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Review

A fresh look at the hygiene hypothesis: How intestinal microbial exposure drives immune effector responses in atopic disease

Eric M. Brown[a,b], Marie-Claire Arrieta[a,b], B. Brett Finlay[a,b,c,*]

[a] Department of Microbiology and Immunology, University of British Columbia, Vancouver, BC, Canada
[b] Michael Smith Laboratories, University of British Columbia, Vancouver, BC, Canada
[c] Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada

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ABSTRACT

There currently is no consensus on which immunological mechanisms can best explain the rise in atopic disease post industrialization. The hygiene hypothesis lays groundwork for our understanding of how altered microbial exposures can drive atopy; yet since its introduction increasing evidence suggests the exposure of our immune system to the intestinal microbiota plays a key role in development of atopic disease. As societal change shifts our microbial exposure, concordant shifts in the tolerant and effector functions of our immune systems give rise to more hypersensitive responses to external antigens. This is contrasted with the greater immune tolerant capabilities of individuals still living in regions with lifestyles more representative of our evolutionary history. Recent findings, buoyed by technological advances in the field, suggest a direct role for the intestinal microbiota-immune system interplay in the development of atopic disease mechanisms. Overall, harnessing current mechanistic studies for translational research into microbiota composition and function in relation to atopy have potential for the design of therapeutics that could moderate these diseases.

1. Introduction

1.1. The hygiene hypothesis: a history

Our thinking of how environmental exposures can be linked to atopic disease can be traced back to a study in the 1970s, where a low prevalence of allergy in indigenous populations in northern Canada was observed when compared to Caucasian Canadians living in urban environments [1]. This was hypothesized to be the result of fewer infections during childhood. A similar observation was made a decade later, with children with elder siblings being less likely to get hay fever [2]. Henceforth the term "hygiene hypothesis" was used to describe the general phenomenon of the association between hygienic conditions and a higher prevalence of allergic disease. Numerous studies have confirmed this relationship, even extending it to the global epidemiology of other immune-mediated diseases, such as type 1 diabetes and inflammatory bowel disease [3,4]. Recently these observations are being linked to the composition of the microbes that colonize our intestinal tract, collectively referred to as our intestinal microbiota. The microflora hypothesis proposes that shifts in composition of our intestinal microbiota caused by early life antibiotic use and dietary changes can lead to a disruption in immune tolerance [5]. An extension of this idea is found in the "old friends" hypothesis, which gives an evolutionary perspective to these observations. This hypothesis proposes that the microbial exposures vital for immune regulation can be derived from our symbiotic relationship with the microbes we have co-evolved with in our environment [6]. The microbiota has co-evolved with the intestinal immune systems for millions of years, during which most encounters involve commensal and mutualistic bacteria rather than pathogenic ones. With a community of cells expressing one hundred times more genes than its host, the microbiota produces significantly more potential antigens that encounter the mammalian immune system than self or pathogen-derived antigens [7].

The emergence of the jaw in early vertebrates came a series of biological advantages, such as an increase in body size and lifespan. Intriguingly, this evolutionary milestone matches the appearance of the adaptive immune system, which not only left early vertebrates better equipped to fight persistent pathogens, but also required the establishment of immune tolerance toward a vast amount of benign and often beneficial microorganisms. The vertebrates capable of mounting immunological memory have a more diverse microbiota than invertebrates, suggesting that the different features of the immune system and the composition of the microbiota are tightly influenced by one another [8]. Given the

* Corresponding author at: Michael Smith Laboratories, University of British Columbia, #301-2185 East Mall, Vancouver, BC V6T 1Z4, Canada.
Tel.: +1 604 822 2210; fax: +1 604 822 9830.
E-mail address: bfinlay@msl.ubc.ca (B.B. Finlay).

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inevitable exposure of the intestine to microbes, the immune system has also evolved dependency on microbial exposure similar to how our body has become dependent on dietary components that we cannot synthesize.

Following industrialization, microbial exposure from our environment has rapidly changed. The notion of a "disappearing microbiota" links post-industrialization habits to a depletion of our ancestral microbiota that we have become dependent on from our environment [9]. As atopic diseases such as food allergy and asthma have become more prevalent since the industrial revolution, the incidence of these diseases could be linked to lifestyle and dietary changes, altering our exposure to microorganisms that educate our immune system. Much of our microbial exposure throughout life is derived from the intestinal microbiota. Thus, analysis of how our immune system interacts with the intestinal microbiota is critical to understand the link between atopy and microbial exposure.

1.2. The intestinal microbiota: our main exposure to microbial antigens

The human intestine harbors a diverse microbial community comprised of mainly bacteria, but also archaea, viruses, and eukaryotes. The intestine is dominated by over 500 species of bacteria, from 7 to 10 phyla, with species from the phyla Bacteroidetes and Firmicutes being the most abundant [10]. These bacteria colonize to levels reaching 100 trillion microbes along the intestinal tract, and bacteria density increases from the small intestine (10^3–10^7 cells per gram of feces) to the colon (10^{12} cells per gram feces) [11,12]. The colonization of the microbiota starting at birth shapes an intimate relationship where host and microbe have co-evolved together for mutually beneficial outcomes. The intestine provides a protected, nutrient rich environment where the microbiota establishes a remarkably stable and resilient ecosystem [10]. In turn, the host uses the metabolic capacity of the microbiota to its advantage. The intestinal metagenome contains roughly 100 times more microbial genes than human genes [13], supplementing the host cells with a "second genome" [14]. Enzymatic products of these genes enhance our digestive capacity of substances such as polysaccharides and complex carbohydrates [15]. Microbial by-products of digestion provide vitamins and nutrients to host cells and contribute to many aspects of host physiology and development, along with conferring colonization resistance to potentially harmful pathogens [16].

While the gut microbiota is remarkably stable throughout life, pronounced differences in bacterial assemblage and gene repertoires have been observed in humans across the globe [17]. Most notably, there are distinct differences in the composition and diversity of the gut microbiota between adults in industrialized versus developing countries [18]. Differing diets, infection rate and other environmental exposures (rather than purely genetic differences) are thought to be the main drivers of these changes, and we are just beginning to grasp the implications the resulting changes in microbial communities have on immune mechanisms relating to atopy [19].

There remain many outstanding questions. Most importantly, could there be a correlation between population based microbial changes and the epidemiological prevalence of atopic diseases? For example, it is well reported that the incidence of atopy in citizens of hunter-gatherer societies is low to non-existent [20]. The microbial exposure from the surrounding environment is significantly different in these societies, and their lifestyle reflects one closely linked to our evolutionary history. How these lifestyle changes feed into the structure and composition of the intestinal microbiota, and influence T helper 2 (Th2) immune responses after exposure to an allergen is the topic of many current studies. In this review, we highlight how certain components of the intestinal microbiota can direct tolerant versus effector immune responses, and then discuss the relevance of this effect on atopic disease. Particular emphasis is placed on the effect the intestinal microbiota have on T helper cell balance and Toll-like receptor signaling. Finally, we introduce plausible hypotheses and mechanisms as to why atopic diseases are absent in geographical regions with poor sanitation, and a lifestyle more reflective of our evolutionary history.

2. The gut microbiota shapes tolerant and effector immune responses

At homeostasis, the gastrointestinal immune environment is one of controlled inflammation and tolerance. Several immune mechanisms are involved in achieving this state. First, most bacteria in the distal small intestine and the colon are not in physical contact with the intestinal epithelial cells (IECs). Only some microorganisms are capable of inhabiting the mucus layer immediately adjacent to the gut epithelium and interacting with epithelial and immune cells. The mucus layer is morphologically divided into inner and outer portions. Bacteria rarely populate the inner mucus layer, while the outer mucus layer provides mucin glycoproteins that facilitate the colonization by a variety of microbial species [21]. This strategy likely accounts for the ignorance of the systemic immune system toward many of the bacterial antigens inhabiting the colon. The proximal small intestine lacks a continuous mucus layer to anatomically contain and compartmentalize the resident microbes. Here, secretion of antimicrobial proteins by Paneth cells, microbiota-specific IgA secretion by B cells and IL-22 production from innate lymphoid plays an important role in regulating the spatial and compositional arrangement of the microbiota [22,23]. Many microbiota-derived antigens do encounter immune cells and those interactions result, in most cases, in immune tolerance. How are tolerogenic responses favored after these encounters? An overview of these mechanisms highlights the many and sometimes redundant strategies that vertebrate hosts utilize to avoid ongoing intestinal inflammation.

As the first layer of cellular defense, IECs secrete thymic stromal lymphopoeitin (TSLP) and tumor growth factor-beta (TGF-β), both of which induce the secretion of tolerogenic cytokines from intestinal dendritic cells [24]. These antigen-presenting cells in turn induce the development of regulatory T cells (Tregs) by secreting TGF-β and retinoic acid [25,26] and promote the differentiation of IgA-producing plasma cells, downregulating the pro-inflammatory arms of mucosal immunity [27]. Tregs are essential for immune tolerance toward the microbiota and their expansion is favored over effector T cells upon microbial neonatal exposure. Treg-deficient mice spontaneously develop IBD due to the overwhelming intestinal pro-inflammatory effector T cell response [28]. This dependence on Tregs to achieve tolerance to the microbiota forces the question of how these cells are trained to specifically respond to these commensal-derived antigens. These cells are selected in the thymus for their ability to suppress T cells with a high affinity to self-MHC molecules, thus preventing autoimmune responses. A recent study provides evidence that Tregs may receive further education from the intestinal microbiota. The microbiota is essential for the peripheral development of colonic Tregs from naïve T cells. Furthermore, there is a much higher heterogeneity in the repertoires of T cell receptor (TCR) α-chain from FoxP3+ Tregs of the colonic lamina propria compared to Tregs from secondary lymphoid organs [29]. Thus, there are post-thymic mechanisms of T cell education that occur peripherally via interactions with the commensal microbiota, which implies that the immune system may have evolved to rely on the microbiota to complete the training of the population of immune cells.

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Tregs promote a tolerogenic environment in part by the overwhelming production of IL-10. This cytokine is essential for gut immune homeostasis and its deficiency also results in spontaneous IBD in mice [30]. It has been postulated that certain bacterial species stimulate the development of Tregs and their production of IL-10. The capsular polysaccharide-A (PSA) from *Bacteroides fragilis* has been described as a potent inducer of IL-10 by stimulating FoxP3 + Treg differentiation. Oral administration of PSA results in the downregulation of the Th17 response in mice that develop autoimmune encephalomyelitis [31]. A group of *Clostridium* species from clusters IV and XIVa also promote Treg differentiation. These clusters encompass ~60% of the strict anaerobe intestinal microbiota and the colons of mice colonized with a mixture of 46 *Clostridium* species have a higher number of Treg cells and an increased secretion of IL-10. Interestingly, this increase in Treg IL-10 production results in a reduction in intestinal inflammation and allergy in experimental animal models [32]. Similarly, mice treated with the probiotic mixture VSL3, which contains a mixture of Bifidobacteria, Lactobacilli and *Streptococcus salivarius* have an increased percentage of colonic Tregs [33].

Alternatively, the microbiota can promote inflammatory Th17 responses. Th17 immune response has been associated with colitis [34,35] and autoimmunity [3,36,37]. This arm of the immune response, however, plays an important role in preventing fungal and bacterial overgrowth and it is essential in the prevention of infection with the murine pathogen *Citrobacter rodentium* [38]. The development of Th17 responses is microbiota-dependent as they are absent in germ-free mice [39]. Specific species of the microbiota have been associated with the expansion of Th17 cells. Segmented filamentous bacteria (SFB) are an unclassified species of bacteria that inhabit the murine ileum and caecum. These bacteria are potent stimulators of Th17 cells and the secretion of the Th17 cytokines IL-17 and IL-23, and of serum amyloid A (SAA), an acute phase protein believed to expand Th17 cells [40,41]. Other mechanisms involved in Th17 cell differentiation and maintenance are the microbiota-derived production of ATP [34], microbiota-stimulation of IL-1β [42] and IL-23 secretion [43]. The presence of distinct microbial species within the microbiota is necessary to achieve a balanced level of tolerant and effector immune cells promoting intestinal homeostasis (Fig. 1). The education of naïve CD4+ T cells imparted by different members of the microbiota may have enormous health implications. With the increasing evidence that modern-age perinatal events, such as cesarean sections and formula feeding, significantly alter the composition of the intestinal microbiota in neonates [44], it is tempting to speculate that the immune regulatory mechanisms dependent on the intestinal microbiota may not occur in the absence of critical bacterial groups, including some *Clostridium* species.

3. Impact of the intestinal microbiota in atopic disease

Atopy is defined as the capacity for one to develop an IgE immune response to an external antigen, leading to hypersensitivity reactions. Clonal expansion of Th2 cells leads to the production of IL-4 and IL-13 which induce B-cell class-switching to IgE and increased likelihood for the development of allergen-specific IgE memory B cells [45]. During a hypersensitivity reaction, this results in the cross-linking of allergen-specific IgE antibodies with mast cells and basophils, leading to degranulation and the release of pro-inflammatory molecules such as vasoactive amines, lipid mediators, chemokines and various cytokines [45]. Allergic inflammation thus develops, with activation and proliferation of Th2 cells playing a central role. Specific immunological mechanisms or characteristics predisposing individuals to develop an atopic disease are not fully understood. As discussed above, colonization of the particular microbial species can drive either tolerant or effector immune responses to antigens. Mounting epidemiological data in humans associates differences in intestinal microbial communities with the development of atopic disease in children [46–50]. Animal models have been used to extend observations made in the human studies by demonstrating that there is a causal relationship between the intestinal microbiota, it's influence on immunity, and subsequent impact on atopic disease [50].

3.1. Perinatal alterations of the intestinal microbiota and T helper cell balance

Mimicking the conditions at birth, germ-free mice and mice treated with high doses of antibiotics exhibit an underdeveloped immune system polarized toward a Th2 response [51–54]. These animals also exhibit an exacerbated disease phenotype in asthma models [55–57]. The transition to a more balanced Th1/Th2 immune response occurs only after microbial colonization. Not surprisingly, different microbes exert different effects in asthma animal models. *Mycobacterium vaccae* [58] and *Helicobacter pylori* [59] both significantly reduce airway disease in mice. Studies have shown that treatment with vancomycin, and not streptomycin, worsened asthma severity in mice [57]. Microbial community analysis in the vancomycin treated animals showed a marked decrease in members of the Bacteroidetes and an increase in members of the *Lactobacillaceae* family, suggesting that the lack of the former and/or the overrepresentation of the latter group may be involved in directing the immune response toward an exacerbated allergic phenotype. The mice treated with vancomycin also had Treg levels which were significantly decreased in the colonic lamina propria [57]. As discussed, various *Clostridium* species have a particular ability to induce Tregs in the colon, and absence of these microbial species could dampen the regulation of Th2 cells. In another study, select *Clostridium* species were critical in the modulation of an allergic response as higher IgE blood titers were observed in mice that were not colonized by these bacteria [32]. Recently, a role for extrathymically produced, induced Tregs (iTregs) in controlling allergic asthma has been described as mice deficient in iTregs show a pronounced Th2 pathology and allergic inflammation in the lung [60]. A function for Tregs in controlling Th2 effector cells and downstream mechanisms of atopy is becoming clear. Tregs have been shown to also directly suppress Th1 and Th17 cell effector function, B-cell IgE production, and the degranulation potential of basophils and mast cells [61]. Given this, Treg induction is important to promote a healthy balanced response to potential allergens by promoting tolerance mechanisms and suppressing effector mechanisms of the immune system. The microbiota has also been shown to modulate the function of invariant natural killer T cells as well [62]. In this study, germ-free mice showed hypermethylation of the gene for the chemokine ligand 16 (CXCL16), a chemokine involved in the migration of the invariant natural killer T (iNKT) cells to mucosal sites. Higher levels of methylation of this gene accounts for the increase in iNKT cells in the mucosa. Microbial colonization reduced the methylation of the CXCL16 gene and the allergic inflammation in these mice, indicating that the microbiota regulates specific components modulating the immune response to potential allergens [62].

There is an apparent 'window of opportunity' during which shifts in the microbiota are critical in immune modulation and atopic disease development [62,63]. Antibiotic treatment of neonate but not adult mice resulted in an increase in disease severity [63], suggesting that the vulnerability of the intestinal microbiota composition occurs early in its development and that later shifts may not result in altered allergic disease phenotypes. Birth cohort studies in humans have found that specific changes in the gut microbiota during the first month of life are associated

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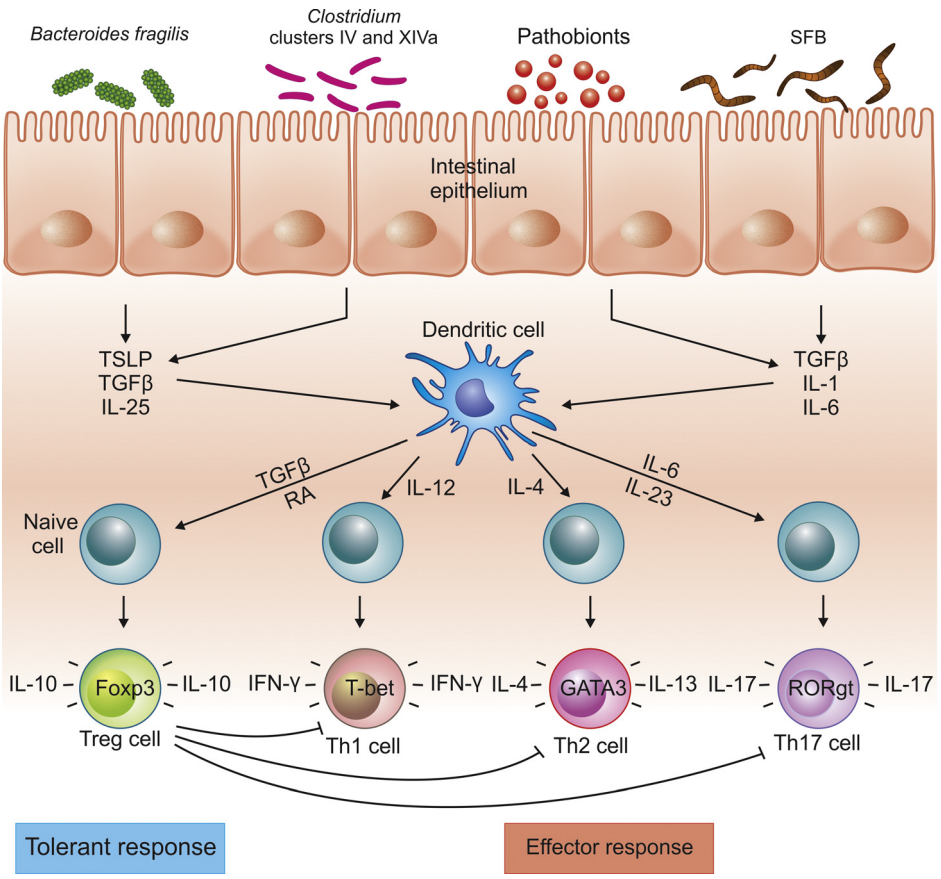

Fig. 1. The intestinal microbiota orchestrates the tone of the intestinal T cell response. Through molecular mechanisms that remain largely unknown, specific microbial cells stimulate the intestinal epithelial cells to produce tolerogenic signals (TSLP, TGFβ, IL-25) or effector signals (TGFβ, IL-1, IL-6). Intestinal dendritic cells (DCs) expressing CD103+ take up microbial antigens in the presence of these signals and subsequently present processed antigens to naïve T cells (Th0) in the context of the major histocompability complex. DCs secrete different 'cocktails' of immune effectors in order to direct a specific type of T cell activation and expansion. Tolerogenic microbes, such as *Bacteroides fragilis* and *Clostridium* species from clusters IV and XIVa, induce DCs to secrete TGFβ and retinoic acid (RA), which trigger the activation of the nuclear transcription factor FoxP3 and the differentiation of Tregs. Other microbes known as segmented filamentous bacteria (SFB) stimulate the activation of the transcription factor RORgt and T cells differentiate into Th17 cells. Many pathobionts stimulate the expansion of Th1 and Th2 cells by the activation of transcription factors T-bet and GATA3, respectively. Pathobionts are normally kept at bay by the host immune response and by the microbiota. When they interact with the intestinal epithelium an inflammatory response follows. In order to regulate or suppress the immune response, T regs secrete the tolerogenic cytokine IL-10. IL-10 has the ability to suppress Th1, Th2 and Th17 cells, making it an essential component that assures immune homestasis. A healthy immune response consists of stimulation of all different arms of the T cell response by microbial cells. However, microbial dysbiosis often results in an overproduction of effector signals and/or an inadequate production of IL-10.

with wheezing later in childhood [64,65]. Mode of delivery, breastfeeding and the use of antibiotics in newborn, among other factors, contribute to altered infant microbiota at one month [66,67]. By bypassing colonization with the maternal vaginal and fecal flora, caesarian sections affect the overall microbiota of infants. These babies have less *Bifidobacterium* and *Bacteroides* species in the gut [68,69] and are more prone to colonization by *Clostridium difficile*, a pathobiont associated with wheezing and atopy in children [66,48]. A meta-analysis of 23 studies concluded that infants born via ceasarian section have a 20% increase in risk of developing asthma during childhood compared to vaginally delivered infants [70].

Among the many benefits already described for breastfeeding, it also appears to confer protection against atopy [71,72] except when there is genetic predisposition to the disease [73,74]. Breastfed babies have higher numbers of *Bifidobacterium* species and a reduction in *C. difficile* colonization [66,69]. Interestingly, breast milk acts not only as a prebiotic in the infant gut but it also contains many species of lactic acid bacteria that are transferred to the infant intestine [75].

Antibiotic use in early infancy is known to induce severe alterations to the infant gut microbiota and to allow the emergence of pathobionts [76–78]. Several studies have found that antibiotic use results in an increased risk of asthma [79–81]. However, since antibiotics are often prescribed for respiratory infections, these findings may be confounded by indication.

3.2. TLR-mediated sensing of the microbiota and mechanisms of allergic inflammation

Atopic diseases are an example of how events that occur within the gut affect distal mucosal sites through the interaction between intestinal bacteria and mucosal immune cells. The theory that mucosal tissues act as a system-wide organ that shares immune cells at different mucosal sites may explain how intestinal immune responses are transferred to the lung and other tissues [82]. However, the cellular mechanisms controlling these transfers are not fully understood. Several immune mechanisms associated with the development of atopy involve innate immunity signaling pathways that interact with the intestinal microbiota. Many of these pathways are triggered by the activation of sensory proteins called pattern-recognition receptors (PRRs). A major class of PRRs is the Toll-like receptors (TLRs), which recognize a variety of conserved microbial associated molecular patterns (MAMPs), and signal for the transcriptional activation of a variety of downstream immune responses mainly through adaptor proteins, including MyD88 [83].

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Historically, it was understood that TLRs expressed on the surface or in the cytosol by host intestinal epithelial cells (IECs) were used to discriminate commensal symbionts from foreign pathogens. However, it is now widely accepted that stimulation of TLRs by commensal microbes occurs in basal conditions in the intestine, and is in fact required to maintain intestinal homeostasis [84]. Studies of germ-free and TLR-deficient mice have provided much of our current understanding of how our intestinal microbiota interact with TLRs. The immune system in germ-free mice lacks many immune components including circulating antibodies, mucus production, antimicrobial protein production, and mucosal T-cells, emphasizing the essential role of PRR recognition of the intestinal microbiota for proper immune function [85]. Germ-free animals fail to develop immune tolerance, and are susceptible to developing hypersensitivity reactions to external antigens, such as allergic airway inflammation [55]. These data demonstrate that the presence of the intestinal microbiota is required to ensure regulation of immune responses to external antigens. Determining which constituents and signals from the microbiota help regulate immunological responses to external antigens will be important for our understanding of atopic diseases. Roles for particular microbial species in rescuing germ-free immune phenotypes are the subject of many current studies. Mono-colonization of germ-free mice with *B. fragilis* revealed that TLR signaling and activation is required to promote tolerance. The ability of *B. fragilis* to establish a symbiotic relationship with the host, and to promote tolerant immune responses, requires signaling through TLR2 by PSA, and *B. fragilis* isolates without PSA lose their symbiotic abilities [86]. This finding implies that evolutionary conserved TLR-MAMP interactions are important to establish host-microbe symbiosis and for promotion of immunological tolerance to commensal bacteria.

A role for TLR4, which detects bacterial lipopolysaccharide (LPS), in mediating Th2 hyper-reactivity to antigens has been well studied. Murine studies of TLR-MAMP interactions derived from the intestinal microbiota have led to important insights into mechanisms of atopy. Optimal Th2 responses to non-pathogenic antigens require TLR4, as mice deficient in TLR4 develop worsened disease in response to a pulmonary challenge of ovalbumin after sensitization [87]. This indicates that TLR4 signaling via MAMPs may be critical in preventing immune dysregulation that leads to allergic airway disease. In mice treated from two weeks with an antibiotic cocktail, intragastric administration of peanut allergen with cholera toxin induces allergen-specific IgE, elevated histamine levels and anaphylactic symptoms in TLR4-deficient mice but not controls [88]. Once the microbiota repopulates post-antibiotic treatment, peanut allergen-specific IgE and Th2 responses are reduced.

Further evidence exists demonstrating TLR-mediated signaling can induce tolerant immune responses, rather than just inflammatory signals. In the small intestine, TLR-signaling can elevate the production of IgA and chemokines which promote the recruitment of B cells to the lamina propria [89,90]. Furthermore, germ-free mice are severely deficient in IgA+ plasma cells, indicating the need for microbial signals to stimulate IgA production [85]. Low levels of IgA antibodies have been linked to allergy and asthmatic conditions early in life [91]. Secreted IgA is preferentially utilized by the immune system to recognize the intestinal microbiota and it possesses a variety of unique properties, including the inability to activate complement pathways, enabling the immune system respond to microbial signals in a tolerant fashion [92]. Host repertoires of IgA cover the majority of the intestinal microbiota without eliciting potent and potentially damaging responses, and seem to be specific for distinct bacterial epitopes of commensals [93]. IgA-mediated effects on tolerating the microbiota could train the immune system to recognize external food antigens in a more tolerant fashion, dampening the risk of food allergy. The extent of TLR-mediated signaling that results in microbiota-specific IgA production or which microbes are required to stimulate this response is unknown.

Mice deficient in MyD88 are used to mimic a situation where particular TLR-signaling events are absent, and many studies link immune defects seen in these mice to an absence in TLR-MAMP signaling cascades. The specificity of MyD88 related phenotypes to TLR signaling deficiencies is not perfect, as these mice are also deficient in IL-1, IL-18 and IL-33 production and not all TLRs signal through MyD88 [94]. Despite this, studies in these mice remain useful to assess whether microbial signals are required for specific immune responses. Antibiotic-induced depletion of the microbiota has been shown to elevate serum IgE concentrations and promotes exacerbated Th2 cell responses to an external allergen [56]. Furthermore, commensal-derived signaling through MyD88 is required to limit serum IgE concentrations from B cells and basophil hematopoiesis in mice, as MyD88-deficient mice have elevated numbers of basophils and a higher concentrations of IgE in the serum [56]. This provides an example of how microbiota-derived signals, through a MyD88 mediated pathway, are required to prevent host susceptibility to Th2-dependent allergic inflammation.

Another immune factor in the development of allergic inflammation is the presence of well-developed gut associated lymphoid tissue (GALT). In germ-free mice, the GALT is immature; Peyer's patches, mesenteric lymph nodes (MLNs) and isolated lymphoid follicles are all small and underdeveloped [95]. It is unclear how microbial signals lead to the development of a GALT. Maturation and development of the GALT is dependent on colonization of the microbiota [96]. MyD88-deficient mice have smaller Peyer's Patches than wild-type mice at 10 weeks post-birth [97]. Recently its been shown that T cell populations in the small intestinal GALT and epithelium are reliant on a species-specific microbiota, indicating that a co-evolved host-specific microbiota is required for immune maturation [98]. When human microbiota was transplanted into germ-free mice, they failed to stimulate intestinal T cell numbers to levels higher than the basal level seen in a germ-free state and their Peyer's Patch sizes were significantly smaller. This suggests that exposure to general commensal MAMPs is not sufficient to induce intestinal immune maturation, and only particular MAMPs will interact with PRRs to signal for a mature immune system. This is concordant with ideas brought forward by the "old friends" and "disappearing microbiota hypothesis," introduced earlier, as an absence of the right gut microbes could shift the balance toward a higher risk for Th2 inflammatory atopic diseases.

Microbial antigens can be sensed by dendritic cells (DCs) and functional mechanisms of antigen uptake by DCs have also been linked to aspects of allergic and inflammatory diseases [99]. Mechanisms for this remain poorly understood; however DCs expressing TLRs seem to get "educated" in distinctive ways by different microbial signals. Under homeostatic conditions, signaling of the microbiota through MyD88 can instruct mononuclear phagocytes to inhibit the transport of luminal commensal bacteria to the MLNs, a key immune inductive site [100]. Loss of MyD88 signaling leads to the trafficking of non-invasive bacteria to the MLNs, promoting more potent inflammatory T-cell responses to microbial antigens [100]. In a homeostatic environment, signaling events from the microbiota through a MyD88-dependent mechanism can thus promote a tolerant response by the intestinal immune system.

The role for TLR-signaling in tolerant responses opens up potentially important avenues for research into how the intestinal microbiota can influence atopic diseases. Furthermore, it attaches possible immune mechanisms to support the epidemiological evidence brought forward by the hygiene hypothesis. Future studies are needed to elucidate mechanisms of how the immune system directs tolerant responses toward specific MAMPs, rather than releasing pro-inflammatory mediators. Taken together, the evidence discussed thus far indicates an optimal microbiota

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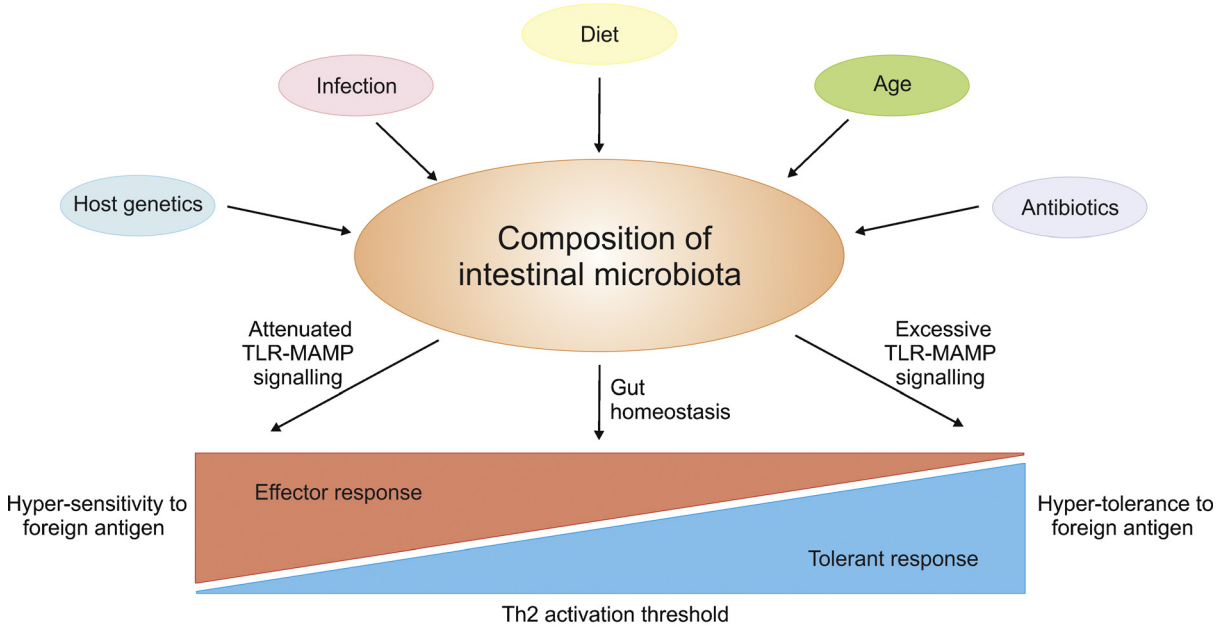

Fig. 2. An explanation for how diet, age, infection, genetic or antibiotic-induced changes in the composition of the intestinal microbiota can alter the sensitivity of the Th2 immune system toward a foreign antigen. The pool of MAMPs provided by the intestinal microbiota can be sensed by TLRs, expressed by the intestinal epithelium and many immune cells, which provide signals to control the responsiveness of Th2 arm of the immune system. In contrast to their pro-inflammatory role during infection, TLR recognition of the intestinal microbiota is critical for maintaining gut homeostasis by stimulating secretion of tolerant signals and regulating inflammatory responses. This can be thought of as an education or conditioning of immune cells to a homeostatic state where only a high dose antigen encounter and/or presence of potent danger signals will activate the potential damaging inflammatory effector response mediated by the Th2 cells. Thus under homeostatic conditions, TLR-MAMP interactions promote a balance between Th2 cell effector responses and tolerant responses induced by regulatory cells. However hypersensitivity responses to a foreign antigen can be brought about by changes in the microbiota composition, leading to an inability for the intestinal microbiota to stimulate TLRs to a level required to provide the regulatory and tolerant signals needed to keep the Th2 effector response in check. On the opposite spectrum, an over-stimulation of TLRs by the intestinal microbiota can promote hyper-tolerance to foreign antigens as the Th2 sensitivity to danger signals becomes elevated to a point where the host becomes unresponsive and tolerant to systemic insults with foreign antigens. Post-industrialization, we may be missing the presence of the "right" microbial signals to balance tolerant and effector responses, leading to atopic disease from a hypersensitive Th2 response. Yet in areas of poor sanitation where atopy is non-existent and vaccines are not as responsive, this could be due to over-stimulation of TLRs by microbial MAMPs, leading to hyper-tolerance to foreign antigens and dampening of Th2 mediated effector responses. Overall, preserving a proper balance between effector and tolerant immune responses is of great important to host fitness and care for your intestinal community of microbes will promote the presence of the TLR-MAMP signals needed to maintain immune responses to foreign antigens that are regulated and controlled.

composition may exist for each particular host, providing the right balance of tolerant and effector signals needed to regulate Th2 mediated immunity (Fig. 2). Despite the evolutionary need for Th2 immune response for recognition and clearance of external (potentially harmful) toxic antigens [101], these responses can be damaging to the host if left uncontrolled.

Given the role of immunomodulatory MAMPs in the microbiota regulating Th2 immunity, potential therapeutics for allergic conditions could involve the use of TLR-agonists, providing tolerant signals to the immune system and dampening the potential of a potent pro-inflammatory response. Large studies are ongoing to explore the use of TLR4 agonists for reducing allergic disease severity [102]. Probiotic, prebiotic and symbiotic therapeutics for atopic disease are also beginning to be discussed, based on recent literature showing the protective effects of these therapies on symptoms of atopy (Table 1). It is unlikely, however, that one single probiotic strain will impart resistance to atopy, thus future studies will focus on ecosystem dynamics, and the possibility of transferring multiple strains of bacteria to a diseased host. Methods of transferring multiple strains of bacteria to a human host are beginning to be utilized to cure intestinal diseases such as *C. difficile* associated colitis and could be relevant as an intervention to reverse symptoms of atopic conditions given the evidence provided thus far [103].

Table 1
Microbiota-based therapeutic interventions to cure atopic conditions.

Treatment	Effect	Reference
Lactobacillus GG	50% reduction in observed atopic eczema in high-risk infants	[129]
L. rhamnosus and *Bifidobacterium anamalis ssp lactis*	Children fed strains in formula for 2 months significantly improved symptoms of atopic dermatitus	[130]
L. rhamnosus and *L. reuteri*	Greater than 50% improvement to atopic dermatitis after 6 weeks of treatment in 1–13 yr olds	[131]
E. coli expressing recombinant peanut proteins	Decreased levels of IgE and Th2 cytokines IL-4, IL-13 in response to a peanut allergen challenge in mice	[132]
L. planatarum	Inhibits dust-mite specific T cell responses in mice	[133]
Galacto-oloigosaccharide and inulin (GOS/inulin)	Infants fed this prebiotic mix for 6 months had a significantly reduced risk for allergic disease, including atopic dermatitis and wheezing	[134,135]
Bifidobacterium breve + GOS/inulin	Reduced incidence of allergy and anaphylaxis in mice after weaning	[136]
L. paracasei	Administration for 30 days significantly reduced symptoms of allergic rhinitis in human adults.	[137]
Fructo-oligosaccharide	Reduction of contact hypersensitivity in mice	[138]

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4. Immune-microbiota interactions in conditions with poor sanitation: the "Reverse Hygiene Hypothesis"

The hygiene hypothesis was initially proposed to explain the rise in the prevalence of hay fever and other allergic diseases in industrialized countries. More recently, the hypothesis has evolved into the concept that an altered composition of gut microbiota could alter immune development, resulting in an increase in atopic diseases. An important, yet unexplained, epidemiological feature of post-industrialization diseases is their relative absence in rural parts of developing countries and their rise in frequency with the adoption of a modern lifestyle [20]. The rise in atopic diseases since the industrial revolution is clear, yet many populations of humans are not effected by these conditions. Understanding the immune mechanisms behind protection from atopy in these populations, rather than just susceptibility, may provide further insight to these complex diseases.

4.1. Chronic infections and environmental enteropathy

Chronic, recurrent infections are a defining feature of populations in the developing world living in areas of poor sanitation and hygiene. Furthermore, many of these individuals are malnourished, and it is well documented that malnutrition can cause a secondary risk of immunodeficiency and a greater susceptibility to infection in humans [104]. Infection itself can also contribute to malnutrition, and this sets up a vicious cycle; an inadequate diet impacts the immune system, increasing the incidence and severity of infection, which leads to an altered metabolism and nutrient loss, exacerbating malnutrition of the host [105]. Concordant with the original observations described in hygiene hypothesis, there is a large body of evidence supporting a potentially protective role of exposures of a wide variety of pathogens against atopy [106–110]. The presence of chronic infections in individuals raised in areas with poor sanitation could alter immune function in a way that protects against atopy, although mechanisms for this protection are unknown.

Individuals living in areas of poor sanitation are also susceptible to developing chronic intestinal inflammation independently of any known infectious etiology. Biopsies from the small intestine of these individuals reveal many distinct characteristics of a subclinical disorder called environmental enteropathy [111,112]. This condition, also known as tropical enteropathy, is a poorly characterized chronic inflammatory disease that primarily affects the small intestine. Environmental enteropathy afflicts individuals who reside for relatively long periods of time in areas with poor sanitation and who have a high exposure to fecal-contaminated water and food [111]. The hallmarks of environmental enteropathy are growth stunting, malabsorption, increased gut permeability, villous blunting, and increased numbers of intraepithelial lymphocytes [113]. Immunologically, individuals with environmental enteropathy show the skewing to a Th1 response in the gut environment, and an increase in intraepithelial CD8+ lymphocytes [114]. Inflammatory cytokines secreted by polarized Th1 cells, such as IFN-γ and TNF-α, can lead to intestinal tissue damage and greater epithelial cell turnover [115]. Studies on adults in Zambia with environmental enteropathy show evidence of increased T cell activation as measured by CD69 and HLA-DR expression [116]. It is therefore likely that histopathological changes observed in environmental enteropathy could be mediated by T cell induced inflammatory damage. While many immunological characteristics of this disease remain poorly characterized, it seems reasonable to postulate that individuals living in regions with high oral exposures to water and food contaminated with microbes will have an altered intestinal immune system than those without these exposures [112]. This altered immune system seen in individuals with environmental enteropathy could function to protect against atopic diseases [117].

Studying how altered immune functions described in these individuals affects the immune response to external antigens could bring fresh insight into the mechanisms of why atopic diseases are extremely rare in areas of poor sanitation. Oral vaccination involves the introduction of an immunogenic antigen or attenuated-organism, and interestingly there is significant heterogeneity in the responses to oral vaccination in children living in industrialized societies compared to children raised in areas of poor sanitation in developing countries [118]. In clinical trials, individuals raised in rural areas of developing countries have shown a lower immunogenicity and efficacy to oral vaccines for polio, rotavirus, *Shigella* and cholera [119]. It was further demonstrated that excessive bacterial growth in the small intestine of children in less developed countries may contribute to the low antibody response to an oral cholera vaccine [120]. The condition, termed small intestinal bacterial overgrowth, is commonly observed in children with environmental enteropathy, indicating how the altered microbiota seen in these individuals could modulate immune function [111].

One possible explanation for the dampened responses to vaccines, and low prevalence of atopy, could be the differing tolerance capabilities of these individuals. Many patients with environmental enteropathy show increased intestinal permeability or a "leaky" gut barrier. This leaky barrier exposes the systemic immune system to a greater variety of potentially harmful and immunogenic antigens translocating the mucosal barrier. This constant exposure of microbial and external antigens to the systemic immune system could lead to systems of tolerance, rather than the immune system eliciting an effector response to attempt to clear the antigen [121]. It is well known that immune tolerance is correlated with increased exposure to an antigen, even one that is typically immunogenic. A good example of this is seen in endotoxin tolerance. Exposure of the immune system to small amounts of endotoxin over a period of time leads to mechanisms down regulating the NF-κB responses to LPS, as these signals are damaging to the host in the face of constant exposure [122]. It is recently been demonstrated that systemic antibodies generated against conserved bacterial polysaccharides dampens allergic airway disease induced by *Aspergillus fumigatus* by significantly down-regulating the influx of allergen associated cell types and cytokine production [123]. This represents a good example of how increased systemic exposure to MAMPs can alter immune mechanisms related to atopic disease. Allergic conditions are triggered by a systemic immune response that is damaging to the host tissue environment, leading to inflammation and mast cell degranulation. In situations where the host is constantly exposed systemically to small levels of external antigens, the threshold of this damaging response could be raised and more tolerogenic mechanisms promoted.

4.2. Dietary and helminth related microbiota alterations

Another explanation for the lack of atopic disease in developing countries also could be a more direct effect by distinct gut microbiota seen in individuals in the developing countries, whose environmental exposures and diet have not changed as dramatically as in developed countries [17]. Post-industrialization has enriched for an altered microbial composition and the implications of this are just beginning to be realized. The "disappearing microbiota hypothesis" echoes this fact, with environmental exposures to antibiotics, toxins and chemicals increased post-industrialization [124]. This is concordant with a dramatic dietary change, more reliant on simple carbohydrates and maize-based products, and less abundant in complex polysaccharides. Studies of the assemblage of the gut microbiota showed marked differences when comparing European versus African subjects, notably a larger portion of Bacteroidetes and Prevotellaceae in adults in Burkina Faso, along

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with bacteria known to be involved in the degradation of complex polysaccharides [18]. Although the focus has mainly been on bacteria, stable communities of viruses and eukaryotes exist in the intestinal tract. Additional tools need to be developed so that they can be extended to study these viral and eukaryotic components. This includes intestinal helminths, which can compete for nutrients within the intestines of infected individuals, and shift the composition of the intestinal microbiota [125]. Interactions between helminths and our immune system have been decreased post-industrialization, and disappearance of these interactions can modulate immune function [126]. Helminths can shape immune function through factors such as excretory-secretory products, which have been shown to modulate cytokine production, basophil degranulation, immune cell recruitment and interference with TLR signaling [112,127]. Colonization of helminths also suppresses Th2 type allergic inflammation in humans, dampening T-cell responsiveness and upregulating tolerant signals to external allergens [128]. There has also been interest in helminth "immunotherapy" to reverse atopic symptoms [128]. Aside from their direct effects on the immune system, the resulting shift in microbiota composition in helminth colonized individuals could potentially alter regulatory immune responses.

5. Conclusions and prospectus

The development of atopic disease is a complex process centered on the development of a distinct, pro-inflammatory Th2 reaction to an external antigen, with susceptibilities based on environmental, immunological and genetic factors. As a result of these complex interactions, a single, unifying mechanistic explanation for the development or prevention of atopic disease is unlikely. Nevertheless, findings described in this review indicate that interactions between the immune system and the intestinal microbiota play a central role in susceptibility to an "over-exuberant" Th2 inflammatory response. Constituents of the intestinal microbiota are immunomodulatory, and colonization of particular subsets of microbes can confer immune signals that are either be inflammogenic or tolerant in nature. These signals feed into immune function by selecting for the development of effector versus regulatory T cells in the intestine, influencing the function and trafficking of DCs, proliferation of allergy-mediating basophils and the maturation of a mature GALT. The compositional nature of the intestinal microbiota is sensed by TLRs, whose signaling events can regulate many aspects of Th2 immunity. Consequently, the presence or absence particular MAMPs in the intestine could influence regulatory components of the immune system.

Through development of a peripheral "tolerance education," the maintenance of a complex microbial community, containing immunomodulatory molecules that mirror those humans have co-evolved with, is of paramount importance. Immune differentiation of danger signals from ubiquitous antigen encounters may require the presence of feedback mechanisms conferred by our external microbial exposure. Dependence of our immune system on microbial exposure is clear, based on studies of germ-free mammals. Yet, each host could require a particular microbial exposure to achieve immune homeostasis, and avoid a dysregulated Th2 immune response to external antigenic insult. Further studies are needed to completely understand the mechanistic and temporal nature of these interactions. Overall, as our diet, antibiotic use and surrounding environment have rapidly changed post-industrialization, tailoring microbial exposure to reflect the distinct evolutionary histories of particular individuals could be an avenue worth pursuing for potential therapeutic intervention. This could be done using probiotics containing import immunomodulatory signals, prebiotics to promote the growth of beneficial microbes or ecosystem therapeutics using bacteriotherapy.

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