

Pathogen Recognition and New Insights into Innate Immunity

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Introduction

The vertebrate immune system is tasked with detection and elimination of “foreign agents” while causing minimal harm to the host. These responses may be clustered as “adaptive” or “innate.” Adaptive immunity is specific and elegant, but slow; recognition of antigens requires random and diverse arrays of antigen receptors (T and B cell receptors) and clonal selection and expansion of effector and antibody-producing cells. Repeat offenders induce more rapid responses due to immunological memory, but newly encountered dangerous organisms could overwhelm the host while differentiation and expansion occurred. In contrast, innate immunity (the most ancient and conserved elements of the immune system) can respond to a range of potentially harmful organisms in a rapid, vigorous, and nonspecific manner; innate responses require no previous exposure but rely on pattern-recognition of “danger signals” for host defense.

Pattern recognition receptors (PRRs) have evolved as an important arm of the innate immune system; examples include mannose receptors (that bind to terminal mannose groups on microbial glycoproteins, facilitating their endocytosis), nucleotide-binding oligomerization domain proteins (NODs, that promote intracellular recognition of microbial peptidoglycans), and the Toll-like receptors (TLRs). TLRs are highly conserved receptors originally identified in *Drosophila* that share both structural and functional characteristics. TLRs are found both on the cell surface and within the cell, where they facilitate recognition of and response to microbes and their components (Pathogen-Associated Molecular Pattern, PAMPs, such as endotoxin [TLR-4], bacterial flagellin [TLR-5], viral RNA, [TLR-3, -7, -8], and bacterial DNA [TLR-9]). Downstream of ligand/TLR engagement are cascades that induce the transcription of cytokines, maturation of inflammatory

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cells, and ultimately engagement of both additional innate and adaptive immune mechanisms.

Mechanisms of Innate Immunity: PAMPs and PRRs

The last decade has witnessed an explosion of information regarding PRRs and the PAMPs that they recognize. PAMPs are conserved products of microbial metabolism that are generally unique to microorganisms and absent from the host (examples include lipopolysaccharides [LPS], lipoproteins, peptidoglycan, and lipoteichoic acids): this allows distinction between “self” and (potentially dangerous) non-self. PAMP recognition emerged early in the evolution of host-defense systems; many PAMPs are detected by innate immune systems of invertebrates and plants. PAMPs are often essential for microbial survival, preventing the generation of escape mutants, and are invariant between microorganisms of a given class, allowing a limited number of germ-line-encoded PRRs to detect nearly any microbial infection. Although the target molecule may differ between microbial species, common molecular patterns can be found in all. For example, the lipid-A portion of LPS is invariant and serves as a PAMP; in contrast, the O-antigen varies between species and is not a strong activator of the innate immune system. Important PRRs that both serve in host defense against microorganisms and also appear to play a role in innate immune responses in atopy and asthma include the TLRs, CD14, and the NODs. These receptors are found both on the cell surface (where they may serve to transduce signals from randomly encountered as well as cell-presented PAMPs) and within the cytosol of cells (recognizing endocytosed PAMPs as well as those that have the ability to cross the cell membrane).

PAMPs and Asthma: The Hygiene Hypothesis

Early life exposure to and recognition of microbes and microbial products (PAMPs) is important for development and programming of immune responses. It has been proposed that a reduction in such exposures, due to modern public health and medical practices as well as social and environmental changes, has had the unanticipated consequence of immune dysregulation, resulting in an increased prevalence of inflammatory disorders. The “Hygiene Hypothesis” speculates that our immune system, evolved to optimize protection from/coexistence with environmental microorganisms, is now faced with a paucity of appropriate targets [39]. Reduced exposures to PAMPs, and thus more limited engagement of PRRs and innate immune responses, has been linked to increased inflammatory disorders of a variety of types.

The prevalence and severity of asthma and atopic disorders have increased substantially over the last 4 decades, especially in developed nations [2]. Currently,

over 20 million people in the United States currently have asthma and over 50 million suffer from rhinosinusitis or other atopic conditions; these disorders may begin early in childhood or later in life. Underdeveloped or third world nations have been relatively spared this epidemic, as are certain populations. Among others groups with reduced risk are included: individuals raised in rural, agricultural settings with early-life exposure to barns [5, 35]; late birth-order children from larger families [43] and those who enter day-care at an early age [39]; and survivors of certain non-respiratory infections [38]. Children at high-risk (e.g., with atopic parents) for developing asthma are somewhat protected if they grow up with a dog in the house [44], and children who are raised in parasite-endemic areas demonstrate increased rates of asthma if they are dewormed [47, 48]. These observations and others suggested a link between childhood infections (e.g., illnesses brought home from school by an older sibling) or microbes and their products (e.g., those found in high concentrations in agricultural settings) and resistance to atopic disorders. In addition to the modern epidemic of asthma and atopy, type I diabetes, multiple sclerosis, and inflammatory bowel disease have also risen dramatically in prevalence; these maladies are most common in industrialized nations and are also on the rise in traditional societies that are undergoing modernization or westernization.

The linking of reduced susceptibility to asthma with early-life exposures to pathogens or microbial products was initially puzzling, as infections and the resulting inflammation have long been associated with asthma exacerbations rather than prevention. Atopic disorders are characterized by a skewing of immune responses towards a Th2 pattern; Th2 cytokines promote eosinophilia, class switching of B cells to the production of IgE antibodies, goblet cell metaplasia and airway mucus hypersecretion, and airway hyperreactivity among other effects. Although the fetal immune system is typically Th2-oriented [32], newborns ordinarily exhibit a rapid decline in this tendency; one hypothesis is that early-life exposure to microbial products (a number of PAMPs have been proposed to account for the Hygiene Hypothesis – see Table 1) induces a Th1 milieu, and since Th1 and Th2 responses are counterregulatory, suppression of Th2 activity may result. It was initially proposed, therefore, that this suppression does not occur (perhaps resulting in a life-long tendency towards Th2 responses to otherwise innocuous antigens) in the absence of early-life infections or related exposures, providing an immunologic basis for the

Table 1 Potential mediators of hygiene hypothesis

PAMP	Source	PRR
Endotoxin	Gram-negative bacteria	TLR-4, CD-14
CpG DNA	Bacteria, viruses, protozoa	TLR-9
Muramic Acid/Peptidoglycans	Bacteria	TLR-2, NOD
ES-62 [phosphorylcholine-containing glycoprotein]	Helminths	???
Glucans	Fungi	
Lipoarabinomannan (LAM)	Mycobacteria	

Hygiene Hypothesis. Considerable doubt has arisen regarding the accuracy of this proposed schema; although increased Th2 responses would account for a higher prevalence of atopy, this would not account for increases in Th1-skewed conditions such as multiple sclerosis and inflammatory bowel disease, nor would they explain the ability of Th2-skewing organisms (e.g., helminths) to protect against atopic disorders. More compelling is an alternative proposal, that a lack of exposure to PAMPs impairs development of appropriate regulatory responses.

Toll-Like Receptors, NODs, and Surfactant Proteins

The family of Toll-like receptors (TLRs) is among the best characterized PRRs. The Toll protein was first identified in *Drosophila* through its control of embryonic dorsal-ventral patterning. It was subsequently found to play an important role in *Drosophila* immunity, as *Toll* mutants were inordinately susceptible to fungal infections. Other related fly proteins, also related to host defense, include 18-wheeler, Tollo, and Tehao [40]; these promote antimicrobial peptide production (defensins) among other activities. The first human homologue (hToll, later TLR-4) was identified in 1997 by Medzhitov [28]; other TLRs have been since recognized, with the current family including 13 mammalian TLRs (TLR1-TLR11) that recognize distinct microbial PAMPs (Table 2) [41].

Members of the TLR family are type I transmembrane PRRs, most of which include a conserved intracellular region, the Toll/IL-1 Receptor (TIR) domain,

Table 2 TLR, ligand [synthetic in brackets], natural source

TLR	Ligand	Source of natural ligand
TLR-1	Triacyl Lipopeptides	Bacteria
TLR-2 [with TLR-1, TLR-6]	Lipopeptides, lipoteichoic acid, peptidoglycan; zymosan, phospholipomannan; GPI Anchor; Envelope Protein	Bacteria, fungi, protozoa, virus
TLR-3	Double stranded RNA; [Poly (I:C)]	Virus
TLR-4	Lipopolysaccharides; mannan, glucuronoxylomannan; Glycoinositolphospholipids; RSV fusion protein	Gram-negative bacterial cell wall, fungi, protozoa, virus
TLR-5	Flagellin	Bacteria
TLR-6	Diacyl lipopeptides; Zymosan	Bacteria, fungi
TLR-7	Single-stranded viral RNA; [Imiquimod]	Virus
TLR-8	Single-stranded viral RNA, Imiquimod	Virus
TLR-9	DNA containing unmethylated CpG motif; Hemozoin	Bacteria, protozoa, virus; protozoa
TLR-10	Unknown	
TLR-11 (murine)	Profilin-like molecule	Protozoa
TLR-12 (murine)	Unknown	
TLR-13 (murine)	Unknown	

which mediates protein–protein interactions with downstream signal transduction elements and varying numbers of extracellular leucine-rich repeats (LRR), involved in ligand binding and TLR dimerization. Supporting its conserved role in innate immunity, TIR is also found in a variety of transmembrane and cytoplasmic proteins in animals and plants that play a role in host defense. In addition to the Toll family, other proteins with TIR domains include IL-1 receptors (IL-1R), Myeloid Differentiation Factor-88 (MyD88), and TIR-containing Adaptor Protein (TIRAP).

Engagement of TLRs by specific, but broadly expressed ligands (PAMPs such as lipoteichoic acid from gram-positive bacteria [TLR-2], lipopolysaccharides in gram-negative bacterial cell walls [TLR-4], and CpG motifs in bacterial DNA [TLR-9]) activate differential, but overlapping signaling pathways. TLRs signal through a group of adaptor proteins that activate downstream kinases and can lead to activation of nuclear factor κ B (NF- κ B), mitogen-activated protein kinases (MAPK) and other cascades, resulting in induction of immune response gene activation. TLR engagement leads to both MyD88-dependent and MyD88-independent responses. Specificity of responses to PAMPs is promoted by differential patterns of receptor expression (including cell surface and intranuclear) and cell types. Some TLRs are broadly expressed whereas others (e.g., TLR-9) demonstrate a more restricted expression pattern.

Well before identification of the TLR system, it was recognized that bacterial DNA is immunostimulatory. More than a decade ago, Krieg first reported that the cytosine–guanine dinucleotide (known, because of the phosphate bond, as CpG), in specific base sequences (CpG motifs) in bacterial DNA, were immunostimulatory and capable of strong B cell activation [24]; we now understand that these effects are due to ligation of TLR-9. Prokaryotic DNA contains the expected frequency of CpG dinucleotides (1:16 base pairs) which are suppressed in eukaryotic DNA (1:50–1:100 base pairs); moreover, when present, the cytosine in eukaryotic DNA is often methylated, which silences or reduces the immunostimulatory properties of the motif. Subsequent studies demonstrated that synthetic oligodeoxynucleotides (ODNs) centered on CpG motifs (CpG ODN) recapitulated the patterns of activation induced by bacterial DNA, setting the stage for the use of CpG ODN as immunotherapeutic agents.

CpG oligodeoxynucleotides are categorized based on their structure; three major classes of CpG-ODN have been described with distinct patterns of activity: A-Class CpG ODNs are based on a phosphodiester backbone; they have phosphorothioate poly-G motifs at the 5′ and/or 3′ ends that can form stable higher-ordered structures. These ODN are strong inducers of IFN- α secretion by plasmacytoid Dendritic Cells (pDCs), and poorly induce B cell proliferation. B-class CpG ODN (of which CpG 7,909, the ODN intended for use in these proposed studies, is a member) consist of a phosphorothioate backbone; these ODN strongly induce B cell proliferation and pDC maturation, strongly induce IL-10, but poorly induce IFN- α secretion from pDCs. C-class ODN have a hexameric CpG motif linked by a T spacer to GC-rich palindromic sequences; they are entirely phosphorothioate and have no poly-G sequences. C-class CpG ODN induce both B cell activation and IFN- α secretion.

When encountered by immune cells, CpG DNA undergoes endocytosis, binds to TLR-9, and is translocated to the nucleus where it induces a series of downstream immune responses. In plasmacytoid dendritic cells (pDCs), TLR-9 activation is dependent on IL-1 Receptor-associated kinase (IRAK)-4 and interferon regulatory factor (IRF)-7 and requires direct interactions between IRF7 and MyD88, TNF- α -receptor activated factor (TRAF)-6, and IRAK-1 [16, 18, 19, 42, 46, 49, 50]. TLR9 activation induces NF- κ B and other intracellular pathways that initiate a rapid innate immune response characterized by cytokines and chemokines including IL-6, TNF- α , type I interferons, IFN-inducible protein-10 (IP-10), and IL-10. Downstream responders to these signals include NK-cells, T cells, and other cells, which amplify and modulate the immune response. Later effects include induction of costimulatory receptors, immunoglobulin isotype switching by B cells, and activation of a cascade of cellular responses. B cells activated via TLR9 are more responsive to antigen stimulation and readily differentiate into plasma cells, thus modulating the adaptive immune response. The sum total of these inflammatory cascades was initially viewed as Th1-skewing; as a result of the induction of IFN- γ from NK-cells [9], IFN- γ , and IL-12 from lymphocytes [22], and IL-12 from antigen presenting cells (APCs) [7], TLR-9 activation can strongly promote Th1 responses [8]. It is increasingly understood, however, that the effect of TLR-9 on regulatory responses (characterized by increased IL-10 and suppression of both Th1 and Th2 effects) may be more important in the Hygiene Hypothesis as well as in developing novel therapies.

Based on the apparent role played by TLR ligand exposure in modulating disease susceptibility, a search for linkages between *TLR* genetic variants and asthma and atopy has been eagerly pursued. Swedish investigators examined a single common *TLR4* polymorphism in a cohort of Swedish school children and found an association with asthma and reduced LPS-induced IL-12 (p70) release [4]. In contrast, two family-based cohorts were evaluated for association between *TLR4* polymorphisms and asthma and found no evidence of association for any of the polymorphisms tested and asthma-/atopy-related phenotypes [33]. Further muddying the waters, a UK family study (including 336 families containing at least 2 asthmatic siblings) found no association between *TLR4* polymorphism and an asthma diagnosis or severity, but did find linkage of atopy severity scores (skin-prick tests and specific IgE), with higher values in subjects with Asp/Gly or Gly/Gly genotypes (1.8 ± 1.1 , $n = 39$) compared to those with Asp/Asp genotype (1.2 ± 1.0 , $n = 279$) ($P = 0.003$) [45]. Similarly, disparate findings have been reported for *TLR2* [11, 34], *TLR9* [3, 25, 30], and *TLR10* polymorphisms [26], among others.

Although critical in innate immunity, TLR engagement also plays a role in controlling adaptive immune responses. Engagement of TLRs on APCs upregulates costimulatory factors (CD80 and CD86) and MHCII molecules and induce cytokines (e.g., IL-12), chemokines, and their receptors, triggering maturation and activation of dendritic cells (DCs). Induction of CD80/86 on APCs by TLRs leads to the activation of T cells specific for pathogens that trigger TLR signaling. B cell activation and immunoglobulin class switching may be modulated both directly by TLR engagement and indirectly, secondary to elaboration of cytokines by other

immune cells. DCs appear to have a significant role in linking innate and adaptive immunity. Their maturation and activation develops in response to engagement of TLRs and other PRRs, such as NOD2, Dectin1 (a β -glucan receptor), and the mannose receptor. These exposures promote a phenotype that skew responses to a presented antigen towards a Th1 or Th2 pattern.

Other important PRRs in innate immunity include the family of NODs and surfactant proteins. NODs are cytosolic proteins that recognize muramic acid and bacterial peptidoglycans (a ubiquitous component of bacterial cell wall) [12]. NOD1 and NOD2 recognize different peptidoglycan motifs, and may confer some specificity to this system (NOD1 appears to respond more to peptidoglycan from gram-negative bacteria). Like TLRs, NOD activation can induce upregulation of nuclear factor- κ B, and also serves to regulate inflammation through enhancement of caspase-1 and caspase-9 actions, which promote apoptosis. NODs interact with other components of innate immunity, such as the TLR system; peptidoglycans can activate TLR2 as well as NODs. Surfactant protein A and B are members of the collectin family of C-type lectins. Like TLRs, they link adaptive and innate immunity; like TLRs, specific polymorphisms have been linked to susceptibility to chronic lung disorders, as well as infections [31].

Novel “Anti-hygenic” Therapeutic Approaches to Asthma and Atopic Disorders

An improved understanding of the innate immune responses associated with an increased prevalence and severity of atopy and asthma (and, perhaps more importantly, those mechanisms which provide protection) has led to the development and investigation of several novel therapeutic approaches. Rook and colleagues have proposed that saprophytic mycobacteria, such as *Mycobacterium vaccae*, along with lactobacillae and certain helminths serve as immunologic “old friends,” historically responsible for immunologic balance and lacking in modern society [36]. Murine studies have found that heat-killed *M. vaccae* can suppress asthmatic inflammation through induction of allergen-specific regulatory cells [51]. Based on these and other studies, a clinical trial was conducted that demonstrated a substantial (but not statistically significant) reduction in late allergic response and a trend towards reduced IgE [6]. Endotoxin exposure can induce Th1 responses and inhibit atopic inflammation in murine models [14, 23], and TLR4 analogs are in development that may prove to provide therapeutic benefit for human asthma [37].

CpG DNA, the TLR-9 agonist, is also being developed as a therapeutic agent for asthma and atopic disorders. We first proposed the potential therapeutic use of CpG-ODN for asthma and atopy based on murine studies in which we were able to prevent atopic sensitization. Using a schistosome egg model of atopic asthma, we found that coadministration of CpG-ODN along with the sensitizing antigen suppressed both pulmonary (airway eosinophilia, bronchial hyperreactivity) and systemic (elevated serum IgE levels, Th2 cytokine production) manifestations of atopic asthma [21].

Although demonstration that CpG ODN administered at the time of allergen sensitization prevents the manifestations of atopic asthma provides a plausible mechanism to explain a potential role for PAMPs in protecting against atopic disorders, this situation is quite different from that encountered in clinical settings. We therefore next explored the effects of CpG ODN on established atopic asthma. Using an ovalbumin (OVA) model of atopic asthma, we examined the effects of treating previously sensitized and challenged mice [20]. We found that mice treated with OVA and CpG-ODN demonstrated nearly complete reversal of eosinophilic inflammation, and marked suppression of bronchial hyperreactivity and IgE responses. Using a mucosal (trans-nasal) approach, mice that received CpG-ODN alone as well as those treated with CpG-ODN and allergen had profound therapeutic responses [17].

There is a growing experience with the effects of CpG ODN in humans; in addition to normal subjects, it has been studied as a treatment or adjunct in infectious disease, malignancy, atopic rhinitis, and asthma. In general, administration of CpG-ODN has been found to be safe. Phosphorothioate oligonucleotides are known to induce sequence-independent backbone-related effects. Chronic dosing of phosphorothioate ODN in rodents results in a dose-dependent mononuclear cell infiltration of the kidney and liver, but these effects have not been seen in monkeys or humans [27]; no adverse effects on renal function has been seen in clinical trials using phosphorothioate ODN. The major dose-limiting acute toxicity of phosphorothioate ODN in primates stems from activation of the alternative complement pathway; leukocyte activation can increase vascular permeability resulting in significant hypotension [15]. This toxicity requires a threshold blood concentration of 40–50 µg/ml, which is generally the result of rapid intravenous administration [29].

CpG-specific toxicity may also result from TLR9 activation. The safety profile of several TLR9 agonists in humans has been observed in clinical trials over a more than 1,000-fold dose range from 0.0025–0.81 mg/kg; a maximal tolerated dose in humans has not been reported to date. The most prominent adverse events are dose-dependent local injection reactions (such as erythema, pain, swelling, induration, pruritus, or warmth at the site of injection) and systemic reactions including headache, rigors, myalgia, pyrexia, nausea, and vomiting. Depending on the dose, systemic symptoms typically develop within 12–24 h of dosing and persist for 1–2 days. At the low doses used in vaccine trials there seems to be a slight increase in the frequency of injection-site reactions, which are generally mild, above the frequency observed with the vaccine alone. Early concerns regarding the risk of inducing autoimmune responses have not been demonstrated in human trials, with no reports of anti-human antibodies or other markers of autoimmune responses. The most common adverse events include injection-site reactions (local pain, erythema, and induration) and mild systemic symptoms (fever, chills, fatigue, myalgias, arthralgias, and headache) [1].

A recent study has evaluated the effect of a novel CpG/allergen immunotherapy (AIC: the ragweed allergen, *Amb a 1*, conjugated to CpG oligonucleotides) for ragweed-induced allergic rhinitis [10]. Subjects were treated with increasing doses of AIC prior to being followed for two sequential ragweed seasons. The study

demonstrated that subjects treated with AIC had significantly better rhinitis scores during the ragweed season that immediately followed the course of therapy as well as during the following year. This study, although limited by a lack of comparison with standard immunotherapy, provides encouragement for the investigation of immune mechanisms invoked by CpG-ODN that are relevant to the induction of tolerance in allergic hypersensitivity.

One published report on the use of CpG ODN in human asthma [13], examined the effect of inhaled CpG-ODN on the pulmonary immune response and airflow obstruction resulting from allergen inhalation challenge. In that study, the group treated with CpG-ODN demonstrated induction of interferon-inducible cytokines and chemokines but no changes in allergen-induced airflow obstruction (neither early nor late) or sputum eosinophilia. These results support the conclusion that CpG-ODN are immunoactive, inducing a pattern of (interferon-related) responses that would be expected to suppress atopic inflammation; the failure to demonstrate reduction in clinically relevant manifestations of the asthma phenotype, however, suggests that these cytokines alone may not be sufficient to provide a therapeutic benefit. Other trials examining CpG-ODN in clinical asthma are ongoing.

Summary

The modern emphasis on the value of hygiene (e.g., “cleanliness is next to godliness”) has led to a remarkable reduction in the epidemic and endemic infectious diseases that were the most significant cause of mortality from the earliest expression of human civilization until the nineteenth century. Resulting measures, including waste management strategies, improved housing, control of the food chain, and both preventive and therapeutic medical practices, may now be leading to unanticipated consequences, most notably an alarming increase in inflammatory disorders, including diabetes, inflammatory bowel disease, multiple sclerosis, and asthma and atopy. The Hygiene Hypothesis of asthma and atopy proposes that early-life exposure to microbes and microbial products (PAMPs) is required for appropriate programming of the developing innate immune system; an absence of such exposures leads to dysregulated inflammation.

The mechanistic basis for the Hygiene Hypothesis is the innate immune PRRs that identify PAMPs by recognition of critical and characteristic motifs. These receptors are believed to have developed secondary to strong evolutionary pressure to survive (coexist with) otherwise dangerous microbes. Recognition of a PAMP by a PRR initiates a cascade of signals that have not only immediate consequences (i.e., engagement of mechanisms that lead to elimination of the threat) but also developmental sequelae (i.e., instruction and programming of the immature immune system), especially when this occurs in early life.

Although numerous PRRs have been identified, the family of TLRs remains the best studied. These receptors (13 of which have been identified in mammals) act individually and in concert to transduce recognition of microbial patterns

into characteristic inflammatory pathways. The same mechanisms that protect against microbes are now being exploited in the development of novel therapeutic approaches to asthma and atopic disorders. CpG-ODN, the TLR-9 ligand, has been shown, in preclinical models, to be effective in the prevention and therapy of atopic asthma. Human studies have supported safety of its use; recent trials suggest benefit in the management of clinical disease.

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