netrin-1 to these receptors is known to activate several signaling pathways including focal adhesion kinase (FAK), protein kinase A (PKA), src kinase, Rac/Rho kinase, phosphor inositol 3-phosphate activated kinase (PI3K), and mitogen activated kinase (MAPK). Death associated protein kinase (DAPK) activation occurs in the absence of ligand binding. Activation of these pathways was associated with changes in cellular function listed in the figure.

receptors transduce a positive signaling pathway leading to cellular proliferation, differentiation, migration, or survival. However, in the absence of their ligand, these receptors are not inactive, like "classical" receptors, but rather induce a "negative signaling" that triggers caspase dependent apoptotic cell death [77, 78]. DCC has classical death domain but not UNC5B. However, both receptors are capable of inducing apoptosis in the absence of ligand netrin-1. Dependence receptor concepts and activity are described in detail elsewhere [76, 77, 79, 80]. In contrast to the secreted netrins, netrin-Gs bind transmembrane proteins called the netrin-G ligands (NGL) and netrin-Gs do not appear to interact with DCC, neogenin, or the UNC5 proteins [61, 81].

Mouse kidney expresses netrin-1, netrin-3, and netrin-4 and their receptors UNC5B, UNC5C, and neogenin [82, 83]. The expression of another receptor mRNA is minimal or negligible. Immunolocalization studies had determined that DCC expression is not detectable in kidney epithelium (unpublished, Ramesh G), whereas UNC5B is expressed only in proximal tubular epithelial cells and vascular endothelial cells. Neogenin is expressed in all segments of nephron but in the basolateral surface similar to netrin-4 localization [82, 83]. UNC5C is localized in the distal tubular epithelium [82]. Similarly, the expression of netrin-1 is undetectable in immune cells, whereas UNC5B and to some extent UNC5A expression but not other receptor expressions were detected in immune cells [13]. The expression of integrins and DSCAM is not determined in the kidney.

4. Regulation of Inflammation by Netrin-1 in Acute Kidney Injury

Netrin-1 was identified as neuronal guidance cue, directing neurons and their axons to targets during development of the nervous system [84]. While netrin-1 is primarily thought of as an axon guidance cue, guidance is unlikely to be its only function since expression studies have shown that netrins are widely expressed outside the nervous system including vascular endothelial cells [83, 85]. Vascular endothelial cells play critical barrier for leukocyte activation and migration into organs by producing repellent factors to leukocytes such as netrin-1. Migration of inflammatory cells to the site of injury is a critical cellular response to initiate the removal of dead cells and induce a regeneration response. However, inappropriate excessive activation and migration of these cells into organs can also result in tissue destruction, and this abnormal influx is thought to be the mechanism involved in many ischemic injuries of organs [13, 31, 86–89]. Barriers to aberrant immune cell activation and migration may exist, for example, the production of immunosuppressive, chemorepellent molecules from the endothelium, and epithelial cells. Downregulation of these chemorepellent factors such as netrin-1 during organ injury may exacerbate inflammation [83, 85, 88]. Initial evidence to support this idea came from in vitro studies. Ly et al. in 2005 [85] demonstrated that netrin-1 inhibits leukocyte migration in response to chemotactic signal. In the same study, it was also shown that sepsis downregulated netrin-1 in endothelial cells which may contribute to increased transendothelial migration of leukocyte. Receptor that mediates in vivo anti-inflammatory function of netrin-1 and whether netrin-1 is also effective in other disease models and tissues was unknown at that time.

Netrin-1 is expressed in many tissues including brain, lung, heart, liver, intestine, and kidney. Kidney is among the highest netrin-1-expressing organs studied [13, 83, 85]. Despite its high expression, the role of netrins in kidney physiology and pathophysiology was unknown. First clue came from animal model of ischemia reperfusion injury of the kidney. We proposed that endothelial netrin-1 represents yet another homeostatic protein whose dysregulation after ischemia contributes to the development of organ failure. Studies from our lab demonstrated some novel insights regarding the roles of netrins in IR injury of the kidney. I/R injury was associated with dramatic changes in abundance and localization of netrin-1. Within 3 h after reperfusion, netrin-1 protein expression was highly induced in tubular epithelial cells with a decrease in peritubular vasculature. By 24 h of IR, netrin-1 expression was seen only

in tubular epithelial cells. However, netrin-1 and netrin-4 mRNA were downregulated despite increased protein expression in epithelial cells at early hours after reperfusion. Moreover, the distribution of netrin-1 changed from being primarily endothelial to being primarily epithelial [83]. Although netrin-1 returned to normal by 48 h after reperfusion, netrin-4 mRNA was persistently downregulated. Similarly, ischemia reperfusion downregulated circulating netrin-1 levels. Moreover, endothelial cells that are subjected to hypoxia also downregulated netrin-1 expression [83]. The functional significance of these changes was assessed by administering recombinant netrins intravenously to mice before I/R injury. Netrin-1 exerted a dose-dependent protection against renal dysfunction [83]. Interestingly, netrin-1 administration reduced neutrophil infiltration into the kidney and the expression of cytokines and chemokines in the kidney. Netrin-4 infusion had no effect on renal function or injury. Since exogenous netrin-1 administration was protective, we speculated that the observed loss of netrin-1 in endothelial cells, rather than increase in epithelial netrin-1, contributes to ischemia reperfusion injury. Since netrin-1 mRNA did not increase in concert with protein abundance, netrin-1 production by epithelial cells may be regulated at the translational level. Subsequently, another study reported a similar anti-inflammatory effect of netrin-1 using whole body hypoxia model [88] and ischemia reperfusion injury of the kidney using a partial netrin-1 knockout (heterozygous knockout) [90]. Interestingly, proximal tubular epithelial cell specific overexpression also protected kidney through suppressing epithelial cells apoptosis [91]. Subsequent studies from our laboratory and other laboratories had demonstrated that administration of netrin-1 reduced inflammation and tissue injury in many different disease models such as acute colitis [92, 93], acute lung injury [94], peritonitis [95], and cisplatin induced AKI [96].

5. Regulation of Inflammation by Netrin-1 in Chronic Kidney Disease

Diabetic nephropathy is a complex chronic disease involving interactions between kidney cells (i.e., endothelial cells, podocytes, and epithelial cells) and immune cells. Its course is accelerated by failure of endogenous renal protective mechanisms in the chronic diabetic milieu. Between 30 and 50% of prevalent patients who are on hemodialysis (HD) have elevated serum levels of inflammatory markers such as C-reactive protein and IL-6 [97, 98]. In some patients, this elevation is chronic, and in some, it is intermittent and generally is associated with breakthrough processes. Furthermore, on many occasions, HD sessions trigger inflammation in a way that is not always identifiable with the conventional markers. Inflammatory markers are powerful predictors of mortality after adjustment for other risk factors [97, 99]. Inflammation also is responsible for other mortality risk factors, such as anemia, malnutrition, vascular disease, and left ventricular hypertrophy [97, 100]. The guidance cue netrin-1 and its receptor UNC5B represent an endogenous anti-inflammatory pathway that is widely expressed in kidney cells and immune cells. The regulation of netrin-1 and its receptor in the kidney during diabetes is not clearly defined. Moreover, its role in the regulation during dialysis is unknown. Previous studies from our lab determined that netrin-1 protein is induced in epithelial cells and excreted in urine during diabetes both in animal model and in humans [101, 102]. In vitro studies had shown that hyperglycemia downregulates netrin-1 expression whereas addition of high concentration of protein BSA induced netrin-1 in the same cells [103]. This induction appears to be a translational mechanism. In addition, diabetes downregulated circulating levels of netrin-1 in two different genetic models. Consistent with downregulation of plasma netrin-1, both netrin-1 and UNC5B mRNA were found to be significantly downregulated in diabetic kidney as compared to control (Figure 3). No change was seen for UNC5B, while netrin-1 protein was undetectable in macrophage RAW264.7 cells (not shown) with/without 30 mM glucose. These findings demonstrate that diabetes downregulates the endogenous netrin-1-UNC5B anti-inflammatory pathway systemically and in the kidney, while proteinuria may upregulate netrin-1 in renal epithelial cells in response to injury [103]. Moreover, overexpression of netrin-1 in tubular epithelial cells before the start of the disease process suppressed diabetes induced increase in infiltration of neutrophils and macrophages, chemokine expression, albuminuria, and tubular epithelial cell apoptosis in kidneys [104]. In addition, diabetes induced a large increase in the excretion of prostaglandin E2 (PGE2) in urine, which was suppressed in netrin-1 transgenic mice. Netrin-1-induced suppression of PGE2 production was mediated through suppression of NFκB-mediated cyclooxygenase-2 (COX-2) in renal tubular epithelial cells [104]. Using a different approach, Eunyong Tak et al. also showed the elevated levels of albuminuria, glomerular filtration rate, and severe loss of kidney function in partial netrin-1 deficient (Ntrn1 $\pm$ mice) diabetic mice, whereas administration of recombinant netrin-1 was associated with attenuated albuminuria and improved histological score for diabetic nephropathy compared to control mice [105]. These results suggest that netrin-1 is a major regulator of inflammation and apoptosis in diabetic nephropathy and may be a useful therapeutic molecule for treating chronic kidney diseases such as diabetic nephropathy.

6. UNC5B Receptor Mediates Netrin-1 Anti-Inflammatory Effects

Infiltration of neutrophils and monocytes is one of the hallmarks of tissue injury in ischemia reperfusion injury of the kidney. Neutrophils were shown to mediate renal ischemia reperfusion injury [26, 106, 107]. Cytokines, such as IL-17 and IFN-γ, produced by neutrophils are known to mediate renal ischemia reperfusion injury [26, 106]. Since only UNC5B receptor expression was seen in a significant amount in leukocytes, we [13] investigated the effect of UNC5B neutralization on netrin-1 mediated suppression of inflammation and chemokine and cytokine production in renal IR injury. The response of the innate immune

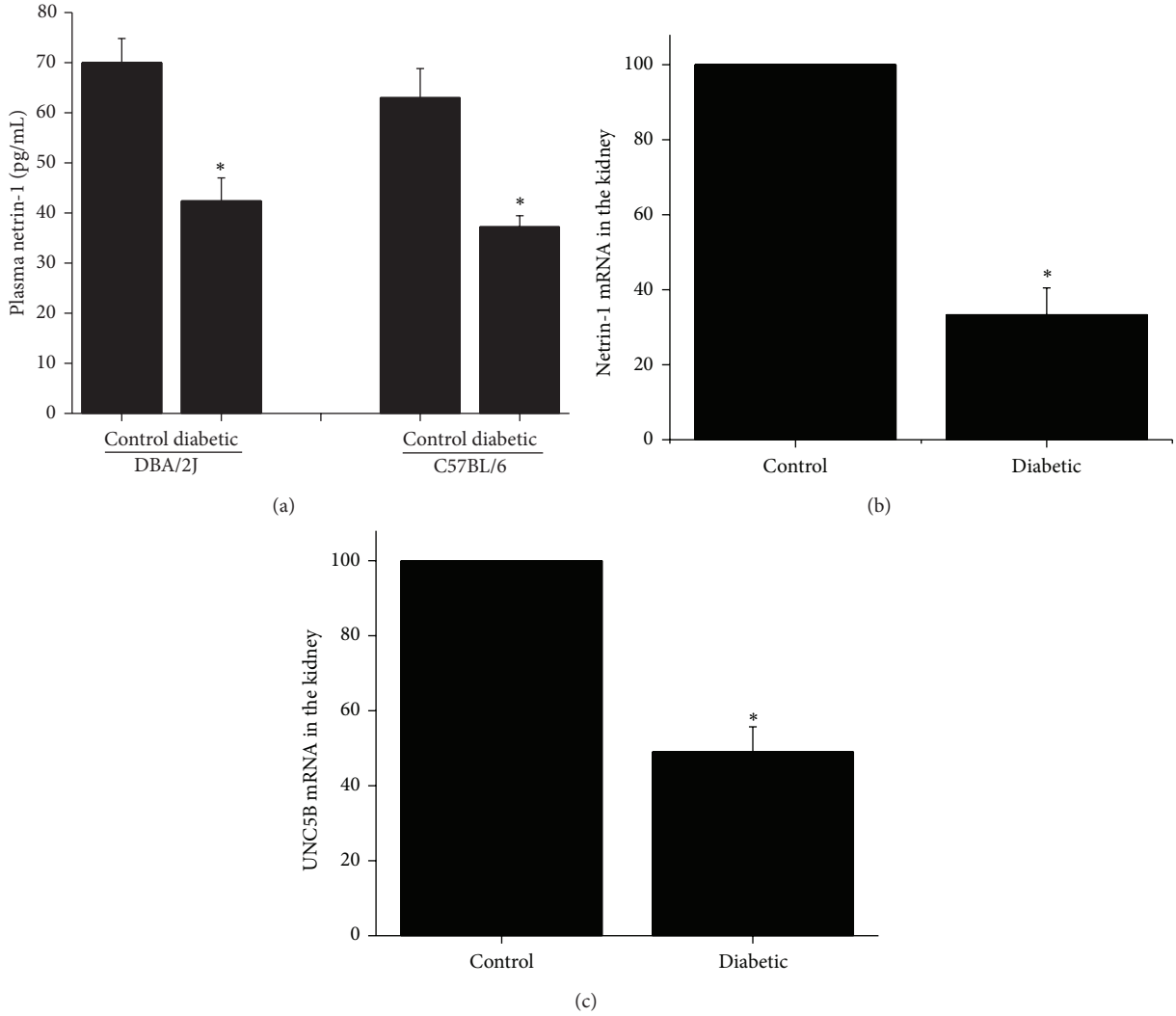

Figure 3: Diabetes downregulates endogenous anti-inflammatory protective pathways. (a) Diabetes (8 weeks after STZ administration) downregulated plasma netrin-1. $^*P < 0.001$ versus nondiabetic control. $n = 5$. Netrin-1 in plasma was quantified by ELISA. (b) and (c) RT-PCR analysis of netrin-1 and UNC5B expression in control and 12-week diabetic mouse. Diabetes downregulated netrin-1 (C) and UNC5B (G) expression in the kidney. $^*P < 0.001$ versus control. $n = 5$.

system to tissue injury is very rapid as seen by a rapid influx of monocytes and neutrophils into the kidney subsequent to renal IRI. Administration of recombinant netrin-1 before or after renal ischemia-reperfusion reduced kidney injury, apoptosis, monocyte and neutrophil infiltration, and cytokine (IL-6, IL-1β, and, TNFα) and chemokine (MCP-1, macrophage-derived cytokine, monokine-induced IFN-γ, keratinocyte-derived chemokine, and chemokine with 6 cysteines) production [13]. Analysis of different netrin-1 receptors expression on leukocytes showed very high expression of UNC5B but not UNC5C, UNC5D, neogenin, or deleted in colorectal cancer [13]. Expression of UNC5A was low. Neutralization of UNC5B receptor reduced netrin-1-mediated protection against renal ischemia-reperfusion injury and increased monocyte and neutrophil infiltration as well as serum and renal cytokine and chemokine production with increased kidney injury and renal tubular cell apoptosis. T cells and dendritic cells content in the kidney were not significantly changed with/without netrin-1 in response to ischemia reperfusion. However, still they may mediate reperfusion injury as shown in many excellent studies [25, 31, 108]. In contrast to the innate immune system, the activation of adaptive immune cells typically takes longer. However, Ag-independent activation of the adaptive immune system is possible [109] which will take only few minutes. For example, a hypoxic condition augments cytokine production in CD4 T cells [110]. CD4 T cell cytokines such as IL-6, IL-17, TNF-α, and IFN-γ contribute to the pathogenesis of IRI of kidney, liver, lung, and intestine [106, 111–114]. The CD4 T cell cytokines are also known to regulate the expression of other soluble mediators of inflammation and function of leukocytes, including monocytes and neutrophils. Investigation into netrin-1's effect on CD4 T cell stimulation showed suppression of Th1/Th2/Th17 cytokine (IL-2, IL-6,

IL-10, IL-13, IL-17, IFN-g, IL-4, and TNFα) production in vitro which was inhibited with UNC5B receptor neutralization [13]. These results had demonstrated that netrin-1 acting through UNC5B receptor reduces renal ischemia-reperfusion injury and its associated renal inflammation. This observation was also confirmed using tissue specific UNC5B knockout mice. Deletion of a single allele or proximal tubular epithelial cell specific deletion of both alleles of UNC5B exacerbated AKI due to ischemia reperfusion and cisplatin. Moreover, neutrophil infiltration and inflammatory cytokine production dramatically increased in the kidney suggesting that UNC5B signaling regulates injury and inflammatory response in AKI [115].

It was not clear whether netrin-1 mediated suppression of neutrophil and monocyte infiltration into the kidney and inhibition of cytokine production are due to its direct effect on neutrophils and monocytes or acting through T cells. This was clarified using a T and B cell deficient mice (RAG-1 knockout). Administration of recombinant netrin-1 to both WT and RAG-1 knockout mice protected kidney from IR injury in both WT and RAG-1 knockout mice by suppressing inflammation, infiltration of neutrophils, and apoptosis [116], suggesting that the effect of netrin-1 on neutrophil infiltration is independent of T and B cells.

7. Netrin-1 Regulates Inflammation through NFκB and COX-2/PGE2 Pathways

The signaling pathway through which netrin-1 suppresses cytokine production is not clear. Netrin-1 is known to activate adenylate cyclase/cAMP pathways [117, 118]. cAMP/protein kinase A-mediated activation of CREB protein suppresses cytokine production from immune cells [119, 120]. Therefore, it is possible that netrin-1-mediated activation of adenylate cyclase/cAMP pathways may inhibit the production of proinflammatory mediators. Recent studies did indicate the increase in cAMP production by netrin-1 in immune cells [85, 94, 95]. However, it is not clear as there are additional pathways that exist for netrin-1 to mediate anti-inflammatory effects. Studies from our lab identified that netrin-1 could regulate inflammation through suppression of NFκB activation through suppression of IκB degradation [104]. Since NFκB is a known regulator of cyclooxygenase pathways, the relation between netrin-1 and COX-2 pathways was examined. Administration of netrin-1 suppressed ischemia reperfusion induced COX-2 expression in tubular epithelial cells as well as neutrophils and macrophages which was associated with reduction in prostaglandin E2 and thromboxane B2 excretion in urine [116]. This suggested that netrin-1 may regulate inflammation through inhibition of COX-2 expression in neutrophils and monocyte. Flow cytometry analysis showed that over 86% of Gr-1 positive neutrophils and 80% of F4/80 positive monocyte/macrophage were positive for COX-2 expression. Very few CD4 T cells were seen in the kidney and 60% of them were positive for COX-2 expression [116]. In addition, neutrophils were colocalized for COX-2, confirming that COX-2-expressing infiltrating cells were indeed neutrophils and macrophages. In vitro studies showed that IFNγ and LPS-induced COX-2 and PGE2 production were suppressed in netrin-1 treated macrophages. Moreover, IFNγ induced increase in MCP-1 and IP-10 production was inhibited by netrin-1 [116]. The netrin-1 mediated suppressive effect on chemokine production was abolished when PGE2 is added to the culture, suggesting that the netrin-1 effect is at the level of COX-2 expression. IL-17 production in neutrophils in response to ischemia is known to initiate IFNγ production and infiltration into kidney [106]. Moreover, IFNγ also regulates IL-17 production in neutrophils. Previous studies have shown that PGE2 increases IL-17 production in both Th17 cells and neutrophils [121]. We determined whether IL-17-induced IFNγ production is dependent on COX-2-mediated PGE2 and that netrin-1 suppresses this pathway, thereby suppressing IFNγ production and neutrophil infiltration into ischemic kidney. When neutrophils were stimulated with IL-17, COX-2 expression and IFNγ production were increased which was inhibited by netrin-1. The suppressive effect of netrin-1 on IFNγ production was abolished by the addition of PGE2, suggesting that the netrin-1 effect is at the level of PGE2 production but is not at the level of its activity [86, 116]. These results suggested that netrin-1 may regulate COX-2 expression through the inhibition of NFκB activation (Figure 4).

8. Netrin-1 Regulates Macrophage Polarization through PPAR Pathways

Macrophages express distinct patterns of surface receptors and metabolic enzymes in response to different stimuli and these ultimately generate the diversity in macrophage functions and phenotypes. Broadly, there are two distinct polarization states of macrophages, M1 and M2, that have been characterized [122–124]. However, more recent studies suggest a spectrum of macrophage polarized state depending on the stimulus characterized by distinct sets of genes that are activated with each stimulus [125]. However, whether such a spectrum of macrophage polarization exists in renal diseases is unknown. LPS and IFNγ can promote macrophage differentiation to a "classical" or M1 phenotype [126]. The M1 activation pattern is associated with tissue destruction and inflammation and is responsible for upregulating proinflammatory cytokines and increasing the production of reactive nitrogen species and reactive oxygen species [127]. In contrast, the "alternative" or M2 activation phenotype of macrophages is induced in response to IL-4 and IL-13. M2-polarized macrophages dampen the inflammatory process by producing anti-inflammatory factors, such as IL-10 and TGF-β1. M2 macrophages also upregulate mannose receptor (MR), C type 2 (Mrc2c), IL-1 receptor antagonist (IL-1RA), and scavenger receptors, such as cluster of differentiation 36 (CD36), as well as increased expression of arginase-1 (Arg-1). The M2 phenotype is thought to promote tissue repair after inflammation and/or injury [122–124, 127].

Improper macrophage activation is pathogenically linked to various metabolic, inflammatory, and immune disorders. Therefore, regulatory proteins controlling macrophage activation have emerged as important new therapeutic targets.

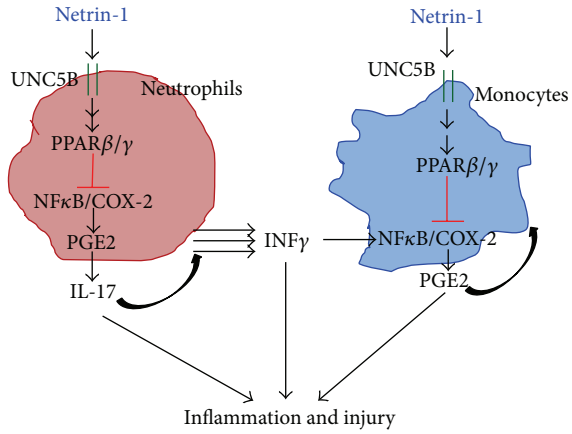

Figure 4: Netrin-1 regulates neutrophil and monocyte mediated inflammation through suppression of COX-2 expression and PGE2 production. Netrin-1 acting through UNC5B receptor induces the activation of PPARγ. Activation of PPARγ and other signaling pathways like PI3K causes suppression of IκB degradation and inactivation of NFκB transcription factor. NFκB activation is required for COX-2 induction. In the absence of NFκB activation COX-2 expression and PGE2 production was suppressed. This leads to reduced IL-17 production and IFNγ production thereby suppresses inflammation and tissue injury during ischemia reperfusion injury.

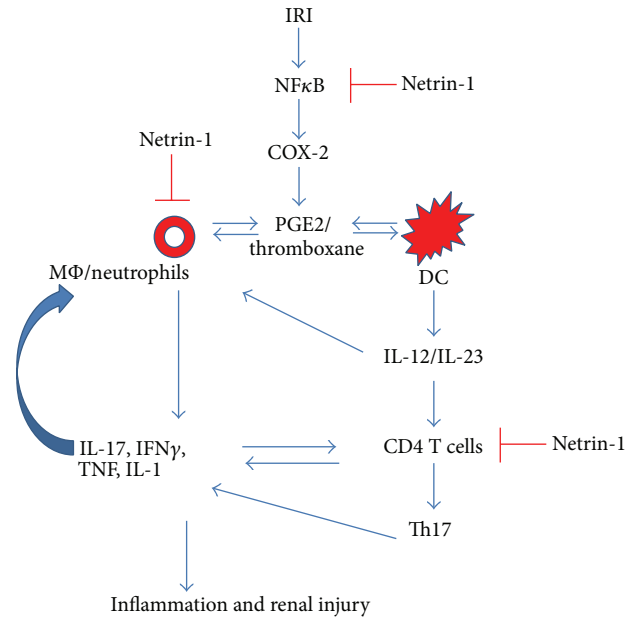

Figure 5: Pathways of inflammatory mediators and their regulation of netrin-1 during ischemia reperfusion injury of the kidney. Ischemic insults initiate inflammation in the kidney through activation of NFκB mediated COX-2 expression and prostaglandin E2/thromboxane production. These mediators, acting through an autocrine or paracrine mechanism, stimulate the production of cytokine and chemokines from monocyte (MΦ), neutrophils, and dendritic cells (DC). T cells may be activated through DC or direct activation by soluble mediators released from other cells or receptor mediated mechanism. Activation and release of soluble mediators from these white blood cells cause vascular leakage, edema, hypoxia, and tubular epithelial cell damage which will be manifested as kidney dysfunction. Netrin-1 reduces the severity of these events by acting at multiple levels through downregulation of COX-2 expression and suppression of inflammatory mediators production.

Previous studies from our lab demonstrated that netrin-1 regulates inflammation and infiltration of monocytes and ameliorates ischemia reperfusion-induced kidney injury [13, 83, 116]. In subsequent studies, we determined whether netrin-1 regulates the phenotype of macrophages and the signaling mechanism involved in the macrophage polarization. Overexpression of netrin-1 increased expression of arginase-1, IL-4, and IL-13 and decreased expression of COX-2 in spleen and kidney, indicating a phenotypic switch in macrophage polarization toward an M2-like phenotype [86]. Moreover, flow cytometry analysis showed a significant increase in mannose receptor-positive macrophages in spleen as compared to wild type. In vitro, netrin-1 increased expression of M2 markers (MR, arginase-1, and IL-10) but suppressed expression of M1 marker (iNOS and IL-1β) in both peritoneal macrophages and RAW264.7 cells [86]. Moreover, netrin-1 suppressed IFNγ-induced M1 polarization and production of inflammatory mediators such as IL-6 and IP-10. Adoptive transfer of netrin-1-treated macrophages suppressed inflammation and kidney injury against ischemia reperfusion. Interestingly, netrin-1 activated three anti-inflammatory pathways in macrophage. These include the PPARs (plasmid has a combination of PPARα, PPARβ/δ, and PPARγ response elements), glucocorticoid response element (GRE), and retinoic acid response elements (RXRs). PPARs are members of a nuclear-hormone-receptor superfamily; they transduce a wide variety of signals, including environmental, nutritional, and inflammatory events into a defined and ordered set of cellular responses at the level of gene transcription. Various types of fatty acid metabolites of arachidonic acid can bind and activate PPARs. Recent evidence has indicated an important role for PPARs in the control of various types of the inflammatory response [128]. These functions are mediated by several mechanisms which include the abilities of the PPARs to transrepress the activities of many activated transcription factors (nuclear factor-κB (NF-κB)), signal transducers and activators of transcription (STATs), activator protein 1 (AP1) and nuclear factor of activated T cells (NFAT), transcriptional upregulation of NFκB inhibitor kappa B (IκB), and the ability of PPAR-RXR heterodimers to inhibit phosphorylation of the MAPK (JNK and p38) cascade [128, 129]. Apart from the transcriptional activation of PPARs, netrin-1 induced the PPARγ ligand 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 in the presence of TLR4 activation in macrophage cell line [86]. Neither TLR4 activation alone nor netrin-1 increases 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 levels in macrophages [86]. Administration of netrin-1 suppressed ischemia reperfusion injury, which was abolished by PPARγ antagonist. In vitro, addition of netrin-1 strongly induced PPARβ and -γ expression and activation suggesting a role for PPARγ in mediating the netrin-1 effects in vitro and in vivo. This is further supported by in vitro studies where netrin-1 treatment increased the expression of M2 markers (MR and

IL-10) whereas IFNγ treatment induced M1 markers (COX-2, iNOS, and IL-6). Addition of netrin-1 with IFNγ suppressed M1 marker expression but enhanced M2 marker expression as compared to cells treated with IFNγ alone [86]. Importantly, when cells were treated with PPARβ and PPARγ antagonist along with netrin-1 and IFNγ, the M2 polarizing effects of netrin-1 were abolished suggesting the important role of PPAR pathways in mediating netrin-1 effects in macrophages [86].

9. Conclusion

Guidance cue netrin-1 is a versatile molecule and its role beyond axon guidance is implicated which includes regulation of immune cell migration, cytokine production, macrophage polarization, and regulation of cyclooxygenase-2 pathways. Studies performed in acute and chronic kidney disease as well as disease of other organs have clearly indicated that netrin-1 is a useful therapeutic agent to control inflammation and tissue injury acting at multiple levels (Figure 5). Future studies will identify new pathways that are regulated by netrin-1 in immune and nonimmune cells. Development of netrin-1 based therapies or small molecule that can activate its receptor UNC5B will be able to treat not only kidney disease but also the inflammatory disease of other organs as well.

Conflict of Interests

All authors declared no competing financial interests.

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Research Article

Efficiency of Double Layered Microencapsulated Probiotic to Modulate ProInflammatory Molecular Markers for the Management of Alcoholic Liver Disease

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Alcohol-related disorders are one of the challenging current health problems with medical, social, and economic consequences. Endotoxemia, oxidative stress, and release of a variety of inflammatory molecules are established mediators in alcoholic liver injury (ALD). Probiotics like *L. plantarum* though were reported to attenuate ALD, their *in vivo* health benefits are limited by their survival and sustenance in the adverse gut conditions. Therefore, to enhance their *in vivo* performance, chitosan coated alginate beads entrapping *L. plantarum* were prepared, characterized, and evaluated for their efficacy against ALD in rats. Following chronic alcohol exposure, rats developed endotoxemia, showed enhanced levels of liver enzyme markers, NF-κB levels, and increased cytokines such as TNF-α and IL12/p40 subunit, and reflected significant histological changes in the intestine and liver. However, cosupplementation with double layered microencapsulated probiotic significantly ($P < 0.05$) reduced the levels of endotoxemia, serum transaminases, NF-κB, and cytokines complemented with restoration of normal histoarchitecture of the intestine and liver. It is being documented here for the first time that the probiotics have the potential to inhibit IL-12/p40 subunit which is a recently explored potential marker for developing novel therapeutic agents. This study reveals that microencapsulation of probiotics may offer a biopharmacological basis for effective management of ALD.

1. Introduction

Alcohol-related disorders (ALD) are one of the challenging current health problems with far reaching medical, social, and economic consequences [1–3]. Several lines of investigations [4, 5] indicate that alcohol abuse induces endotoxemia, activation of transcription factor NF-κB, and release of a variety of inflammatory mediators including TNF-α, IL-1β, and IL-6 responsible for mounting oxidative stress culminating into liver injury.

Use of probiotics to manage the alcohol-induced endotoxin mediated liver injury is attributed to a variety of its health benefits including immunomodulatory and anti-inflammatory effects. A randomized trial carried out by Kirpich et al. in 2008 and 2012 showed an improvement of liver function tests and restoration of normal bowel flora upon administration of probiotics for the treatment of alcoholic liver disease. Further to this, *Lactobacillus* GG also ameliorates oxidative stress and intestinal permeability in alcoholic liver injury. Probiotics such as *Lactobacillus plantarum*, *Lactobacillus* GG, and *Bifidobacterium bifidum* have demonstrated a significant reduction of oxidative stress and restoration of normal bowel flora [6–11].

Several important mechanisms by which the probiotic provides various health benefits include modification of the gut microbiota, competitive adherence to the mucosa and epithelium, strengthening of the gut epithelial barrier, and modulation of the immune system of the host. Evidence also demonstrates that probiotics communicate with the host by pattern recognition receptors, such as toll-like receptors

which modulate key signaling pathways, such as nuclear factor-κB which either enhances or suppresses the downstream pathways [12].

However, major concern for the use of probiotics *in vivo* is that they must survive and sustain transit through the extreme conditions of the gut in large quantities to facilitate their colonization in the host to confer these benefits. In this context, encapsulation techniques may ensure greater survival of probiotic bacteria under gastric conditions. This is a method by which bacteria are protected from detrimental factors of environments such as high acidity (low pH), bile salts, molecular oxygen in case of obligatory anaerobic microbes, bacteriophages, and chemical as well as antimicrobial agents [13–15]. However, to the best of our knowledge, improved efficacy, if any, of microencapsulated probiotic against liver damage has not been assessed so far.

Amongst the encapsulation devices, microencapsulation in calcium alginate microparticles has been widely used owing to its ease of handling, nontoxic nature, and low cost. *Alginate* is a linear heteropolysaccharide extracted from algae, with two structural units consisting of D-mannuronic and L-guluronic acids and it forms hydrogels in the presence of divalent ions such as Ca^{2+}. However, certain disadvantages are attributed to alginate beads including the susceptibility to acidic environments and deterioration of beads when subjected to monovalent ions or chelating agents which absorb calcium ions. These limitations can be efficiently compensated by coating alginate with suitable compounds so as to impart it with mechanical strength. *Chitosan* is a linear polysaccharide with negative charge arising from its amine groups which are obtained by deacetylation of chitin [16]. Chitosan has been used for coating the alginate capsules to provide strength and for continuous sustainable release of bacteria. Another advantage of chitosan, which adds to its use as a coating material, is its mucoadhesive properties, which prolongs the residence time of dosage allowing a sustained drug release at a given target site. Furthermore, mucoadhesive polymers can guarantee an intimate contact with the absorption membrane, providing the basis for a high concentration gradient as a driving force for passive drug uptake [17]. Anticipating the added advantage of encapsulated probiotics, in the present study, efficacy of these probiotic bacteria microencapsulated in a double layer of polymers was evaluated for the management of ALD.

2. Materials and Methods

2.1. Agents. Absolute ethanol (99.9%) was purchased from Brampton, Ontario. The probiotic microorganism *Lactobacillus plantarum* (MTCC 2621), used as a probiotic, was acquired from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh (India).

L. plantarum was cultivated in *Lactobacillus* MRS broth (1% inoculum) at 37°C for 18 hours. The cells were harvested by centrifuging the culture at 8000 rpm for 15 min at room temperature. The cells were washed twice with 0.1% peptone water and were suspended in 1 mL of the same.

2.2. Microencapsulation of Cells. *L. plantarum* was encapsulated in calcium alginate by the method of Krasaekoopt et al. 2003, 2004 [18, 19] via extrusion technique. Briefly, the suspended cells were dispersed in prepared 1%, 2%, and 3% sterile sodium alginate and kept for overnight stirring. The dispersion of *L. plantarum* in a solution of sodium alginate was then dropped into 1% sterile calcium chloride with stirring on a magnetic stirrer. The beads were left for hardening by continuous stirring for 2 hours. The formed *L. plantarum* encapsulated alginate beads (AL beads) were then coated with medium molecular weight chitosan (Sigma, India) using the method of Krasaekoopt et al. 2003 [18], in which the beads were suspended in chitosan solution (0.4 g of chitosan in 90 mL distilled water acidified with 0.4 mL of glacial acetic acid to achieve a final concentration of 0.4% w/v; pH 5.7–6) and were stirred for 1 hour. The beads were then filtered using Whatman filter paper 1 and were freeze dried (−60°C) under vacuum for storage ($n = 6$). *The alginate loaded microparticles were named as AL and AL beads coated with chitosan were referred to as AL-CA.*

2.2.1. Characterization and Evaluation of AL and AL-CA Beads

(1) Determination of Particle Size. The particle size of the formed beads (AL and AL-CA beads) was determined in triplicate and mean size was recorded using particle size analyzer (Malvern instruments limited, Malvern, UK).

(2) Scanning Electron Microscopy (SEM) for Surface Morphology. Freeze dried calcium alginate beads (AL and AL-CA beads) were coated with gold film under vacuum to modify the conducting materials and surface morphology was studied.

(3) Drug Entrapment Efficiency (DEE) of Probiotic Beads. For bacterial enumeration, the beads (AL and AL-CA beads) were crushed in 1% sodium citrate solution while stirring for 60 minutes. Serial dilutions were made in 0.1% peptone water and spread plate on MRS Agar and incubated for 48 hours at 37°C ($n = 6$) and number of colonies forming units (cfu) was counted. Thereafter percentage of entrapment was calculated using the following formula:

$$\text{DEE\%} = \frac{\log \text{cfu/100 g of prepared beads} \times 100}{\log \text{cfu/mL initially loaded in the alginate mix}}. \quad (1)$$

(DEE = drug entrapment efficiency; cfu = colony forming units).

(4) Viability of Bacteria Postentrapment. To assess the viability of entrapped bacteria the beads (AL and AL-CA beads) were crushed in 1% sodium citrate solution while stirring for 60 minutes. Serial dilutions were made in 0.1% peptone

water and 0.1 mL was spread plated on MRS Agar plates and incubated for 48 hours at 37°C.

(5) Determination of Porosity. Beads (AL and AL-CA beads) were filled in a 10 mL graduated measuring cylinder up to the mark. The cylinder was tapped 500 times and the final volume was noted. Initial volume was kept the same in all the cases and the final volume gave the tap volume. The porosity was calculated according to the following equation and mean % porosity and standard deviation were recorded [16]:

$$\text{Porosity} = \frac{V_b - V}{V_p} \times 100 \quad (2)$$

V_b: bulk volume of the beads = 10 mL; V_p: true/tap volume of the beads;

$$V = V_b - V_p. \quad (3)$$

(6) Viability of Entrapped Probiotic Bacteria in Simulated Gastric Fluid (SGF) and Sequentially in Simulated Intestinal Fluid (SIF). Free probiotic cells and the beads (AL and AL-CA beads) were incubated in SGF without pepsin (dissolved 2.0 g of sodium chloride in 7.0 mL of hydrochloric acid and sufficient water to make 1000 mL, pH 1.2) for 4 hours and the samples were then sequentially transferred to SIF without pancreatin (dissolved 6.8 g of monobasic potassium phosphate in 250 mL of water, mixed, and added 77 mL of 0.2 N sodium hydroxide and 500 mL of water and adjusted the resulting solution to a pH of 6.8 ± 0.1, diluted with water to 1000 mL) for 2 hours and viable count was studied as described earlier [14].

(7) In Vitro Release Studies. Release study of probiotic beads (AL and AL-CA beads) was carried out in SIF under aseptic conditions. 100 mg beads were incubated at 37°C in test tubes containing 10 mL of SIF for 3 hours. At intervals of 1 hour, the supernatant from each tube was analyzed for cell count. For bacterial enumeration, serial dilutions of the supernatant were made in 0.1% peptone water and spread plate on MRS agar and incubated for 48 hours at 37°C.

(8) Bile Salt Tolerance. The viability in the presence of bile salts was assessed by suspending the free cells and beads (AL and AL-CA beads) in MRS broth supplemented with 0.3% (w/v) bile salts (sodium deoxycholate and sodium taurocholate) for 4 hours and bacterial count was estimated as described earlier.

2.3. In Vivo Studies

2.3.1. Ethics Statement. The experiment protocols were approved by the Institutional Animal Ethics Committee (approval ID: IAEC/282/dated 30/8/2012) and performed in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, on animal experimentation. All efforts were made to minimize the suffering of animals.

2.3.2. Animals. Female Wistar rats (200–250 g) were procured from Central Animal House, Panjab University, Panjab University, Chandigarh (India). The animals were housed under standard laboratory conditions, maintained on a 12 : 12 h light : dark cycle, and had *ad libitum* access to food (Ashirwad Industries, Pvt, Ltd., Punjab, India) and water. It has been reported in the literature that females develop more severe liver injury than males due to smaller amount of body water and lower activity of alcohol dehydrogenase enzyme (ADH) in stomach [20].

2.3.3. Dosing

Alcohol Dosing. Rats were administered 10 g/kg of body weight/day of 35% (v/v) ethanol by oral gavage in double distilled water for two weeks. Thereafter, the dose was increased to 14 g/kg of body weight/day and was continued for 10 weeks through oral gavage [21].

Probiotic Dosing. 10^{10} cfu/mL of *Lactobacillus plantarum* was dispersed in 1 mL of PBS (pH 7.2). Chitosan coated alginate beads containing equivalent *L. plantarum* were dispersed in 1% carboxymethyl cellulose and were administered to rats through oral gavage.

2.3.4. Experimental Design (Figure 3). After an acclimatizing period, rats were randomly divided into the following four groups each comprising 10–12 rats as depicted in Figure 3. The dose of the alcohol group was selected on the basis of the previous study [21]. At the end of the experimental period (after 12 weeks), the rats were sacrificed by cervical dislocation. Livers were removed quickly, rinsed in cold phosphate buffer saline (0.05 M, pH 7.4), and stored at −62°C.

2.3.5. Measurement of Blood Alcohol. After 10 weeks of alcohol administration, blood was taken from the tail vein 1.5 h and 2.5 h after gavage. Blood alcohol levels (BAL) were measured using the alcohol dehydrogenase kit from Sigma Chemical Co., USA.

2.3.6. Plasma Endotoxin Assay. Endotoxin level in the plasma samples was measured using Toxin Sensor Chromogenic LAL Endotoxin Assay Kit (Hycult Biotech). Briefly, 50 μL of plasma was incubated with 50 μL of limulus amebocyte lysate (LAL) at 37°C for 45 min. After several subsequent reactions, the samples were read spectrophotometrically at 405 nm. The plasma endotoxin levels were calculated against a standard curve of endotoxin concentrations of 0.1, 0.04, 0.02, 0.01, and 0.005 EU/mL.

2.3.7. Markers of Liver Damage

Assessment of Liver Function. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzyme activities in serum were determined using ERBA test kits (ERBA Diagnostics, Mannheim, Germany). Alkaline phosphatase (ALP) was estimated using Enzopak Diagnostic kit (Reckon Diagnostics, India).

Histological Studies. Liver and intestine tissues removed aseptically from the animals were cut into small pieces and fixed in 10% buffered formalin. Samples were dehydrated in different grades of alcohol, washed with xylene, and embedded in paraffin wax, and the sections were stained with hematoxylin-eosin and examined under the light microscope. Histological interpretation was done by Dr. B. N. Datta, Ex-Professor of Pathology, Post Graduate Institute of Medical Education and Research, Chandigarh (India).

Mechanistic Studies. Livers removed aseptically from the rats were rinsed in 0.05 M phosphate buffer saline (pH 7.4) (PBS). A 25% (w/v) tissue homogenate was prepared in PBS using a Potter Elvehjen homogenizer.

Assay for NF-κB p50 Subunit. Assay for NF-κB/p50 subunit in the nuclear extracts was performed in all the groups by commercially available Transcription Factor Assay kit (Upstate Biotechnology, NY, USA) according to the manufacturer's instructions. This assay combines the principle of the electrophoretic mobility shift assay (EMSA) with the 96-well based enzyme linked immunosorbent assay (ELISA). Briefly, cellular extracts were prepared from liver tissue using Chemicon's Nuclear Extraction kit. During the assay, the capture probe, a double stranded biotinylated oligonucleotide containing the consensus sequence for NF-κB, was mixed with the nuclear extract in the transcription factor assay buffer provided directly in the streptavidin coated plate and incubated for 2 h at room temperature. Plates were then washed to remove the unbound material. The bound NF-κB transcription factor subunit p50 was detected with rabbit anti-NF-κB p50 (specific primary antibody). HRP conjugated secondary antibody was then used for detection using 3,3,9,5,5,9-tetramethybenzidine (TMB/E) as the substrate and absorbance was read at 450 nm. Positive and negative controls were also run simultaneously [21].

Assay for Tumor Necrosis Factor-α (TNF-α). Assay for TNF-α was performed by ELISA in the liver homogenates using commercially available cytokine assay kit (R&D Systems, USA) according to the manufacturer's instructions described by us earlier [21]. Briefly, standards and tissue homogenates were dispensed in the 96-well microtiter plates precoated with monoclonal antibody specific for rat TNF-α. To each of the designated wells, 50 μL of assay diluent was added; the plates were sealed with acetate plate sealer and incubated at room temperature for 2 h. Plates were then washed five times with the wash buffer and 100 μL of rat TNF-α conjugate was dispensed into each well. Plates were again sealed and incubated at room temperature for 2 h, after which they were washed five times with the wash buffer and 100 μL of substrate solution was dispensed into each well. Plates were finally incubated at room temperature (in dark) for 30 min. 100 μL of the stop solution was added into each well to stop the reaction and absorbance was read at 450 nm. The results were expressed as picogram/mL of the TNF-α released. The ELISA was sensitive to 5 picogram/mL of the TNF-α released.

Table 1: Size of *L. plantarum* alginate beads (AL) and chitosan coated alginate beads (AL-CA).

Bead type	Size of bead in μm
Alginate beads	69.2 ± 6.9 μm
Chitosan coated alginate beads	80.4 ± 1.5 μm*

All values are represented as mean ± standard deviation. *$P < 0.05$ versus AL beads.

Assay for IL12/p40 Subunit. To check the levels of IL12/p40 subunit a double antibody sandwich ELISA was performed using the commercially available kit (Qayee-bio, China) according to the manufacturer's instructions. Briefly, standards (50 μL) and test samples (10 μL of liver homogenate + 40 μL of sample diluent) were added to the 96-well microtiter plates precoated with monoclonal antibody specific for rat IL-12/p40 subunit. To these designated wells, 50 μL of HRP labelled IL12/p40 subunit antibody was added and incubated for 60 minutes at 37°C. Following incubation, the excess liquid was discarded, dried, and washed five times with washing buffer. 50 μL each of chromogens A and B was dispensed and incubated for 10 minutes at 37°C away from light. The assay was stopped by adding 100 μL of stop solution and the absorbance was read at 450 nm.

3. Results

3.1. Characterization of Beads

3.1.1. Size of Microparticles and Scanning Electron Microscopy (SEM) Images. The alginate (AL) microparticles (Figure 1(a)) were spherical in shape with a wrinkled surface. The wrinkled surface can probably be due to the loss of water content during the lyophilization process. On the other hand, incorporation of medium molecular weight chitosan not only significantly ($P < 0.05$) increased the size of the probiotic loaded beads but also smoothened the surface of alginate microparticles (Figure 1(b)). The mean size of the AL beads was 69.2 ± 6.9 mm which was significantly ($P < 0.05$) lower than the AL-CA beads 80.4 ± 1.5 mm (Table 1).

3.1.2. Entrapment Efficiency. Viable count determination of the used probiotic was repeated six times ($n = 6$) and the mean viable count was calculated. In all the cases, the initial cell count was kept in the range of 10.2–10.73 log cfu/mL. Maximum cell entrapping was observed to be 80% in the probiotic loaded beads where the concentration of sodium alginate was kept as 2% (Table 2). The viable cell count obtained was 8.15 ± 0.20 log cfu/100 mg in alginate beads. Further, on coating with chitosan, 77% entrapment efficiency was obtained.

3.1.3. Porosity. The chitosan coated alginate beads entrapping probiotic (AL-CA) were more porous (95%) as compared to the alginate (AL) probiotic beads (84%).

3.1.4. Viability of Entrapped Probiotic Bacteria in Simulated Gastric Fluid (SGF) and Sequentially in Simulated Intestinal Fluid (SIF). To assess the likelihood of microencapsulated

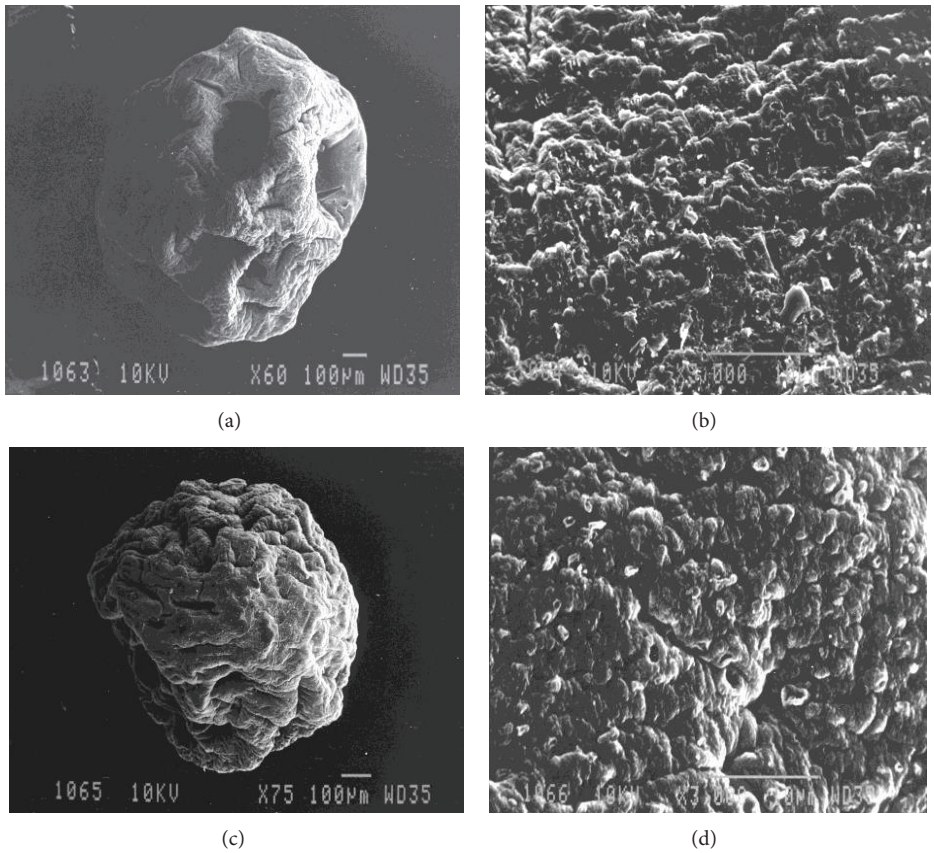

Figure 1: Scanning electron micrographs of (a) alginate beads containing probiotic (60x), (b) alginate beads containing probiotic (300x), (c) AL-CA beads containing probiotic (60x), and (d) AL-CA beads containing probiotic (3000x).

Table 2: The drug entrapment efficiency (DEE) of probiotic AL and AL-CA beads ($n = 6$).

S. NO	Alginate conc.	Initial no of bacteria loaded (cfu/100 mg)	No of bacteria entrapped (cfu/100 mg)	EE%
1	1%	10.73 ± 0.17	6.28 ± 0.44	58.5%
2	2%	10.20 ± 0.11	8.15 ± 0.20	80.00%*
3	3%	10.66 ± 0.19	6.61 ± 0.31	62.00%

All values are represented as mean ± standard deviation. * $P < 0.01$ versus 1%, 3%.

* 2% sodium alginate was chosen for entrapment as it maximum entrapment efficiency was obtained. The beads were further coated with chitosan and now the entrapment efficiency obtained was 77%.

and free probiotic bacteria surviving passage through the stomach following oral administration, they were tested in simulated gastric fluid (SGF; pH-1.2) without pepsin for four hours and sequentially in simulated intestinal fluid (SIF; pH-7.4) without pancreatin for two hours. Table 3 shows a significant decrease ($P < 0.05$) in cell count for free probiotic than AL beads and AL-CA beads. However, chitosan coated probiotic alginate beads (AL-CA) provided the best protection (71% of the bacteria survived after six hours in alkaline environment) than the AL (69.4% survival rate) and free probiotic (56.8% survival rate). The cell count of free probiotic gradually decreased by ≈6 log units with incubation in SGF without pepsin for four hours. A decrease in cell count (≈4 log units) was also observed with AL beads. However, the viability of *Lactobacillus plantarum* was reduced by only ≈2 log units when double encapsulation was provided with chitosan over alginate beads. In the alkaline environment, the cell viability of the free probiotic further was reduced to ≈2 log units which shows that the free probiotic cannot tolerate the harsh environment of gastrointestinal pathway.

3.1.5. In Vitro Release Study. The *in vitro* release studies (Figure 2) showed that there was constant release of probiotic from both types of beads during the four-hour duration in the SIF. No significant difference ($P < 0.001$) was observed between the AL-CA and AL probiotic beads (~80%). The AL-CA beads could not affect the release of probiotic rather it offered better stability.

3.1.6. Bile Salt Tolerance. Chitosan coated alginate microparticles encapsulating *L. plantarum* were the most effective ($P < 0.05$; 7.95 ± 0.87) in providing protection against bile salts (Table 4).

As the chitosan coated alginate beads (AL-CA) provided better protection in all the above-mentioned parameters to

Table 3: Growth of microencapsulated *L. plantarum* ($\log_{10}$ cfu) in Simulated Gastric fluid (SGF) and Simulated Intestinal fluid (SIF) ($n = 6$).

	Simulated gastric fluid				Simulated intestinal fluid		
Time	0 hr	1 hr	2 hr	4 hr	5 hr	6 hr	%
Unencapsulated probiotic	8.76 ± 0.11	8.06 ± 0.14	7.79 ± 0.11	7.38 ± 0.2	6.73 ± 0.22	4.25 ± 0.31	56.8%
AL beads	8.59 ± 0.39	8.14 ± 0.23	7.5 ± 0.43	7.2 ± 0.38	6.26 ± 0.34	5.97 ± 0.09[a]	69.4%
AL-CA beads	8.81 ± 0.12	8.4 ± 0.37	8.02 ± 0.16	7.52 ± 0.42	6.41 ± 0.41	6.29 ± 0.13[b,c]	71%

Beads at the end of 4 hours were shifted to SIF. All values are represented as mean ± standard deviation. [a]$P < 0.05$ versus unencapsulated probiotic; [b]$P < 0.05$ versus AL beads; [c]$P < 0.05$ versus AL beads.

Table 4: Bile salt tolerance of *L. plantarum* ($\log_{10}$ cfu) in alginate (AL) and alginate coated chitosan beads (AL-CA) ($n = 6$).

	Initial count	1 hr	2 hr	3 hr	4 hr
Unencapsulated probiotic	8.92 ± 1.23	7.97 ± 0.5	6.91 ± 0.89	6.85 ± 1.54	6.57 ± 0.77
AL beads	8.74 ± 0.37	8.25 ± 0.18	7.85 ± 0.54	7.74 ± 0.16	7.55 ± 0.67[a]
AL-CA beads	8.88 ± 0.65	8.74 ± 0.45	7.95 ± 0.14	7.62 ± 0.49	7.95 ± 0.87[b,c]

All values are represented as mean ± standard deviation in. [a]$P < 0.05$ versus unencapsulated probiotic; [b]$P < 0.05$ versus unencapsulated probiotic; [c]$P < 0.05$ versus AL beads after 4 hours of incubation in 0.3% bile salts.

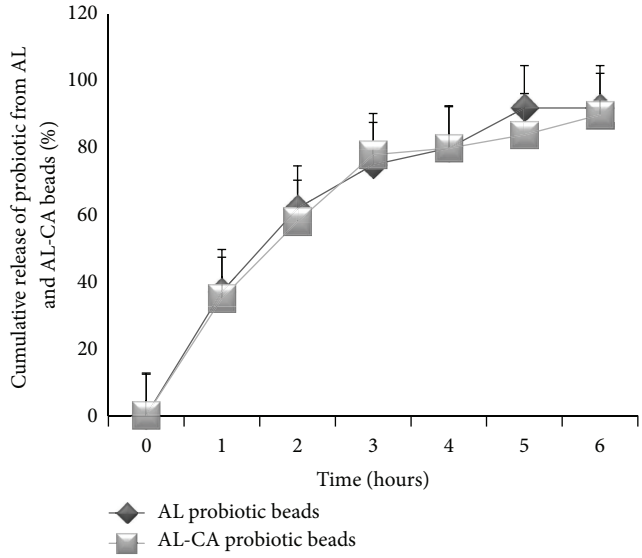

Figure 2: The *in vitro* release study for AL and AL-CA beads up till 6 hours. No significant difference was found for the two types of beads.

the probiotic than the alginate beads (AL), therefore, the former were used for further *in vivo* studies for chronic alcohol consumption.

3.2. In Vivo Studies

3.2.1. Blood Alcohol Levels. After 12 weeks of regular alcohol administration, the blood alcohol levels were found to be significantly increased in the alcohol supplemented group when compared to other groups. Blood alcohol levels (BAL) 1.5 h and 2.5 h after ethanol administration by gavage in the alcohol group were 243.2 mg/dL and 198.6 mg/dL, respectively. BAL in the alcohol treated and chitosan coated probiotic alginate beads supplemented group were 214.7 and 176.1 mg/dL after 1.5 and 2.5 h of alcohol administration, respectively.

3.2.2. Plasma Endotoxin Levels. The alcohol administered rats (group 2) suffered from significant endotoxemia (0.54 EU/mL) as compared to the control rats (0.16 EU/mL). The plasma endotoxin levels in alcohol administered and AL-CA supplemented group were significantly ($P < 0.01$) lower (0.312 EU/mL) than group 2 (Figure 4).

3.2.3. Assessment of Liver Functions. Estimation of alanine, aspartate aminotransferases, and alkaline phosphatase is considered as biochemical markers for liver damage. Therefore, in the present study levels of ALT, AST, and ALP were determined in the serum samples obtained from different groups. The levels of alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase were significantly elevated in the alcohol administered groups (104.3 ± 17.56 IU/L; 299.45 ± 21.40 IU/L; 259.4 ± 16.40 IU/L, resp.) as compared to the control groups (41.45 ± 15.45 IU/L; 260.34 ± 5.6 IU/L; 155.45 ± 23.40 IU/L, resp.). However, the levels were attenuated in the alcohol treated, free probiotic fed group. More pronounced ($P < 0.05$) results were obtained with AL-CA beads when coadministered during alcohol abuse (31.45 ± 14.59 IU/L; 254.67 ± 38.56 IU/L; 147.65 ± 35.67 IU/L, resp.) (Table 5). The entrapped probiotic appears to cause complete attenuation in the liver markers as no significant difference was observed from the control group.

3.2.4. Tissue Architecture

Liver. The liver sections of alcohol administered rats showed vacuolar degeneration, micro- and macrofollicular fatty changes, and focal collection of lymphocytes. Portal tract inflammation (portal triaditis) was also observed (Figures 5(b) and 5(c)). No morphological alteration was observed in the probiotics (free (Figure 5(f)) and encapsulated (Figure 5(g))) group and control group (Figure 5(a)). The probiotic administration in both free and encapsulated groups after alcohol administration showed an improvement

Table 5: Effect of free probiotic and encapsulated probiotic on hepatic markers in the serum of control and alcohol-administered rats.

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
ALT (IU/L)	41.45 ± 15.45	104.3 ± 17.56*	57.67 ± 10.56	31.45 ± 14.59#	40.62 ± 26.22#$	39.56 ± 22.44
AST (IU/L)	260.34 ± 5.6	299.45 ± 21.40*	278.56 ± 32.45	254.67 ± 38.56#	265.98 ± 37.56#$	258.98 ± 16.80
ALP (IU/L)	155.45 ± 23.40	259.4 ± 116.40*	185.67 ± 40.21	147.65 ± 35.67#	158.343 ± 29.56#$	152.34 ± 22.45

All values are represented as mean ± standard deviation of eight different observations.
*$P < 0.05$ versus group 1, group 5, group 6.
#$P < 0.05$ versus group 2.
$$P < 0.05$ versus group 3.

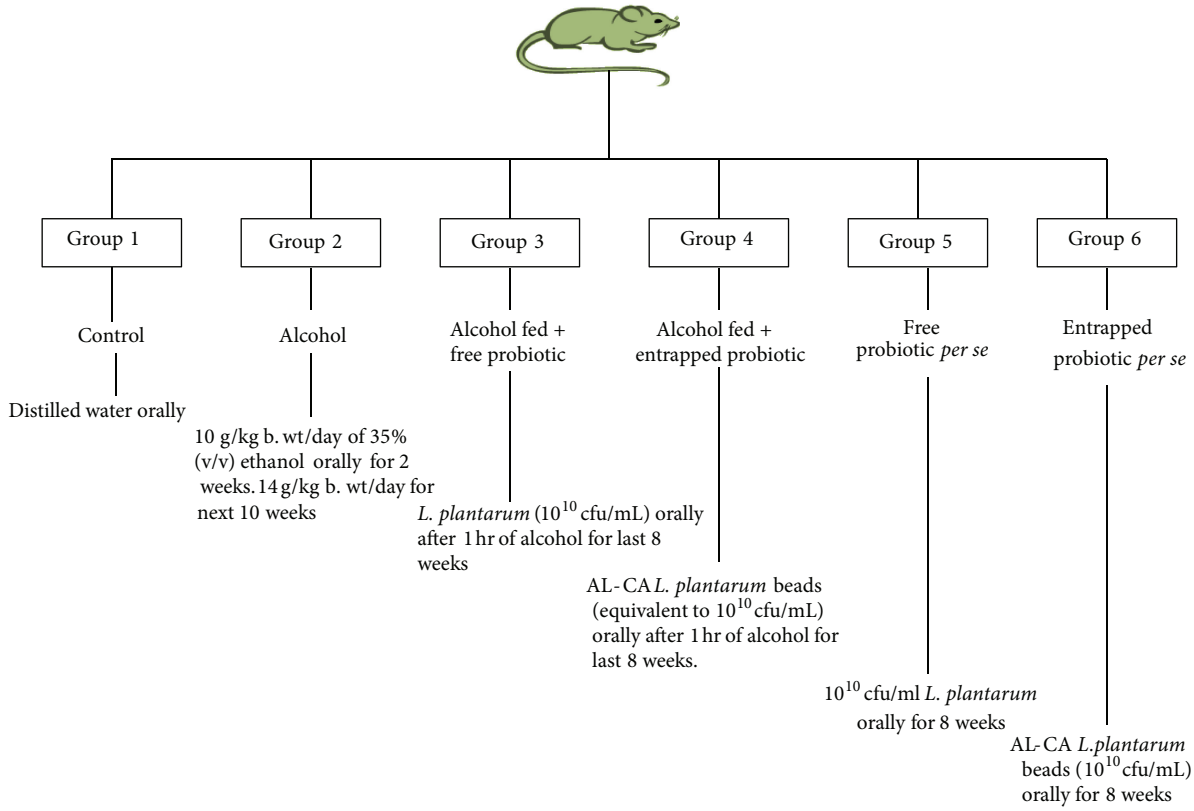

Figure 3: Diagrammatic representation of various treatment groups made for *in vivo* studies.

in the liver histology (Figure 5(d)). Slight kupffer cell hyperplasia was seen in the free probiotic supplemented group (Figure 5(e)).

Intestine. The control group showed normal intestine (Figure 6(a)). The intestine sections of alcohol administered group showed chronic active colitis with excess of lymphocytes especially in the superficial zones of the mucosa. The normal lymphoid follicles seemed enlarged with generally normal glands. However, occasional areas showed necrosis (Figures 6(b) and 6(c)). The intestine of probiotic (free (Figure 6(d)) and encapsulated (Figure 6(e))) treated groups restored the normal intestinal histology with goblet secreting mucous cells. The *per se* group of both the free probiotic supplemented group (Figure 6(f)) and encapsulated probiotic group (Figure 6(g)) showed normal intestine.

3.2.5. Assay for NF-κB. The alcoholic cellular extract showed elevated levels of NF-κB p50 subunit ($O.D._{450\,nm}$—2.601) as compared to control group ($P < 0.001$; $O.D._{450\,nm}$—0.652), whereas the supplementation of encapsulated probiotic significantly lowered the levels of NF-κB ($O.D._{450\,nm}$—0.890). However, the entrapped probiotic decreased the level of NF-κB significantly as compared to the alcohol abused rats and rats coadministered with free probiotic ($O.D._{450\,nm}$—1.470) (Figure 7).

3.2.6. Assay for TNF-α and IL-12/p40 Subunit. The levels of both the proinflammatory cytokines, that is, TNF-α and IL-12/p40 subunit, decreased significantly ($P < 0.05$) with the cosupplementation of encapsulated probiotic *L. plantarum* as compared to the alcoholic group. Alcohol consumption caused a 2.9-fold rise in the levels of both the cytokines (35.5 ± 2.4 pg/mg protein of TNF-α and 34.64 ± 1.5 pg/mg

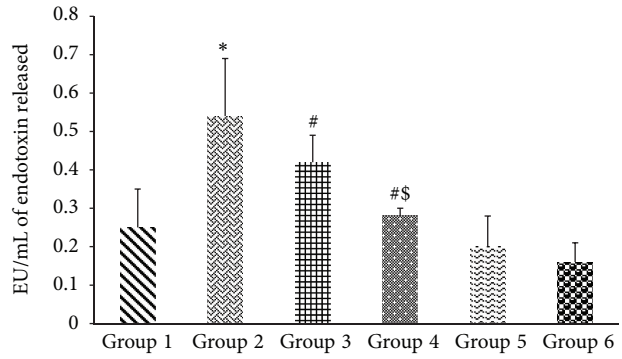

Figure 4: Effect of probiotic (free and encapsulated) on endotoxin levels in alcohol administered rats. Values are expressed as mean ± S.D. of eight different observations. $^{*}P < 0.05$ versus group 1, group 5, and group 6; $^{\#}P < 0.005$ versus alcohol (Alc) (group 2); $^{\$}P < 0.05$ versus group 3.

protein IL12/p40) as compared to the control value (12.08 ± 0.98 pg/mg protein TNF-α and 11.66 ± 0.56 pg/mg protein IL-12/p40) shown in Figures 8 and 9 but the encapsulated probiotic attenuated the cytokines levels to 13.66 ± 3.2 pg/mg protein of TNF-α and 8.46 ± 0.76 pg/mg protein of IL12/p40, respectively. The encapsulated probiotic caused 2.5-fold in the levels of TNF-α and 4-fold decrease for IL12/p40 subunit as compared to alcoholic rats.

4. Discussion

Bioencapsulation consists of entrapment of a biologically active material inside a microparticle, providing immobilization, safety, and controlled release as well as physical structure or functions. Therefore, in the present study, probiotic was microencapsulated and evaluated for its efficacy in alcohol-induced endotoxin-mediated liver injury.

SEM images revealed that the alginate beads entrapping probiotic have a wrinkled surface. This may be explained by the fact that the particles usually are heterogeneous with a dense surface layer and a loose core which results in their collapse and hence wrinkled shape [22]. The size of the microparticles was found to be in accordance with the earlier study by Lee et al. and Mokarram et al. [22, 23].

AL-CA beads encapsulating the probiotic were found to be more spherical and had a smoother surface than the AL beads. Due to low viscosity, chitosan diffuses rapidly into the microparticles and uniformly distributes itself through the whole shell giving the bead a spherical shape and smoother surface [22]. On coating the alginate beads with chitosan, there was a slight loss of entrapment efficiency which may be due to the removal of superficial probiotic present on the surface of alginate beads during the coating process.

In order to extrapolate the properties of microencapsulated probiotic from *in vitro* to *in vivo* animal models, the beads were tested for their potential to withstand and grow under the simulating conditions encountered in stomach (extreme acidic pH) and intestine (microaerophilic conditions, bile salts). Hence free and encapsulated *L. plantarum* beads were exposed to these conditions. The bacterial population was maintained when encapsulated with alginate and further coated with chitosan, whereas a significant reduction in the count of free probiotic was observed. The free probiotic could not resist the harsh extreme dual environment of the gut which is in accordance with the earlier study [19]. The results are in concordance with Lee et al., 2004 [22], which states that the gastric juice enters the less protected microparticles resulting in a decline in bacterial growth. This indicates that the chitosan coating protected the bacterium from the harsh acidic environment and thus the bacterial population was maintained. Likewise, AL-CA beads have the potential to absorb bile by an ion exchange reaction that takes place between chitosan and bile salts, thus limiting the diffusion of bile salts into the beads and protecting the entrapped probiotic from interacting with the bile salts [24]. Krasaekoopt et al. and Chávarri et al. [19, 25] also reported that the microencapsulation technique provides protection to the probiotic in the harsh gut environments. Chitosan coating did not affect the release of bacteria from within the microparticles. Therefore, it may be concluded that the AL-CA beads provided better protection, stability, and survivability to the probiotic without affecting its release. Thus, these beads were used for subsequent *in vivo* studies.

In the present study, the observed BAL in the rats confirmed the appropriate alcohol consumption which is broken down in the liver generating potentially dangerous by-products in the presence of alcohol dehydrogenase. Perhaps more so than alcohol itself, these products contribute to alcohol-induced liver damage. Moreover, chronic alcohol consumption mediates endotoxemia which occurs due to alterations in the gut microbiota (dysbiosis) as well as compromised gut barrier function leading to increased intestinal permeability. This indicates that the therapeutic strategies targeting the gut microbiome may be effective in the treatment of ALD [26].

In this context, probiotics are being explored extensively in view of their potential to maintain the composition of normal bowel flora, in addition to their competition for nutrient and adhesion sites, production of inhibitory compounds such as bacteriocins, and lowering of cationic pH by the production of short chain fatty acids [27]. It has been documented that the mucoadhesive microparticles such as alginate or chitosan adhere to the intestinal walls thereby increasing the time of absorption. Chitosan, a polycationic polymer, is known to modulate tight junctions controlling the transport process. These factors ensure better bioavailability and sustainability of microparticles inside the gut [28].

In this study, the probiotic beads, because of their nature of controlled release as mentioned above, were found to be better in restoring liver and intestine histology, reducing endotoxemia, and attenuating inflammation. The entrapped probiotic significantly lowered the levels of transcription factor NF-κB after alcohol consumption as compared to the free probiotic which might have reduced TNF-α levels. Blocking of NF-κB resulting in the downregulation of TNF-α has been reported by us earlier [21]. The results are in accordance with an earlier study where *Lactobacillus delbrueckii* and *Lactobacillus fermentum* ameliorated the inflammation by

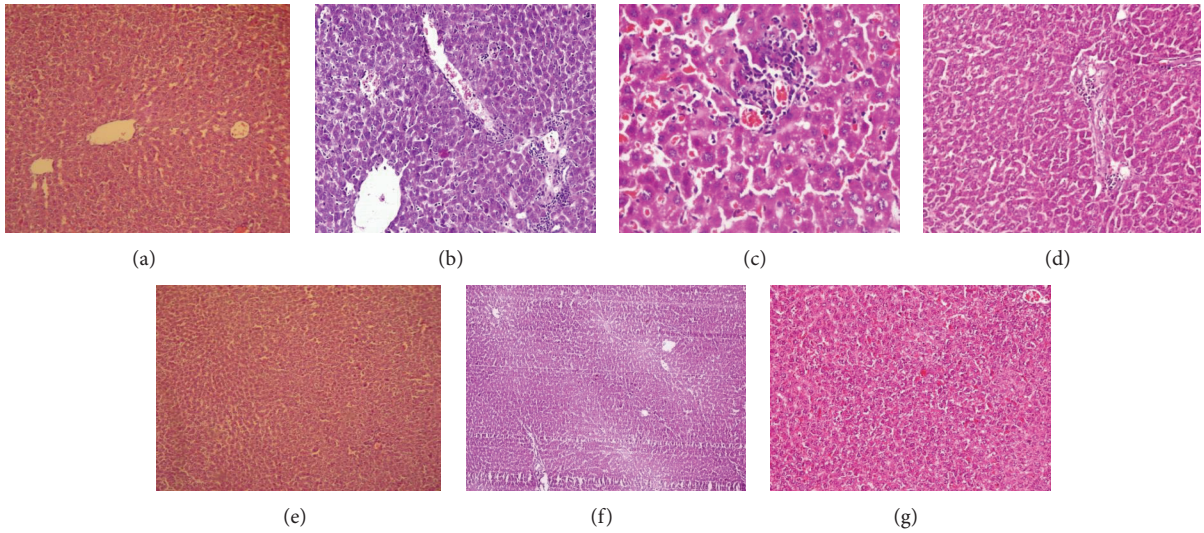

Figure 5: Representative photomicrographs of hematoxylin-eosin stained rat liver sections. (a) Normal rat liver (100x); ((b), (c)) liver section from rat administered 10–14 g/kg of body weight of 35% alcohol orally for 12 weeks showing vacuolar degeneration, microvesicular fatty change, focal collection of lymphocytes, and vascular congestion (200x, 400x), respectively; (d) photomicrograph of alcohol administered cosupplemented with free probiotic group showing normal histology with little hyperplasia of Kupffer cells (100x); (e) photomicrograph of alcohol administered cosupplemented with encapsulated probiotic group showing normal histology (100x); (f) photomicrograph of free probiotic *per se* group showing normal histology (100x); (g) encapsulated probiotic *per se* group showing normal histology (100x).

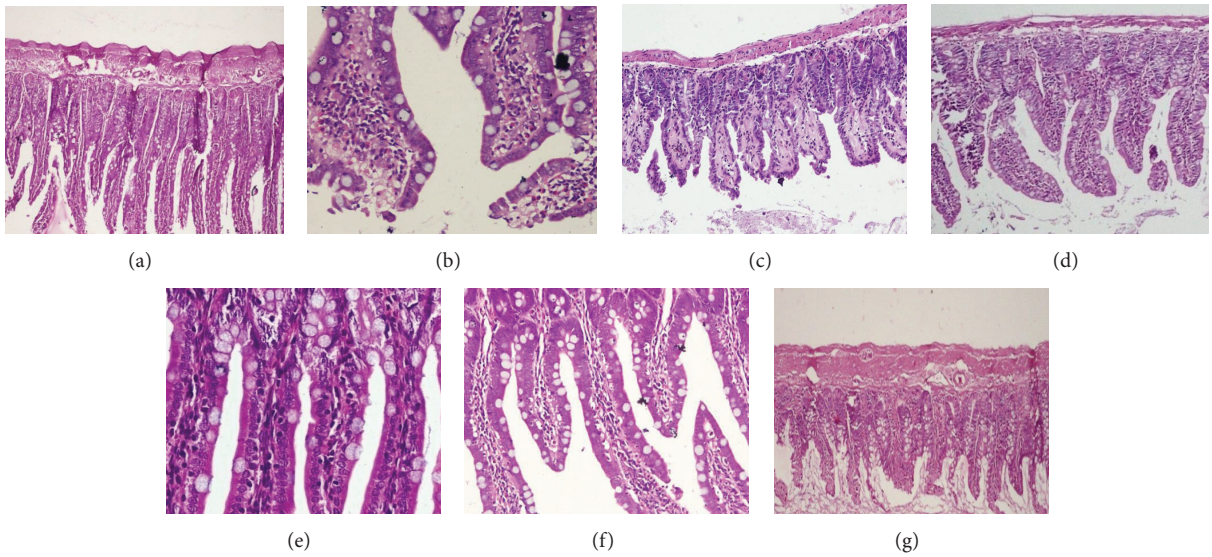

Figure 6: Representative photomicrographs of hematoxylin-eosin stained rat intestine sections. (a) Normal intestine (100x); ((b), (c)) photomicrographs of damaged intestine, severe colitis, and infiltration of lymphocytes in alcohol administered group (400x, 100x); (d) photomicrograph of alcohol administered cosupplemented with free probiotic group showing normal intestine with slight inflammation (200x); (e) photomicrographs of alcohol administered cosupplemented with encapsulated probiotic group showing normal intestine (400x); (f) photomicrograph of free probiotic *per se* group showing normal intestine (200x); (g) photomicrographs of encapsulated probiotic *per se* group showing normal intestine (100x).

decreasing concentration of IL-6 and expression of TNF-α and NF-κB p65 in ulcerative colitis [29, 30]. VSL#3, a combination of five probiotics, also lowered the expressions of iNOS, COX-2, NF-κB, TNF-α, IL-6, and p-Akt and increased IL-10 expression in colonic tissues in acute colitis [31].

The use of antitumor necrosis factor (TNF) therapies has been a huge success in immune-mediated inflammatory diseases (IMIDs) [32]. However, the major limitation with the anti-TNF α therapy is that it lacked the efficacy and loss of response in some patients and led to potential adverse

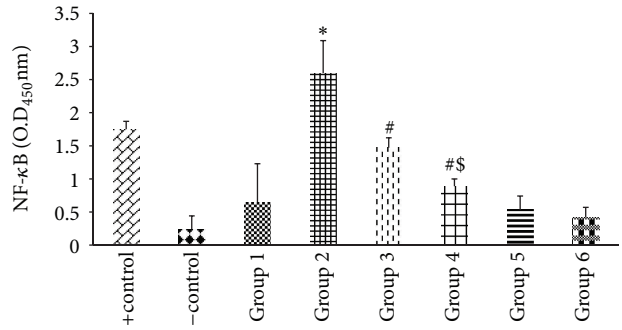

Figure 7: Effect of probiotics on alcohol-induced activation of NF-κB in liver. Values are expressed as mean ± S.D. of five different observations. $^{*}P < 0.001$ versus group 1, group 5, and group 6; $^{\#}P < 0.01$ versus alcohol (Alc) (group 2); $P < 0.05$ versus group 3. Positive control (+control) refers to the TNF-α treated HeLa whole cell extract; negative control (−control) refers to the biotinylated double stranded nonspecific competitor oligonucleotide probe which does not contain the NF-κB consensus sequence. Note: values are not corrected for the protein content. Values are not the same according to the protein content as the value for protein content varying by 14 ± 5.00 mg/mL of tissue for all the samples.

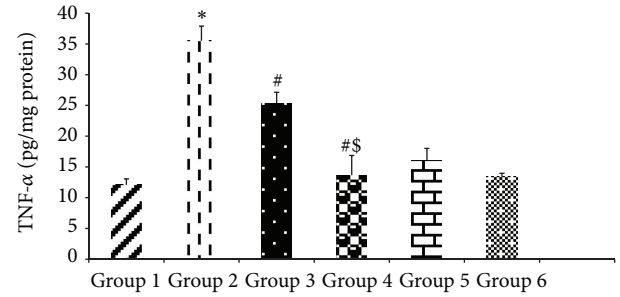

Figure 8: Effect of probiotics on hepatic TNF-α levels in alcohol-fed rats. Values are expressed as mean ± S.D. of eight different observations. $^{*}P < 0.05$ versus group 1, group 5, and group 6; $^{\#}P < 0.05$ versus alcohol (Alc) (group 2); $^{\$}P < 0.05$ versus group 3.

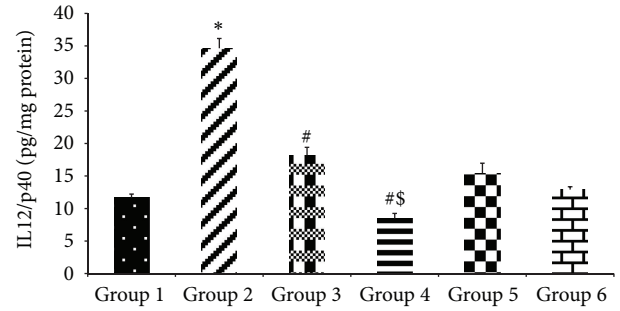

Figure 9: Effect of probiotics on the expression of IL12/p40 on alcohol-induced liver. Values are expressed as mean ± S.D. of eight different observations. $^{*}P < 0.05$ versus group 1,group 5, and group 6; $^{\#}P < 0.05$ versus alcohol (Alc) (group 2); $^{\$}P < 0.05$ versus group 3.

effects [33]. Thus, recent attention turned to other significant cytokines released during inflammatory response which are safer and prove to be a pharmaceutical basis for therapeutic intervention. One of the most potent cytokines turns out to be interleukin- (IL-) 12 family of cytokines in the pathogenesis of inflammatory mediated diseases. Interleukin-12 is composed of subunit IL-12 p40, which interacts with the IL-12Rb1 receptor. The cytokines IL-12 and IL-23 share the same subunit IL-12/p40. Blockade of this subunit has been reported to supress both the cytokines [34]. The encapsulated probiotic efficiently reduced the levels of IL-12/p40 subunit which is recently being considered as a potential target for developing novel strategies against ALD. Thus anti-NF-κB, anti-TNF-α, and anti-IL-12/p40 subunit activity of the probiotic correlated well with the functional activity of transaminases resulting in restoration of clinical manifestations of the disease in terms of tissue architecture.

5. Conclusions

To the best of our knowledge, this is the first report wherein the improved efficacy of probiotic after being microencapsulated in liver damage has been demonstrated. Further, it may be noticed that microencapsulated probiotic ameliorated ALD by suppressing molecular inflammatory markers particularly IL-12/p40 subunit which remained unexplored earlier.

Conflict of Interests

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

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