

Toll-like receptor ligands and atopy: A coin with at least two sides

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Allergic disease prevalence rates have increased dramatically in affluent countries over the last half century. One proposed explanation is that decreased exposures to microbes caused by modern public health practices has led to deficiencies in an important source of immune education and a consequent increase in the risk of pathogenic immune responses to environmental antigens. Recently, it has become clear that innate responses to microbes are mediated in large part by Toll-like receptors (TLRs), which recognize a diverse family of ligands produced by viruses, bacteria, and fungi. In this perspectives article we will review experimental evidence suggesting that TLRs also play a dominant role in innate responses to noninfectious immunostimulatory materials present in environments of daily living. We will further discuss how ligands for different TLRs can polarize the T_H bias of adaptive responses in opposing directions. Finally, we will consider how TLRs might contribute to the genesis of atopy and the clinical potential of pharmacologic interventions that target TLRs for the prevention and treatment of allergic diseases. (*J Allergy Clin Immunol* 2006;117:1133-40.)

Key words: *Hygiene hypothesis, Toll-like receptor, peptidoglycan, LPS, immunostimulatory sequence oligodeoxynucleotide, CpG motif, asthma, Allergy*

Despite a well-developed understanding of the molecular and cellular mechanisms of atopy, in many parts of the world, the health burden of allergic diseases has increased steadily for many decades. In part, failure to reverse current allergic disease trends is a consequence of the limited efficacy of the therapeutic options available. Although several medications are highly effective at preventing and treating symptoms, therapies directed at the prevention

Abbreviations used

AIC:	Allergen/ISS-ODN conjugate
BMDDC:	Bone marrow–derived dendritic cell
HDE:	House dust extract
IDO:	Indoleamine 2,3-dioxygenase
ISS-ODN:	Immunostimulatory sequence oligodeoxynucleotide
PAMP:	Pathogen-associated molecular pattern
PGN:	Peptidoglycan
TLR:	Toll-like receptor

and reversal of allergic hypersensitivities, including dietary restrictions, environmental controls, and traditional immunotherapy, have not proved particularly efficacious. However, our growing appreciation that microbial exposures have a significant influence on immune development and allergic risk offers hope that their immunomodulatory activities can be harnessed in the development of therapeutic agents that have a substantive effect on the natural history of allergic diseases.

TOLL-LIKE RECEPTORS AND THEIR LIGANDS

Our understanding of the molecular basis for innate immune recognition of microbial pathogens has increased substantially in the last decade. Mononuclear cells, particularly antigen-presenting cells, express a range of preformed pattern-recognition receptors that identify potential pathogens.¹ These receptors interact with microbial components that are often referred to as pathogen-associated molecular patterns (PAMPs). As originally defined, PAMPs were thought to meet certain criteria, including (1) their expression by microbes but not by mammalian cells, (2) conservation of structure across a wide range of pathogens, and (3) the capacity to stimulate innate immunity. However, although the term PAMP has been accepted into the vocabulary of immunology, it is important to remember that PAMPs are not unique to pathogens and are produced at equivalent levels by microbes that do not cause disease. Toll-like receptors (TLRs) have been identified as having a central role in the detection of PAMPs and in host protection against microbial infections.² Interestingly, although TLR ligands are not readily

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generated by mammalian cells under physiologic conditions, recent reports suggest that under conditions of tissue inflammation, oxidative stress, and/or necrosis, molecules with TLR stimulatory activities (heat shock proteins, modified RNA and DNA species, and potentially others) can be generated and released by dying cells.³

At least 11 mammalian TLRs have been identified.^{2,4} TLR2 interacts with peptidoglycan (PGN), a constituent of all bacterial cell walls, as well as additional components of gram-positive bacteria, mycobacteria, and fungi; TLR3 responds to double-stranded RNA; TLR4 recognizes most species of LPS; TLR5 is activated by bacterial flagellin; TLR7 (mouse and human) and TLR8 (human) recognize several synthetic imidazoquinolones, as well as viral single-stranded RNA sequences; TLR9 is activated by DNA sequences that are rare in mammalian genomes but common in the genetic material of bacteria, fungi, and DNA viruses; and TLR11 (mice) recognizes an undefined PAMP associated with uropathogenic bacteria and a profilin-like protein associated with *Toxoplasma gondii*. Certain TLRs (ie, TLR1, TLR2, and TLR6) are also recruited into the phagosome, where they heterodimerize with other TLRs.^{2,5} Such interactions between different TLRs might serve to expand the variety of PAMPs that can be recognized by this family of proteins and therefore the number of microbes to which the innate immune system can respond.

Signaling pathways activated by TLR ligands lead to NF- κ B and mitogen-activated protein kinase activation, cytokine gene transcription (eg, IL-6, IL-10, and IL-12), and costimulatory molecule expression (eg, CD40 and B7).^{2,5} Interestingly, despite their common use of several signaling molecules, qualitative differences exist in the cellular responses elicited by ligands for different TLRs.^{5,6} It is believed that collateral signaling pathways are responsible for this diversity. For example, molecular studies have shown that Toll/IL-1 receptor homologous (TIR) domain-containing adapter protein (TIRAP), TIR domain-containing adapter protein inducing IFN- β (TRIF), and TRIF-related adapter molecule (TRAM) participate in signaling through only a limited number of TLRs.^{2,5} Moreover, ligands for some TLRs (ie, TLR3, TLR4, TLR7, and TLR9) induce type 1 IFN production in responsive cells by upregulating expression of IFN regulatory factors, whereas ligands for TLR2 and potentially other TLRs do not.⁷ Finally, unlike all other TLRs investigated, MyD88 does not participate in signaling through TLR3.⁵ Because a comprehensive discussion of TLRs is beyond the scope of this article, readers are referred to several excellent reviews on the subject, including the review by Kaisho and Akira in this month's issue of *The Journal of Allergy and Clinical Immunology*.^{2,5,8,9}

TLRs, LIVING ENVIRONMENTS, AND ALLERGIC RISK

Because microbes are ubiquitous in the world in which we live, it has been suggested that TLR ligand exposures

through the intestines, inspired air, and skin provide an important molecular link between microbes, normal immune development, and the atopic phenotype.¹⁰⁻¹² Unfortunately, although both intellectually attractive and popular, this hypothesis is far from proved. In the following paragraphs we will consider the experimental evidence further.

Aeroallergen exposure is a clear prerequisite for the development and persistence of respiratory allergic diseases.¹³⁻¹⁵ Nonetheless, although for some allergens (ie, cockroach and mites) higher exposure levels have been associated with an increased risk of sensitization, for other allergens (ie, dogs, cats, and molds) this correlation has not been found.^{14,16} Furthermore, for allergens associated with animals, increased levels of exposure have been associated with decreased atopic risk in several studies.^{15,16} These and other observations suggest that aside from allergens themselves, other immunomodulatory elements within living environments influence the balance between immune homeostasis and dysregulation. In support of this view, endotoxin (TLR4) has been reported to be present at higher concentrations in homes with regular exposures to animals than in homes without animal exposures.^{12,17} Moreover, infants raised in homes with high ambient endotoxin levels have been found to be at low relative risk for development of allergic hypersensitivities in many, although not all, published reports.^{12,18} Further comment on studies linking environmental TLR ligand exposures with allergic risk can be found in articles by Schaub et al¹⁹ and Perzanowski et al,²⁰ also included in this month's issue of *The Journal of Allergy and Clinical Immunology*.

In consideration of the apparent association between ambient endotoxin exposure levels and allergic risk, it is important to note that endotoxin-rich environments also generally contain increased levels of other immunostimulatory microbial products. These include ligands for TLR2 and TLR9.^{21,22} Furthermore, several man-made pollutants are thought to promote the development of allergic hypersensitivities.²³ Although much has been learned in recent years, such complexity in the content of daily exposures has hampered efforts to develop a holistic understanding of their effect on allergic risk. In this regard we reasoned that direct study of the immunologic activities of unpurified but clinically relevant environmental samples might prove enlightening. In recently published studies we found that sterile house dust extracts (HDEs) stimulate bone marrow-derived dendritic cells (BMDDCs) to upregulate costimulatory molecules and to produce IL-6 and IL-12p40 at high levels²⁴ but little IL-12p70 (unpublished observations). Moreover, relative bioactivities of HDEs were found to correlate with their endotoxin content. Finally, we observed that HDE immunostimulatory activities were partially dependent on TLR2, TLR4, and TLR9 and almost completely dependant on MyD88. These findings provide direct evidence that TLRs play a dominant role in mediating proinflammatory responses to particulates present in environments of daily living.

LPS (TLR4) AND ATOPY

In the laboratory LPS has been found to have divergent effects on the allergic phenotype. It has long been known that *Escherichia coli* LPS (TLR4) promotes murine IgE synthesis *in vitro*.²⁵ However, in an *in vivo* rat model LPS inhibited the development of allergen-specific IgE and asthma if delivered within 6 days of allergen sensitization. Paradoxically, in these same studies LPS exposure after this period led to an exaggeration of the asthmatic phenotype.²⁶ Eisenbarth et al²⁷ have also reported on the seemingly schizoid immunologic activities of LPS. These investigators found that LPS induced either T_H1- or T_H2-biased responses to intranasally coadministered ovalbumin when delivered at high or low doses, respectively; low-dose LPS vaccinations also primed mice for development of the asthmatic phenotype. In our own unpublished studies we have confirmed that at low and high doses, LPS functions as a T_H2- and T_H1-biasing adjuvant, respectively. The paradox of LPS's bipolar adjuvant activities has yet to be resolved, but experiments conducted by Kaisho et al²⁸ with MyD88-deficient dendritic cells might be relevant to its understanding. These investigators demonstrated that MyD88-deficient dendritic cells activated with LPS induced T_H2 cell differentiation, whereas LPS-activated wild-type dendritic cells induced T_H1 cell differentiation. Considered in context with the studies of Eisenbarth et al,²⁷ these observations suggest that a MyD88-independent LPS signaling pathway induces T_H2 cell development and is responsive to LPS at low concentrations, whereas the MyD88-dependent pathway dominates at high LPS concentrations and favors T_H1 cell development.

IMMUNOSTIMULATORY SEQUENCE OLIGODEOXYNUCLEOTIDE (TLR9) AND ATOPY

Viral and bacterial DNA have inherent immunostimulatory activities not shared with mammalian DNA.^{29,30} The molecular basis for this effect lies in the high content of unmethylated CpG dideoxynucleotides found in microbial DNA, whereas these sequences are infrequent in mammalian genomes. In addition to microbial DNA, synthetic immunostimulatory sequence oligodeoxynucleotides (ISS-ODNs; also known as CpG motifs) activate TLR9. However, murine and human homologs of TLR9 appear to have unique flanking DNA sequence requirements.²⁹ There are many excellent reviews of the antiallergic activities of ISS-ODNs,³⁰⁻³² and only brief mention of these will be made here. Fig 1 provides a schematic summary of how ISS-ODN (CpG motif)-based interventions are currently thought to inhibit allergic immune dysregulation and end-organ hypersensitivity responses.

ISS-ODN has been found to induce an innate response dominated by cytokines (eg, IL-10, IL-12, type 1 IFNs, and IFN- γ) that are known to inhibit the allergic phenotype.³³⁻³⁵ Moreover, mice injected with ISS-

ODN have increased serum cytokine levels for a week or longer.³⁴ As an allergen-independent therapeutic agent given to T_H2-sensitized mice within hours of allergen challenge, ISS-ODN has proved more effective than corticosteroids in attenuating the hypersensitivity responses of asthma, allergic conjunctivitis, and allergic rhinitis.^{30,36} It is generally believed that the innate cytokine response to ISS-ODN is responsible for its rapid attenuation of allergic manifestations. However, we have observed that ISS-ODN also inhibits IL-4 receptor expression³³ and IL-4 responsiveness (signal transducer and activator of transcription 6 phosphorylation, unpublished data). Hayashi et al³⁷ have additionally reported that ISS-ODN induces alveolar macrophages, pulmonary dendritic cells, and resident lung epithelial cells to produce high levels of indoleamine 2,3-dioxygenase (IDO), an enzyme that degrades tryptophan into immunoinhibitory metabolites. Moreover, ISS-ODN-mediated IDO induction was shown to be IFN- γ and IL-12 dependant, and ISS-ODN-mediated inhibition of T_H2-biased airway hypersensitivity responses in turn was found to be at least partially IDO dependant. It must also be noted that although allergen-independent ISS-ODN therapy potentially attenuates the allergic phenotype in T_H2-sensitized mice, the effect is only temporary, and underlying allergen hypersensitivities are not irreversibly extinguished.³⁸ Taken together, experimental evidence suggests that although allergen-independent ISS-ODN delivery rapidly inhibits the activities of effector T_H2 cells and other cells that contribute to the allergic hypersensitivity response, this intervention does not efficiently re-educate the memory B and T cells that maintain allergen-specific hypersensitivities.

Applying the vaccination paradigm to its study, ISS-ODN has been shown to be a potent systemic and mucosal adjuvant, promoting long-lived and T_H1-polarized adaptive responses in both animals and human subjects.^{39,40} Recent observations further suggest that ISS-ODN-activated plasmacytoid dendritic cells might also promote the differentiation of antigen-specific T regulatory cells.^{41,42} In murine models of asthma and anaphylaxis, allergen/ISS-ODN vaccination has been reported to elicit antigen-specific responses that both prevent and reverse the T_H2-polarized hypersensitivities associated with allergen-alum vaccination.^{30-32,43} Unlike the rapid, allergen-independent, but relatively short-lived T_H2 inhibition elicited directly by ISS-ODN, the adaptive response induced by allergen/ISS-ODN vaccination is believed to mature over weeks, to be allergen specific, and to imprint on memory lymphocytes and their subsequent responses to allergen encounter.

Recognizing the clinical potential of ISS-ODN, Drs Eyal Raz and Kenji Takabayashi began developing allergen/ISS-ODN conjugates (AICs) at the University of California, San Diego, approximately 10 years ago. In collaboration with other investigators at their institution and Johns Hopkins University Medical School, these modified allergens were found to be far more immunogenic than allergen mixed with ISS-ODN in mice.^{44,45}

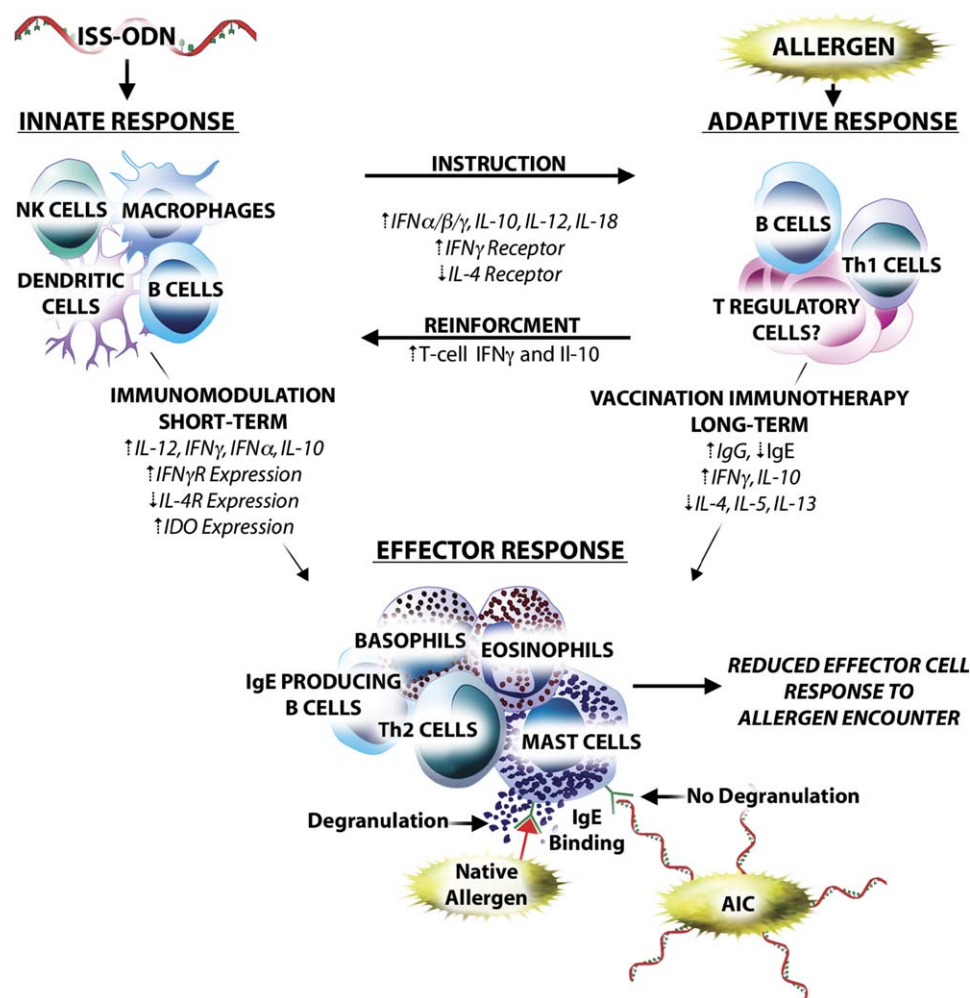


FIG 1. Schema for the antiallergic activities of ISS-ODN (also known as CpG motifs). ISS-ODN directly activates cells of the innate arm of the immune system to produce a number of antiallergic and T_H1 -promoting cytokines. Additionally, ISS-ODN induces IDO and IFN- γ receptor expression while inhibiting IL-4 receptor expression by responsive cells (see text for details). This immediate response to ISS-ODN directly inhibits the effector arm of the allergic hypersensitivity response, including the response of precommitted T_H2 cells. Over time, ISS-ODN-induced innate immune activation imprints on adaptive immunity by promoting the development of T_H1 -biased and inhibiting T_H2 -biased adaptive responses to allergens. ISS-ODN-activated plasmacytoid cells might also promote T regulatory cell responses. Thus ISS-ODN activates innate immunity to provide rapid and allergen-independent protection against T_H2 -biased hypersensitivity responses while concurrently promoting the development of long-lived and allergen-specific adaptive responses that are resistant to T_H2 dysregulation. AICs are ideally suited to the treatment of allergic hypersensitivities because (1) AIC is far more immunogenic than native allergens or allergen/ISS-ODN mixtures and (2) ISS-ODN conjugation masks IgE binding sites on AIC, rendering AIC hypoallergenic compared with native allergens (see text for details). Adapted with permission from Horner and Raz.³⁰

However, of potentially greater importance, oligodeoxynucleotide conjugation was found to render allergens resistant to binding by allergen-specific IgE. As a consequence, AIC challenge of T_H2 -sensitized mice elicited far weaker hypersensitivity responses than challenge with native allergen in models of asthma and anaphylaxis.^{43,44} Furthermore, Amb A 1 AIC was more than 100-fold less allergenic than native ragweed allergen in end point dilution skin testing studies of patients with ragweed allergy.⁴⁶ A recent clinical trial report further

demonstrated that after 6 weekly injections of Amb A 1 AIC, *ex vivo* ragweed-specific cytokine responses of PBMCs obtained from treated allergic patients were shifted away from T_H2 and toward T_H1 cytokine production.⁴⁷ Finally, in the only efficacy trial published to date, patients with ragweed allergy treated with the 6 Amb A 1 AIC injection schedule were found to have attenuated nasal allergen challenge responses several months after the final AIC injection.⁴⁸ Moreover, although clinical improvement was not noted during the ragweed season

immediately after AIC treatment, fewer respiratory symptoms were reported by patients during the following ragweed season. Although quite preliminary, these findings offer hope that immunotherapeutics like AIC might prove safe and effective for the desensitization of allergic patients.

TLR2 LIGANDS AND ATOPY

Studies comparing the immunologic activities of LPS isolated from different bacterial species have made it clear that although LPSs from a majority of gram-negative bacteria species signal through TLR4, LPSs from select bacteria signal through TLR2. Moreover, when used at the same dose in vaccination studies, TLR2 (*Porphyromonas gingivalis*)– and TLR4 (*E coli*)–specific LPSs were shown to induce T_H2- and T_H1-biased adaptive responses, respectively.⁴⁹ In our own investigations the immunologic activities of PGN (TLR2) were compared with those of ISS-ODN (TLR9).⁶ Mice intranasally immunized with antigen and PGN were again found to have T_H2-biased adaptive responses and were primed for T_H2-biased airway hypersensitivity responses on subsequent intranasal antigen challenge. In contrast, intranasal antigen/ISS-ODN vaccination induced T_H1-polarized adaptive responses but failed to prime mice for allergen-specific hypersensitivity responses. Another TLR2 ligand (lipopeptide Pam-3-Cys) has been found to promote human T_H2 cell development in *in vivo* cultures.⁵⁰ However, in previously T_H2-sensitized mice, treatment with lipopeptide Pam-3-Cys has also been reported to inhibit allergen-specific airway hypersensitivity and T_H2 cytokine responses, whereas allergen-specific IFN- γ responses were induced.⁵¹ This discordance in experimental results will require further investigation to be reconciled.

The quality of innate responses elicited by TLR2 ligands might help to explain why they generally favor development of T_H2-polarized adaptive responses. For example, LPS has been shown to elicit a far stronger IL-12 response (T_H1 promoting) from dendritic cells than TLR2 ligands, whereas TLR2 ligands were found to induce greater mast cell degranulation and release of T_H2-promoting cytokines.^{52–54} Likewise, we have observed that BMDDCs activated with PGN expressed higher levels of OX40L and produced more IL-6 (T_H2 promoting) but less IL-12 than BMDDCs activated with ISS-ODN.⁶ Mechanistic studies additionally suggest that TLR2 agonist activation of extracellular signal-regulated kinase 1/2-dependent pathways contribute to their T_H2 adjuvant activities.⁵⁰

From a clinical perspective, patients with atopic dermatitis are generally heavily colonized by TLR2-expressing bacteria (*Staphylococcus aureus*) and display evidence of T_H2-biased immune dysregulation.⁵⁵ Moreover, *S aureus* colonization and infections can precipitate atopic dermatitis flairs, for which antibiotic therapies are often effective. Finally, investigators have found that

application of *S aureus*–derived lipoteichoic acid (TLR2) to murine skin induces an eczematoid inflammatory response.⁵⁶ Given these observations and our knowledge of the immunologic activities of TLR2 ligands, it is reasonable to speculate that microbial interactions with TLR2 could contribute to the genesis of eczema and other allergic diseases. However, further studies will be required to validate this theory.

OTHER TLR LIGANDS AND ATOPY

Although it remains to be determined whether TLR3, TLR5, and TLR7/8 ligands can significantly influence the allergic phenotype, their immunomodulatory influence on adaptive immunity has been investigated to some extent. TLR3 ligands have been found to serve as T_H1-biasing adjuvants.⁵⁷ As with TLR2 and TLR4 ligands, conflicting reports exist as to the influence of flagellin (TLR5) on adaptive immunity. It was originally described as a T_H1 adjuvant, but more recent studies suggest that highly purified flagellin might actually favor T_H2-biased adaptive responses.⁵⁸ Finally, ligands for TLR7 and TLR8 (ie, R848 and its derivatives) have consistently been described as T_H1-polarizing adjuvants. Furthermore, R848 has been reported to inhibit T_H2 cell cytokine production while promoting production of IFN- γ by human T_H2 cells.⁵⁹ Along with other studies reviewed in this perspectives article, these investigations attest to the wide spectrum of immune responses that can be elicited by ligands for different TLRs.

TLR LIGAND-CONTAINING MICROBES AND ATOPY

Not only are microbes a rich source of TLR ligands and other PAMPS, but the hygiene hypothesis proposes that at least some infections have the potential to protect against the genesis of atopic diseases. Therefore investigations have considered how exposures to specific microbes influence the development of allergic hypersensitivities and diseases. Several reports suggest that purified protein derivative–positive children are at lower atopic risk than purified protein derivative–negative children.^{60,61} Furthermore, in murine studies vaccination with either live or killed mycobacteria effectively prevented development of allergic manifestations.^{62,63} Moreover, BCG vaccination has been associated with a reduced allergic risk in some, but not all, human clinical trials.^{64–66}

In other investigations heat-killed *Listeria* species was found to be a potent adjuvant for the induction of T_H1-biased and the inhibition of T_H2-biased responses in mice and dogs.^{67,68} Furthermore, rectal vaccination with heat-killed *E coli* expressing peanut allergen has been reported to reverse allergen-specific hypersensitivities in T_H2-sensitized mice.⁶⁹ Finally, live transgenic *Mycobacterium vaccae* and *Lactobacillus plantarum* expressing house dust

allergen are reported to be effective vaccines for inducing T_H1 -biased allergen-specific responses in mice.^{70,71} Because interventions with both live and dead bacteria have proved protective against the allergic diathesis, these experimental findings provide additional credence to the view that TLR and other PAMP receptor interactions with microbes play an important role in their ability to influence allergic risk.

Interestingly, the intestinal flora of infants born in countries with low and high prevalence rates for allergic diseases have been shown to differ.⁷² The intestinal flora of nonatopic and atopic infants have also been suggested to be unique.⁷³ Along with these observations, ingestion of probiotics has been found to improve the skin status of patients with eczema and prevent the development of other allergic manifestations both in human subjects and mice.^{74,75} Although these observations are quite intriguing and offer a novel approach for the prevention and treatment of atopic diseases, they are fairly preliminary and, at present, poorly understood. In particular, there is no experimental evidence to suggest that the TLR content of intestinal microflora associated with the provocation and prevention of atopy are unique. Therefore the mechanistic basis for specific intestinal microflora influencing allergic risk remains to be elucidated.

CONCLUSIONS

The present epidemic of allergic diseases experienced in developed countries is likely to be due to environmental factors associated with modern living because prevalence rates remain low in the third world, and these epidemiologic trends have developed too rapidly to have a simple genetic basis. 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. Relatively recently, it has become evident that TLRs and their ligands provide an important molecular interface for cross-talk between microbes and mammalian immunity. Therefore it is logical to consider whether environmental and pharmacologic exposures to TLR ligands might influence the pathogenesis of allergic diseases. Experimental evidence in support of this view has been reviewed extensively in this perspectives article.

Clearly, more study will be required to develop a complete understanding of how physiologic TLR ligand exposures influence immune maturation and allergic risk. Nonetheless, studies presented herein demonstrate that TLR ligands have the potential to both protect against and precipitate the development of allergic manifestations. Finally, clinical experience with AIC suggests that therapeutics that activate TLR9 and potentially other PAMP receptors could prove safe and effective for the prevention and treatment of allergic diseases.

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Correction

With regard to the September 2005 article entitled "Predictors of asthma-related health care utilization and quality of life among inner-city patients with asthma" (2005;116:636-42): The following acknowledgment should have appeared at the end of the article:

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