

Toll-like receptor ligands: hygiene, atopy and therapeutic implications

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Purpose of review

Allergic and certain other inflammatory diseases have become more common in industrialized countries over the past few decades. One potential explanation for such trends is that with a decreased incidence of microbial exposures, as a result of modern public health practices, an important source of immune stimulation has been lost, with a consequent increase in inflammatory responses and their associated diseases. This review will focus on our current understanding of how microbial exposures impact on host immunity and the pathogenesis of allergic diseases.

Recent findings

In the past decade, it has become clear that a number of molecular interactions between immunocytes and microbial compounds are mediated by Toll-like receptors on host cells. Moreover, recent investigations have suggested that ligands for different Toll-like receptors have the potential both to inhibit and promote the development of allergic hypersensitivities and diseases.

Summary

On the basis of studies discussed herein, we speculate that physiological exposures to Toll-like receptor ligands have important yet complex effects on immune homeostasis and host susceptibility towards atopic diseases. Moreover, we anticipate that a fuller understanding of how physiological Toll-like receptor ligand exposures impact on immune development will lead to novel therapeutic interventions for the prevention and treatment of atopic diseases.

Keywords

allergy, asthma, CpG motif, hygiene hypothesis, immunostimulatory sequence oligodeoxynucleotide, lipopolysaccharide, peptidoglycan, Toll-like receptor

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Abbreviations

ISS immunostimulatory sequence
PAMP pathogen-associated molecular pattern
TLR Toll-like receptor

Introduction

In its simplest iteration, the 'the hygiene hypothesis' proposes that modern public health practices have led to the relative sterilization of living environments in the developed world and a consequent deficit in microbial stimulation of host immunity [1,2]. As a result, an imbalance in immune homeostasis is thought to exist, which predisposes towards the development of allergic diseases and other diseases of immune dysregulation.

Strong circumstantial evidence for the concept of hygiene impacting on the pathogenesis of allergic diseases comes from epidemiological studies. First, changes in atopy prevalence rates in the developed world have occurred in temporal association with the implementation of public health measures that protect against the spread of infections. These include indoor plumbing, decontamination of water supplies, pasteurization and other food sterilization procedures, routine vaccination against common childhood infections, and the ready availability of antibiotics [3–6]. People living in countries without such health standards continue to be plagued by infections that have been all but eradicated from wealthy nations, whereas allergic disease prevalence rates remain low [4–6].

Within developed countries, antibiotics, vaccinations, and living conditions that reduce the frequency of community-acquired infections have been suggested to increase the relative risk of the development of allergic manifestations [5–7]. In contradistinction, anthroposophic lifestyles that include a microbe-rich diet, limited vaccinations, and infrequent antibiotic use, have been associated with protection from atopy [8], as have infections with tuberculosis, measles and hepatitis A [9–12]. However, although results from many epidemiological investigations support a role for infections and non-invasive microbial exposures in protecting against the pathogenesis of allergic diseases, not all studies have reached the same conclusion. In particular, infections with several different respiratory viruses, in particular respiratory syncytial virus, have been suggested to precipitate the development of asthma [13–15]. Furthermore, *Staphylococcus aureus*-infected skin is a known cause of eczema flares in patients, whereas the application of *S. aureus*-derived lipoteichoic acid to shaved skin facilitates allergen sensitization and the pathogenesis of experimental eczema in mice [16,17,18•].

As originally proposed, the hygiene hypothesis suggested that infections induce T helper cell type 1-biased immunoresponsiveness, rendering the host resistant to diseases fueled by T helper cell type 2-biased immune dysregulation. However, this supposition has recently been challenged by several observations. For example, parasitic infections that induce high serum IgE levels and robust T helper cell type 2-biased cellular responses are associated with protection from the development of allergen-specific IgE and atopic manifestations [4,19,20]. Furthermore, whereas the prevalence rates for diseases of T helper cell type 2-biased immune dysregulation are increasing in industrialized countries, so too are prevalence rates for inflammatory bowel disease and several T helper cell type 1-biased autoimmune diseases. Of particular relevance to the hygiene hypothesis, diabetes and other autoimmune diseases appear to develop more frequently in children with lower levels of microbial exposure [4,21,22]. These findings suggest that the T helper cell type 1/2 paradigm may not explain why microbial exposures protect against atopy and other diseases of immune dysregulation [4].

On the basis of published literature, we and other investigators believe that non-invasive microbial exposures and infections are 'generally' protective against the development of allergic hypersensitivities and diseases, but important questions remain. For example, can select microbial infections also enhance the genesis of allergic diseases? In addition, although the molecular pathways by which microbes activate innate immunity have been fairly well characterized, it is less clear how these pathways affect the pathogenesis of allergic diseases. This review will focus on these issues, offering evidence that cell activation through Toll-like receptors (TLRs) have profound yet complex and potentially bipolar effects on host immunity and the pathogenesis of allergic diseases.

Microbes elicit innate immune responses via Toll-like receptor activation

In the past decade, we have learned that polymorphonuclear and mononuclear cells express a range of pattern recognition receptors that discriminate between potential pathogens and self. These receptors interact with microbial components that have been termed 'pathogen-associated molecular patterns' (PAMPs) [23]. TLRs have recently been identified as playing a central role in the detection of PAMPs and the early host response to microbial infections [23,24*,25]. To date, ligands for eight of the 11 known mammalian TLRs have been identified. TLR2 recognizes various components of Gram-positive bacteria, mycobacteria, and fungi; TLR3 senses double-stranded RNA; TLR4 recognizes lipopolysaccharide; TLR5 recognizes bacterial flagellin; TLR7 (mouse and human) and TLR8 (human) recognize viral

single-stranded RNA sequences and several synthetic imidazoquinolines; TLR9 recognizes CpG DNA sequences that are rare in mammalian genomes but not the genomes of most bacteria, fungi, and DNA viruses; and TLR11 has recently been shown to play a role in innate responses to uropathogenic bacteria [26*] (Table 1).

Signaling pathways used by all TLRs lead to nuclear factor kappa B and mitogen-activated protein kinase activation. Some of the TLRs also activate members of the IRF family. Most of the TLRs signal through a shared molecular pathway that utilizes MyD88. However, recent work has shown that some adaptor molecules, including TIRAP, TRAM and TRIF, are used by only a limited number of TLRs. Such differential utilization of these and potentially other signaling molecules may explain why ligands for different TLRs have been found to elicit divergent innate and adaptive immune responses [24*,25].

Immune regulation in functionally Toll-like receptor-deficient mice

In previous studies, mice raised under germ-free conditions were shown to have a deficit in their capacity for mucosal tolerance and a predisposition towards mounting T helper cell type 2-biased responses [27]. MyD88 plays a critical role in signaling via all TLRs characterized to date [24*]. However, unlike other TLRs, ligands for TLR3 and TLR4 continue to activate MyD88 knockout mononuclear cells [24*]. Therefore, to a great but not complete extent, the MyD88 knockout mouse approximates immune development in the absence of TLR ligands. With these considerations in mind, the finding that MyD88 knockout mice spontaneously developed higher levels of serum IgE than wild-type mice is quite provocative [28]. Furthermore, in these same investigations, the T helper cell type 1

Table 1. Toll-like receptors and their ligands

Receptor	Microbial and synthetic ligands
TLR1	Triacyl lipopeptides (e.g. Pam3Cys),
TLR2	Lipoproteins, lipopeptides (e.g. Pam3Cys), peptidoglycan, zymosan, lipoteichoic acid, atypical lipopolysaccharide, lipoarabinomannan
TLR3	Double-stranded RNA (e.g. poly I:C)
TLR4	Lipopolysaccharide, taxol, viral F-protein (e.g. respiratory syncytial virus)
TLR5	Flagellin
TLR6	Diacyl lipopeptides, zymosan, lipoteichoic acid
TLR7	Imidazoquinoline, loxoribine, single-stranded RNA viruses
TLR8	Imidazoquinoline, single-stranded RNA viruses
TLR9	CpG DNA and oligonucleotides
TLR10	Not determined
TLR11	Ligands derived from uropathogenic bacteria

For further information see Medzhitov and Janeway [23], Akira and Takeda [24*], and Beutler [25].

responsiveness of MyD88 knockout mice to antigen in Freund's adjuvant was lost, whereas these mice developed normal T helper cell type 2-biased responses to antigen/alum vaccination. In unpublished studies, we also found that unimmunized MyD88 knockout mice have elevated serum IgE levels and T helper cell type 2-biased IgG subclass levels (more IgG1 and less IgG2a) compared with wild-type mice. Moreover, relative to wild-type CD4 cells, MyD88 knockout CD4 cells expressed higher levels of GATA3 (an important T helper cell type 2 regulatory factor) and developed a more T helper cell type 2-biased cytokine response to anti-CD3/CD28 stimulation.

Given its central role in TLR ligand signaling and the results just cited, it is tempting to speculate that deficiencies in physiological TLR stimulation are responsible for the intrinsic T helper cell type 2-biased immunoreactivity of MyD88 knockout mice. However, alternative explanations exist, as MyD88 not only participates in TLR signaling but also in signaling through the IL-1 and IL-18 receptors [28]. Although mice with unrelated IL-1 and IL-18 signaling deficiencies do not appear to share the immunological phenotype of MyD88 knockout mice [28,29], MyD88 is likely to play as yet unidentified roles in immunocyte functionality that are unrelated to its role in TLR signaling. In addition, whereas IRAK-4, like MyD88, plays a central role in TLR signaling [30,31], three patients described as having IRAK-4 deficiency, although predisposed to pyogenic infections, were not noted to be atopic [30]. All points considered, it still remains reasonable to suggest that deficiencies in physiological immune activation by ambient TLR ligands may contribute to the intrinsic T helper cell type 2 bias of MyD88 knockout mice.

Epidemiological studies link Toll-like receptor ligand exposure levels with relative atopic risk

Because of the ubiquitous distribution of microbes in our environment, we and others suggest that TLR ligand exposures via the intestines, inspired air, and skin are likely to provide an important molecular link between microbes and normal immune development. Unfortunately, to date, epidemiological studies of TLR ligand exposures and their association with allergic disease pathogenesis have focused primarily on lipopolysaccharide (endotoxin). Nonetheless, these epidemiological investigations have proved quite enlightening.

von Mutius [6] found that compared with control family homes, endotoxin levels are higher in the homes of farmers with livestock, and their family members have a lower incidence of allergic diseases. This inverse association between endotoxin exposure and atopy rates

was also been found for infants living in more urban environments [32]. Additional evidence for environmental endotoxin influencing the pathogenesis of allergic diseases comes from genetic studies that have linked CD14 (lipopolysaccharide binding protein) polymorphisms with the severity of the atopic phenotype [33].

Although the case for lipopolysaccharide remains, it is important to remember that other immunologically active microbial products, including ligands for other TLRs, are also abundant in living environments. Investigations have already determined that compared with homes with lower levels of endotoxin, homes with higher endotoxin levels also have elevated levels of TLR2 and TLR9 ligands [34,35]. Therefore, it remains to be determined whether lipopolysaccharide directly protects against atopy or whether higher home endotoxin levels are an indirect measure of exposures to additional TLR ligands or other microbial products with more potent anti-allergic activities. In order to address this issue, future epidemiological studies will need to consider the many immunostimulatory microbial products that are likely to be present in homes and other environments of daily living.

TLR4 ligand has paradoxical effects in murine asthma models

In the laboratory, lipopolysaccharide has been found to have divergent effects on the allergic phenotype. It has long been known that *Escherichia coli* lipopolysaccharide (TLR4) promotes murine IgE synthesis *in vitro* [36]. However, in an in-vivo rat model, *E. coli* lipopolysaccharide inhibited the development of allergen-specific IgE and asthma if delivered within 6 days of allergen sensitization, whereas lipopolysaccharide exposures after this period led to an exaggeration of the asthmatic phenotype [37]. Recently, Eisenbarth and colleagues [38] also reported on the seemingly schizoid immunological activities of *E. coli* lipopolysaccharide. The investigators found that *E. coli* lipopolysaccharide induced either T helper cell types 1 or 2-biased responses to intranasally co-administered OVA when delivered at high or low doses, respectively. Low-dose lipopolysaccharide vaccinations also primed mice for the development of the asthmatic phenotype. The paradox of differential T helper cell types 1 and 2 adjuvant activity with high and low-dose lipopolysaccharide has yet to be resolved, but experiments conducted by Kaisho *et al.* [29] with MyD88 knockout dendritic cells may be relevant to its understanding. The investigators demonstrated that MyD88 knockout dendritic cells activated with *E. coli* lipopolysaccharide induced T helper type 2 cell differentiation, whereas lipopolysaccharide-activated wild-type dendritic cells induced T helper type 1 cell differentiation. Considered in context with the studies of Eisenbarth *et al.* [38], these

observations suggest that a MyD88-independent lipopolysaccharide signaling pathway induces T helper type 2 cell development and is responsive to lipopolysaccharide at low concentrations, whereas the MyD88-dependent pathway dominates at high lipopolysaccharide concentrations and favors T helper type 1 cell development.

TLR9 ligands prevent and reverse the allergic phenotype in mice and humans

The genetic material from a variety of fungi, bacteria, and DNA viruses has been found to have inherent immunostimulatory activities that are not shared by mammalian DNA [39]. The molecular basis for microbial genomes having immunostimulatory activities lies in their content of unmethylated CpG dideoxynucleotides, which are far less frequent in mammalian genomes. There are many excellent reviews of the immunostimulatory and anti-allergic activities of synthetic immunostimulatory sequence (ISS) oligodeoxynucleotides (also known as CpG oligodeoxynucleotides) [40–42], and only brief mention of these will be made here.

ISS oligodeoxynucleotides have been found to induce an innate response dominated by cytokines (e.g. IL-10, IL-12, type 1 and type II IFNs) that are known to inhibit the allergic phenotype [40–42]. Moreover, as an allergen-independent therapeutic agent given to T helper cell type 2-sensitized mice just before allergen challenge, ISS oligodeoxynucleotide has proved more effective than corticosteroids in attenuating the hypersensitivity responses of asthma [40,42], allergic conjunctivitis [43], and allergic rhinitis. It is believed that the innate response to ISS oligodeoxynucleotide is responsible for its rapid attenuation of effector cell functions in an allergen-independent manner. Interestingly, although allergen-independent ISS oligodeoxynucleotide therapy can potentially attenuate the allergic phenotype in T helper cell type 2-sensitized mice, the effect is only temporary, as underlying allergen hypersensitivities are not irreversibly extinguished [40,44].

Applying the vaccination paradigm to its study, ISS oligodeoxynucleotide has also been found to be a potent systemic and mucosal adjuvant that promotes highly T helper cell type 1-biased immune responses [45,46]. Moreover, in murine models of asthma and anaphylaxis, ISS oligodeoxynucleotide has shown great promise as an anti-allergic vaccine adjuvant [47–49]. In this context, the allergen-specific response induced by immunization with ISS oligodeoxynucleotide prevents and reverses T helper cell type 2-biased hypersensitivities at the level of adaptive immunity, and the effect appears to be both allergen specific and long lasting [40]. Pre-clinical investigations with ISS oligodeoxynucleotide-based vaccines have been so promising that phase I/II clinical

trials with allergic rhinitis patients have already been conducted, with preliminary but favorable results [50,51].

TLR2 ligands promote asthma pathogenesis in mouse models

Studies comparing the immunological activities of lipopolysaccharide isolated from different bacteria have made it apparent that whereas many lipopolysaccharide species signal through TLR4, other atypical lipopolysaccharide species signal through TLR2. Moreover, when used at the same doses in vaccination studies, TLR2 (*Porphyromonas gingivalis*) and TLR4 (*E. coli*)-specific lipopolysaccharide were found to induce T helper cell types 2 and 1-biased adaptive responses, respectively [52]. In other investigations, ligands for TLR2 and TLR4 were observed to elicit unique innate dendritic cell and mast cell responses. TLR4 stimulation induced stronger IL-12 production from dendritic cells, whereas TLR 2 stimulation favored mast cell degranulation and T helper cell type 2 cytokine production [53–55]. In our own recently published investigations, the immunological activities of TLR9 ligand were directly compared with those of Pam-3-cys (a synthetic and TLR2-specific agonist) [56] and peptidoglycan (a derivative of bacterial cell walls with both TLR2-dependent and independent immunostimulatory activities) [57]. We found that these TLR2 ligands induced bone marrow-derived dendritic cell responses that were phenotypically similar (i.e. high IL-6/low IL-12/low IL-13 production) to one another but distinct from bone marrow-derived dendritic cell responses to ISS oligodeoxynucleotide stimulation (i.e. high IL-6/high IL-12/no IL-13 production) [56,57]. Moreover, mice immunized with antigen and Pam-3-Cys or peptidoglycan were found to develop T helper cell type 2-biased adaptive responses and were primed to develop T helper cell type 2-biased airway hypersensitivity responses upon airway allergen challenge [56,57]. In contrast, mice immunized with antigen and ISS oligodeoxynucleotide developed robust T helper cell type 1-biased responses but did not develop significant airway hypersensitivity responses after allergen challenge.

In line with studies just reviewed, other investigations have demonstrated that murine skin exposure to *S. aureus*-derived lipoteichoic acid (a known TLR2 ligand) primes mice for the development of experimental eczema [17,18]. Moreover, atopic dermatitis patients are generally heavily colonized by TLR2 expressing *S. aureus*, and low-grade skin infections with this bacteria precipitate atopic dermatitis flares for which antibiotic therapies are often effective [16]. Given these clinical observations and our knowledge of the immunological activities of TLR2 ligands, we speculate that under some conditions, environmental exposures to TLR2

ligands may facilitate the development of allergic diseases. In further support of this position, TLR2 polymorphisms have already been linked to atopy in epidemiological studies.

The immunological influence of other Toll-like receptor ligands

Although it remains to be determined whether TLR3, TLR5, and TLR7/8 ligands can significantly influence the allergic phenotype, their immunological activities have been studied to some extent. In several studies, TLR3 ligands have been found to promote T helper cell type 1-biased adaptive responses [58]. As with TLR4 ligands, conflicting reports exist as to the influence of flagellin (TLR5) on adaptive immunity. Flagellin was originally reported to be a T helper cell type 1 adjuvant [59], and recently it was reported to favor a T helper cell type 2-biased adaptive response [60]. Finally, ligands for TLR7 and TLR8 (i.e. R848 and its derivatives) have been consistently described as potent T helper cell type 1 adjuvants [61,62]. Furthermore, R848, has been found to convert T helper cell type 2 effector cells towards a T helper cell type 0 or even a T helper cell type 1 phenotype *in vitro* [63]. Although not directly applicable to the hygiene hypothesis, these reports further highlight the range of immunological responses that can be elicited by ligands for the different TLRs.

Conclusion

The rapid increase in allergic disease prevalence rates experienced in developed but not underdeveloped countries over the past 50–100 years is likely to be a consequence of environmental factors, as these trends have developed far too rapidly to have a simple genetic basis [4,5]. The hygiene hypothesis proposes that as a result of modern public health practices, individuals living in the industrialized world experience a relative deficiency in immune stimulation by microbes, rendering them vulnerable to the development of allergic hypersensitivities and their associated diseases [1–4]. Fairly recently, it became evident that TLRs and their associated ligands provide an important molecular interface for crosstalk between microbes and man [24*,25]. Therefore, it is logical to consider whether TLR ligands might influence the pathogenesis of allergic diseases. TLR9 ligands have already been identified as having potent anti-allergic activities in mice and humans, and ongoing investigations may find that ligands for additional TLRs also have utility for the prevention and treatment of atopic conditions.

Clearly, additional investigations will be required to develop a complete understanding of the physiological role of TLR ligands in shaping host immunity and the predisposition towards atopy. However, epidemiological

studies and laboratory investigations presented herein suggest that TLR ligands have the potential both to protect against and precipitate the allergic phenotype. The hypothesis put forth by Stachan in 1989 [2] that microbial exposures can protect against atopy has been both provocative and fruitful. Nevertheless, we suggest that the hygiene hypothesis is likely to require modification in order to accommodate the spectrum of immunological activities that can be induced by TLR ligands. Finally, although TLR-dependent PAMPs were the focus for this article, a number of PAMPs stimulate additional immunocyte receptors, suggesting that microbial products may also impact on host allergic disease susceptibility via TLR-independent signaling pathways.

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