

Do microbes influence the pathogenesis of allergic diseases? Building the case for Toll-like receptor ligands

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The prevalence and severity of allergic diseases and other diseases of immune dysregulation are increasing in industrialized countries. One explanation for these trends is that decreased exposure to microbes, due to modern public health practices, has resulted in the loss of a main source of immune provocation, and a consequent increase in pathogenic immune responses and their associated diseases. It is now clear that molecular interactions between immunocytes and microbes are mediated largely by Toll-like receptors (TLRs) on host cells and a diversity of ligands produced by viruses, bacteria and fungi. Physiological exposures to TLR ligands are also likely to have an important yet complex role in host immune homeostasis and predisposition towards atopy.

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Abbreviations

DC	dendritic cell
ISS-ODN	immunostimulatory sequence oligodeoxynucleotide
LPS	lipopolysaccharide
PAMP	pathogen-associated molecular pattern
Th	T helper
TLR	Toll-like receptor

Introduction to the hygiene hypothesis

The hygiene hypothesis was initially proposed by Strachan in 1989 [1] and has been championed subsequently by Martinez and Holt [2], and Liu and Murphy [3], among others. In its simplest iteration, the theory suggests 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. As a result, an imbalance in immune homeostasis is thought to exist, which predisposes individuals towards the development of allergic diseases.

Strong circumstantial evidence for the concept that hygiene has an impact on atopy (a state of hypersensitivity towards environmental allergens) comes from epidemiological studies. First, current trends towards an increasing prevalence of allergic disease in the developed world coincide with the widespread implementation of public health measures to protect against the spread of infections [3,4]; such measures 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. People living in countries without such health standards continue to be plagued by infections that have been all but eradicated from wealthy nations, but the prevalence of allergic disease in these countries remains low [4,5]. Second, in developed countries, antibiotics, vaccinations and living conditions that reduce the frequency of community-acquired infections have been suggested to increase the relative risk for developing allergic manifestations [6,7]. In contradistinction, households in which hygiene standards are poor, coupled with an anthroposophic lifestyle that includes a microbe-rich diet, limited vaccination and infrequent antibiotic use, have been associated with protection from atopy [8,9], as have tuberculosis, herpes simplex, measles, hepatitis A and other infections [7].

Although results from many epidemiological investigations support the idea that infections and other microbial exposures are involved in protecting against the pathogenesis of allergic diseases, not all studies have reached the same conclusions. In particular, the local inflammatory effects of infections with several different respiratory viruses have been proposed to precipitate the development of asthma [10,11]. Furthermore, in one relatively old but well-designed prospective study, infants hospitalized with respiratory syncytial virus infections were found to have an increased risk for developing allergen-specific hypersensitivities [12]. Although we favor the possibility that some infections — particularly if early in life — can precipitate the development of allergic hypersensitivities, except for the above-cited study there is, unfortunately, little evidence at present to support this idea [10].

As originally proposed, the hygiene hypothesis suggested that infections induce T helper 1 (Th1)-biased immunoresponsiveness, which in turn protects the host from diseases fueled by Th2-biased immune dysregulation [4]. Recently, however, this supposition has been challenged by several observations. For example, parasitic infections that induce high concentrations of serum IgE

and a robust Th2-biased cellular immune response are associated with protection from the development of allergen-specific IgE and the clinical manifestations of atopy [4,5]. In addition, the prevalence of diseases related to Th2-biased immune dysregulation has been increasing in the industrialized world, but so too has the prevalence of inflammatory bowel disease and several Th1-biased autoimmune diseases [4,5]. Of particular relevance to the hygiene hypothesis, diabetes and several other autoimmune diseases seem to develop more frequently in children who have had less exposure to microbes [4,13,14].

On the basis of the published literature, we and many other investigators believe that microbial exposures and infections are generally protective against the development of allergic hypersensitivities and diseases. But important issues remain unresolved. For example, although respiratory viral infections are likely to contribute to the pathogenesis of asthma via direct pulmonary effects, it is unclear whether respiratory and/or other infections can fuel the immune dysregulation that precipitates the development of allergen-specific hypersensitivities. Furthermore, the Th1–Th2 paradigm seems to fall short of explaining how microbes protect against atopy. Wills-Karp *et al.* [4] have proposed an alternative paradigm, in which IL-10 and other anti-inflammatory cytokines, which are generated during most infections, maintain host tolerance by inhibiting both Th1- and Th2-biased pathogenic responses to non-pathogenic antigens. Although attractive, however, this theory has not been rigorously challenged, and the means by which microbes protect against atopy remain speculative at present. Finally, whereas 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. In this review, we address this final issue, discussing evidence that Toll-like receptor (TLR) ligand–receptor interactions can have profound yet complex effects on host immunity and the allergic phenotype.

TLRs and their ligands

In the past decade, much has been learned about the molecular basis for innate immune recognition of microbial pathogens. It has been established that mononuclear cells, and in particular antigen-presenting cells, express numerous pre-formed pattern recognition receptors that can identify potential pathogens. These receptors interact with microbial components that have been termed ‘pathogen-associated molecular patterns’ (PAMPs) [15]. By definition, PAMPs meet specific criteria including, first, production by microbes but not the mammalian host; second, conservation of structure across a wide range of pathogens; and third, the capacity to stimulate innate immunity. Recently, TLRs have been identified as having a central role in the detection of PAMPs and in the early host response to microbial infections.

Ten mammalian TLRs and several of their ligands have been identified [16•]. TLR2 interacts with peptidoglycan, lipopeptides and other products from Gram-positive bacteria, acid fast bacteria and fungi, as well as lipopolysaccharide (LPS) from select bacterial species, whereas TLR4 recognizes LPS produced by most Gram-negative bacteria [16•]. Further diversification in microbial recognition is provided by TLR5, which recognizes bacterial flagellin [17], and by TLR3, which recognizes double-stranded RNA produced during the course of many viral infections [18]. Although natural ligands have not been identified for TLR7, several synthetic analogs have been shown to activate immunity via this receptor [19]. TLR9 is the last of the well characterized TLRs and it recognizes specific DNA sequences that are significantly underrepresented in mammalian genomes but that occur at predicted frequencies in most bacterial and viral genomes [20–22]. Some TLRs (TLR1, TLR2 and TLR6) are also known to be recruited into the macrophage phagosome, where they heterodimerize with other TLRs [16•,23]. Such interactions may serve to expand further the repertoire of PAMPs that can be recognized by the TLR family of proteins and, therefore, to increase the number of microbes to which the innate immune system can respond.

Signaling pathways used by TLRs universally lead to activation of the transcription factor NF- κ B and mitogen-activation protein kinases, general production of cytokines such as IL-6, IL-10 and IL-12, and expression of costimulatory molecules including CD40 and B7 [16•]. However, despite the fact that all the TLRs that have been studied signal through shared molecules (such as MyD88 and NF- κ B), their ligands seem to be capable of inducing very divergent immunological responses [16•]. It is thought that there are collateral signaling pathways that are responsible for the unique features of immune activation induced through the various TLRs. Several accessory signaling pathways, used by some but not all TLRs, have been already identified and it seems likely that even more will be found in the years to come.

Epidemiological links between exposure to LPS and protection from atopy

Because of the ubiquitous distribution of microbes in our environment, we and others believe that exposures to TLR ligands via the intestines, inspired air and skin are likely to provide an important molecular link between microbes, normal immune development and the atopic phenotype. Unfortunately, to date epidemiological studies of exposures to TLR ligands and their association with allergic disease pathogenesis have focused exclusively on LPS, because detection assays for other TLR ligands have not been standardized and are not readily available.

In a recently published study, Braun-Fahrlander *et al.* [24•] found that levels of endotoxin were higher in the

homes of farmers with livestock than in control family homes, and that the farmers' family members had a lower incidence of allergic diseases. This inverse association between endotoxin exposure and atopy rates has been also found for infants living in more urban environments [25]. Additional evidence that endotoxin has a role in protecting against the development of allergic hypersensitivities and diseases comes from genetic studies linking polymorphisms in the gene encoding CD14 (the LPS co-receptor) with the severity of the atopic phenotype [26]. Interestingly, genetic analyses of individuals suffering from Crohn's disease, another disease associated with immune dysregulation, have recently demonstrated disease linkage to NOD2, another protein that contributes to LPS signaling [27,28].

Although a case can be made for high levels of environmental LPS protecting against atopy, it is important to consider that other immunologically active microbial products, including ligands of other TLRs, are also likely to be more abundant in environments rich in LPS. Thus, it remains to be determined whether LPS is directly protective against atopy or whether higher levels of LPS are an indicator of increased exposure to microbial products in general. In this vein, although no epidemiological studies have explored the potential association between exposures to other TLR ligands and atopy, investigators have clearly established that, in addition to LPS, other TLR ligands including those of TLR2 [29] and TLR9 (AM Schiltz *et al.* abstract 484 in *J Allergy Clin Immunol*, 2003, 111:S189), are ubiquitous in our environment. Thus, a complete understanding of how TLR ligands shape adaptive immunity and predisposition towards allergy will require additional epidemiological studies of all TLR ligands found to be present at biologically significant levels in living environments.

MyD88 and atopy

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 Th2-biased responses [30]. MyD88 has been found to be important for signaling by all TLRs characterized so far, with the possible exception of TLR3, which does not appear to incorporate MyD88 into its signaling cascade [16•]. Unlike other TLR ligands, however, ligands of TLR3 and TLR4 continue to induce residual and functional signaling by MyD88-knockout mononuclear cells [16•]. To a great but not complete extent, therefore, the MyD88-knockout mouse approximates immune development in the absence of TLR ligands. With these considerations in mind, Schnare *et al.*'s [31] finding that MyD88-knockout mice spontaneously develop higher concentrations of serum IgE than do wild-type mice is thought-provoking. Furthermore, the same investigators found that the Th1 responsiveness of MyD88-knockout mice to vaccination with antigen in Freund's adjuvant is

lost, although the mice develop a normal Th2 response to vaccination with antigen in alum [31].

In unpublished studies (A Horner, D Chisholm, L Libet, E Raz, unpublished data), we have confirmed that MyD88-knockout mice have increased serum concentrations of total IgE and Th2-biased IgG subclasses (more IgG₁ and less IgG_{2a}) as compared with wild-type mice. Moreover, we observed that MyD88-knockout CD4⁺ cells had a more Th2-biased cytokine response to anti-CD3/CD28 stimulation and expressed larger quantities of GATA3, an important Th2 regulatory factor, than did wild-type CD4⁺ cells. In additional immunization studies, we found that MyD88-knockout mice also developed a more Th2-biased adaptive response to protein immunization without adjuvant than did wild-type mice.

Given the central role of MyD88 in TLR ligand signaling, it is tempting to suggest that, owing to deficiencies in physiological activation by TLR ligands during immune maturation, MyD88-knockout mice are intrinsically Th2-biased in their immunoreactivity. Caution must be exercised in coming to this conclusion, however, because MyD88 contributes not only to TLR ligand responsiveness but also to signaling by other soluble factors including IL-1 and IL-18 [31]. Although mice with deficiencies in IL-1 and IL-18 signaling do not seem to share the immunological phenotype of MyD88-knockout mice [31,32], MyD88 is likely to have as yet unidentified roles in immunocyte functionality that are unrelated to its role in TLR signaling. Furthermore, whereas IRAK-4, like MyD88, has a key role in TLR signaling [33,34], three individuals described as having IRAK-4 deficiency were not noted to have allergic manifestations although they were predisposed to pyogenic infections [33]. The above points taken into account, it nevertheless still remains tempting to consider the possibility that the immunological phenotype of MyD88-knockout mice is, at least in part, reflective of deficiencies in TLR activation during immune maturation.

Experimental allergic disease models

Lipopolysaccharide

In the laboratory LPS has been found to have divergent effects on the allergic phenotype. It has long been known that *Escherichia coli* LPS (a ligand of TLR4) promotes the synthesis of murine IgE *in vitro* [35]. In an *in vivo* rat model, however, *E. coli* LPS was found to inhibit the development of allergen-specific IgE and asthma if administered within 6 days of allergen sensitization. Paradoxically, *E. coli* LPS exposure after this period led to an exaggeration of the asthmatic phenotype in the same studies [36]. Recently, Eisenbarth *et al.* [37•] also reported on the seemingly schizoid immunological activities of *E. coli* LPS. They found that *E. coli* LPS induced Th1- or Th2-biased responses to intranasally co-administered OVA peptide when delivered at a high or low dose,

respectively; vaccinations with low-dose LPS also primed mice for developing the asthmatic phenotype.

The paradox of differential Th1 and Th2 adjuvant activity with high- and low-dose LPS has yet to be resolved, but experiments conducted by Kaisho *et al.* [32] in MyD88-knockout dendritic cells (DCs) may be relevant to its understanding. These investigations demonstrated that DCs from MyD88-knockout mice activated with *E. coli* LPS produced Th2 cell differentiation, whereas wild-type DCs activated with LPS produced Th1 cell differentiation. Considered in context with the studies of Eisenbarth *et al.* [37], these observations suggest that a MyD88-independent LPS signaling pathway induces Th2 cell development and is responsive to LPS at low concentrations, whereas the MyD88-dependent pathway dominates at high LPS concentrations and favors Th1 cell development.

Immunostimulatory sequence oligodeoxynucleotides

Microbial DNA has inherent immunostimulatory activities that are not shared by mammalian DNA [22]. The molecular basis for viral and bacterial genomic DNAs having immunostimulatory activity lies in their content of unmethylated CpG dideoxynucleotides, which are far less frequent in mammalian genomes. Immunostimulatory sequence oligodeoxynucleotides (ISS-ODNs), containing CpG dideoxynucleotides, are the natural ligands of TLR9, but murine and human homologs of this receptor have unique sequence requirements [38]. There are many excellent reviews of the anti-allergic activities of ISS-ODNs [39,40,41], and we mention them only briefly here.

ISS-ODN has been found to induce an innate response dominated by cytokines, such as IL-10, IL-12, type 1 interferons (IFN- γ), that are known to inhibit the allergic phenotype [39]. Moreover, as an allergen-independent therapeutic agent given to Th2-sensitized mice just before allergen challenge, ISS-ODN has proved to be more effective than corticosteroids in attenuating the hypersensitivity responses of asthma, allergic conjunctivitis [39] and allergic rhinitis (CS Rhee, L Libet, D Chisholm, A Horner, E Raz, unpublished data). It is thought that the innate response to ISS-ODN is responsible for its rapid attenuation of effector cell functions in an allergen-independent manner. Interestingly, although allergen-independent ISS-ODN therapy can potentially attenuate the allergic phenotype in Th2-sensitized mice, the effect is only temporary as the underlying allergen hypersensitivities are not irreversibly extinguished [39].

Used in vaccination models, ISS-ODN has been also found to be a potent systemic and mucosal adjuvant that promotes immune responses that are highly Th1 biased [42,43]. Furthermore, in murine models of asthma and anaphylaxis, ISS-ODN has shown great promise as an

anti-allergic vaccine adjuvant [42,44,45]. In this context, the allergen-specific response induced by immunization with ISS-ODN prevents and reverses Th2-biased hypersensitivities at the level of adaptive immunity, and the effect seems to be both allergen-specific and long lasting [39,45]. Pre-clinical investigations with ISS-ODN-based vaccines have been so promising that phase I and phase II clinical trials in individuals affected with allergic rhinitis have been already conducted, with preliminary but favorable results (P Creticos, personal communication).

TLR2 ligands

Studies comparing the immunological activities of LPS isolated from different bacteria have made it apparent that many LPS species signal through TLR4, whereas other LPS species signal through TLR2. When used at the same doses in vaccination models, TLR2-specific LPS (*Porphyromonas gingivalis*) and TLR4-specific LPS (*E. coli*) were found to induce Th2- and Th1-biased adaptive responses, respectively [46]. In other investigations, ligands of TLR2 and TLR4 were also found to elicit unique innate DC and mast cell responses: TLR4 stimulation induced stronger production of IL-12 from DCs, whereas TLR2 stimulation favored mast cell degranulation and the production of Th2 cytokines [47–49]. These innate response profiles may help to explain the Th2- and Th1-biased adjuvant activities attributed to *P. gingivalis* LPS and *E. coli* LPS, respectively [46]. Consistent with these results, we have recently found that intranasal vaccination with peptidoglycan (TLR2) not only induced highly Th2-biased adaptive responses to co-administered ovalbumin, but also primed mice for the development of allergen-induced asthma. By contrast, intranasal vaccination with ovalbumin and ISS-ODN (TLR9) induced a Th1-biased immune response but did not prime mice for allergen-induced Th1- or Th2-biased airway hypersensitivity responses (D Chisholm, L Libet, A Horner, unpublished data).

From a clinical perspective, individuals affected with atopic dermatitis are generally heavily colonized by TLR2-expressing bacteria (*Staphylococcus aureus*) and show evidence of Th2-biased immune dysregulation [50]. *S. aureus* colonization and infections can also precipitate atopic dermatitis flare-ups for which antibiotic therapies are often effective. Given these clinical observations and our knowledge of the immunological activities of TLR2 ligands, we speculate that these microbial products may participate in the pathogenesis of eczema. Further studies will be required to determine the validity of this theory.

Conclusions

The present epidemic of allergic diseases experienced in developed countries is likely to be due to environmental factors unique to affluent societies, because their prevalence remains low in the third world and has increased 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 [1–3]. Relatively recently, it has become evident that TLRs and their associated ligands provide an important molecular interface for cross-talk between microbes and mammalian immune systems [16]. It is therefore logical to consider whether TLRs and their ligands might also influence the pathogenesis of allergic diseases.

Clearly, more study will be required to develop a complete understanding of the physiological role of TLR ligands in shaping host immunity and predisposition towards allergic diseases. The epidemiological data and laboratory results discussed herein suggest, however, that TLR ligands have the potential both to protect against and to precipitate the allergic phenotype. The hypothesis put forth by Strachan in 1989 [1] that microbial exposures can protect against atopy has been both provocative and fruitful [2]. Nevertheless, we suggest that if TLR ligands and potentially other PAMPs provide the molecular means for microbial impact on the allergic phenotype, the hygiene hypothesis may require modification to accommodate the spectrum of immunological activities induced by these molecules.

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