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Toll-like Receptors as Sensors of Pathogens

MIKKO HALLMAN, MIKA RÄMET, AND R. ALAN EZEKOWITZ

Department of Pediatrics [M.H., M.R.] and Biocenter Oulu [M.H., M.R.], University of Oulu, 90220 Oulu, Finland, and Laboratory of Developmental Immunology, Mass General Hospital for Children and Harvard Medical School, Jackson 14; GRJ 1402, 55 Fruit Street, Boston, MA 02114, USA [M.R., A.E.]

ABSTRACT

Initial recognition of microbes, as they enter the body, is based on germ line–encoded pattern recognition receptors that selectively bind to essential components of pathogens. This allows the body to respond immediately to the microbial invasion before the development of active immunity. The signal-transducing receptors that trigger the acute inflammatory cascade have been elusive until very recently. On the basis of their genetic similarity to the Toll signaling pathway in *Drosophila*, mammalian Toll-like receptors (TLRs) have been identified. By now, nine transmembrane proteins in the TLR family have been described. Mammalian TLR4 is the signal-transducing receptor activated by the bacterial lipopolysaccharide. The activation of TLR4 leads to DNA binding of the transcription factor NF-κB, resulting in activation of the inflammatory cascade. Activation of other TLRs is likely to have similar consequences. TLR2 mediates the host response to Gram-positive bacteria and yeast. TLR1 and TLR6 may participate in the activation of macrophages by Gram-positive bacteria, whereas TLR9 appears to respond to a specific sequence of bacterial DNA. The TLRs that control the onset of an acute inflammatory response are critical antecedents for the development of adaptive acquired immunity. Genetic and developmental variation in the expression of microbial pattern recognition receptors may affect the individual's predisposition to infections in childhood and may contribute to susceptibility to severe neonatal inflammatory diseases, allergies, and autoimmune diseases. **(*Pediatr Res* 50: 315–321, 2001)**

Abbreviations

CpG, cytosine phosphate-guanosine
IL-1RI, IL-1 type I receptor
IRAK, IL-1 receptor-associated kinase
LPS, lipopolysaccharide
LRR, leucine-rich repeat (segment of extracellular part of TLR)
MBL, mannose-binding lectin
NF, nuclear transcription factor
SP, surfactant protein
TIR domain, Toll-IL-1 receptor domain (cytoplasmic part of TLR, IL-1 and IL-18)
TLR, Toll-like receptor
TNF, tumor necrosis factor alpha

Infections account for a significant proportion of morbidity in children, despite active immunization programs and the use of antibiotics. An acute inflammatory response in the absence of adaptive immune defense is essential for survival. An inappropriate innate immune response may lead to serious organ damage, allergies, or autoimmune diseases. A central question relevant to all mammals, and particularly germane to children, is the dynamic interplay between the host and potential pathogens.

Recent investigations led to the discovery of a class of cell surface–signaling molecules termed TLRs, which provide the missing link in the immune response. Several of them control the proinflammatory activation of macrophages and the onset of acute inflammatory cascades. It is becoming increasingly clear that innate immunity is more than the first line of host defense. It is actually a necessary antecedent for the development of an adaptive immune response. In this review, we will highlight the discovery of mammalian Toll receptors and speculate as to the potential implications of these findings for pediatric practices.

ACTIVATION OF ADAPTIVE IMMUNITY

The adaptive immune response is a critical component of the host's response to pathogens. The key features of adaptive immunity are the clonal expansion of lymphocytes in response to a particular antigen and the ability to evoke an immunologic memory. In the adaptive immune system, specific T- and B-cell receptors for each clone of cells respond to a specific antigen. Each clonal receptor in both B and T cells is structurally unique and not encoded in the germ line. A clonal receptor is not predestined to recognize any particular antigen, either. These antigen-specific receptors are not passed to the next generation and therefore have to be established by every generation,

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Correspondence and reprint requests: Mikko Hallman, M.D., Ph.D., Department of Pediatrics, University of Oulu, Kajaanintie 52A, PL 5000, 90220 Oulu, Finland; e-mail: mikko.hallman@oulu.fi
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mostly during early childhood. This process is inadequate for protection of the young host. First, there is a lag period of more than a week for a primary response, or several days in a primed host, before a full adaptive immune response takes effect. The host's capability to limit the infection before adaptive immunity develops used to be a matter of life and death before the introduction of immunizations and antibiotics. Another critical issue is that the virtually limitless number of antigen receptors emerging as a result of random genetic mechanisms may potentially be capable of reacting not only with infectious microorganisms but also with various environmental antigens or even self-antigens. This may lead to allergies or autoimmune diseases.

INNATE IMMUNITY AND PATTERN RECOGNITION RECEPTORS AND MOLECULES

Innate immunity plays a primary role in acute host defense. It critically contributes to destroying the pathogen, determining the localization and extent of the challenge, and facilitating the adaptive immune response. Innate immunity, or first-line host defense, primarily serves to restrict the infectious challenge during the lag period required for adaptive immunity to develop. The innate component of immune recognition is based on a germ line–encoded recognition system. Pattern recognition receptors and molecules selectively recognize membrane-derived structures in pathogenic microorganisms. The latter include, among others, LPS (also called endotoxin) from the cell wall of Gram-negative bacteria, peptidoglycans, teichoic acids and lipoteichoic acid from Gram-positive bacteria, lipoglycan from *Mycobacteria tuberculosis,* and mannans from yeast and the outer surface proteins that adorn viruses.

The total number of receptors involved in innate recognition of antigens is limited to approximately 100. However, microbes are extremely heterogeneous and have very high mutation rates. The response of the innate immune system has been focused on the highly conserved and essential structures collectively present in many types of microorganisms. Many of the mammalian pattern recognition receptors date back hundreds of millions of years.

The absence of microbial markers on the surface of the host cells constitutes the basis of self *versus* nonself discrimination (1, 2). This requires receptor-ligand interaction between the host's pattern recognition molecules and specific molecular patterns in the microorganism. Although certain postreceptor events signal proliferation and differentiation of inflammatory cells, many host defense functions are activated instantaneously or at least within less than a day (1–3). The expression of the pattern recognition receptors is not clonal, and a given receptor expressed by a given cell type has a defined specificity to a certain group of microbes. The receptors of the innate immune system are preferentially expressed on monocytes and macrophages, but also on dendritic cells, B cells, and even in other cell types not traditionally considered in host defense.

Structurally, pattern recognition receptors have LRR domains, calcium-dependent lectin domains, or scavenger-receptor protein domains (1). Functionally, pattern recognition receptors can be divided into three classes labeled as secreted, endocytotic, or signaling molecules. The best-characterized secreted receptor is MBL, a member of the collagen-containing lectin or collectin family that includes SP-A, SP-D, and others. MBL is synthesized in liver and secreted into serum as a component of the acute-phase response. MBL binds to carbohydrates on Gram-positive and Gram-negative bacteria, yeast, and some other microorganisms. This leads to activation of MBL-associated proteases and consequent activation of the complement cascade by a mechanism not requiring antigen-antibody complexes (3).

Endocytic pattern recognition receptors occur on the surface of phagocytes. These receptors recognize the pathogen-associated molecular patterns and thereby move the pathogen into intracellular lysosomes of macrophages, where it is destroyed. Components of the pathogen may then be processed to be presented by major histocompatibility complex molecules on the surface of the macrophage. Macrophage mannose receptor and macrophage scavenger receptor are among the endocytic pattern recognition receptors identified thus far.

Signaling receptors recognize the pathogens and induce the expression of a variety of acute-phase reaction products, including the inflammatory cytokines. Receptors for these molecules are present in a variety of inflammatory and somatic cells and are capable of inducing a number of the mediators involved in acute host defense (the cytokine cascade). Some of the inducible molecules influence the presentation of the antigen to immunocompetent clonal cells. Epithelial cells, macrophages, natural killer cells, and antigen-presenting cells prominently line the mucous surfaces and express specific pattern recognition receptors. A long-term elusive problem has been the lack of knowledge of the signaling receptors that mediate the onset of the inflammatory cascade.

FAMILY OF TLR

The *Toll* gene was first described in the context of a pathway that prescribes the dorsal–ventral development in the *Drosophila* embryo (4). The detection of the link between this embryonic pathway and mammalian innate immunity is largely because of the recognition of the conserved pathways and motifs between insects and mammals. The similarity between the dif/dorsal pathway in *Drosophila* and the NF-κB pathway in mammals led to the realization that the *Toll* pathway also specifies antifungal responses at the later developmental stages of this insect (5). This study revealed remarkable homology in the cytoplasmic domains of two transmembrane proteins, namely *Drosophila Toll* and the mammalian IL-1RI, as shown in Figure 1 (6–9). These observations, in turn, linked the signal transduction pathways in *Drosophila* and mammals and led to the discovery of a human TLR (now termed TLR4). Ligation of TLR4 induced NF-κB activation, cytokine release, and induction of costimulatory molecules on macrophages that, in turn, assisted the activation of naïve T cells (6).

To date, at least nine mammalian genes encoding mammalian TLR molecules have been identified (6–10). As depicted in Figure 1, TLRs are type 1 membrane proteins. The extracellular domain (ectodomain) of TLR contains LRR. All TLRs have a single transmembrane domain and a cytoplasmic do-

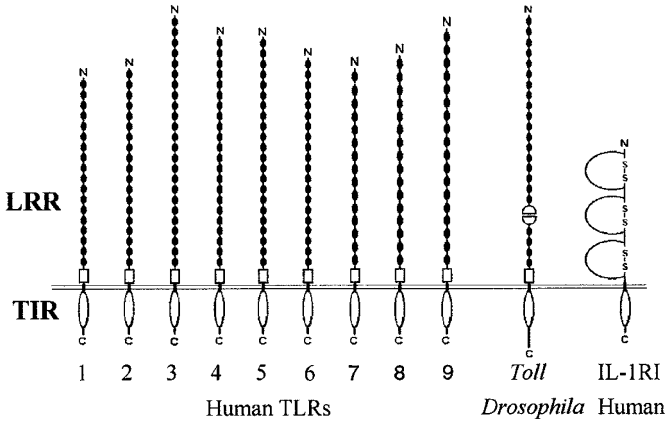

Figure 1. Schematic representation of the protein structure of human TLRs (6–9), *Drosophila melanogaster* Toll, and vertebrate IL-1RI. Individual 20- to 29-amino acid LRR are indicated as black circles. The ectodomain consisting of 18–24 LRR shows variation among different human TLRs and among different species. A sequence of approximately 60 amino acids, including two to four conserved cysteines, is shown as a white box. This domain is conserved in several other human proteins, such as platelet glycoprotein IX and oncofetal antigen 5T4 (Genebank accession numbers X52997 and N86494, respectively). A cysteine-rich domain of approximately 90 amino acids present in *Drosophila Toll*, but missing from human TLRs, is depicted as two white semicircles opposite to each other. The ectodomain of IL-1RI consists of three immunoglobulin-like domains. An intracellular signaling domain of approximately 200 amino acids common to all TLRs and vertebrate IL-1RI is indicated as a gray ellipse. This TLR/IL-1R or TIR domain is highly conserved among different TLRs and IL-1RI and among different animals.

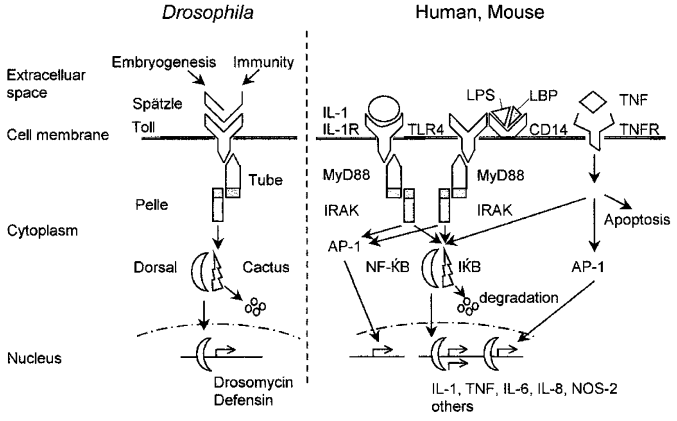

Figure 2. The pathway for the innate immune response in insects and in mammals. The homology between insects and mammals is illustrated by the similar shapes of parts of the molecules. Otherwise, the shapes are arbitrary. The following homologies between insects and mammals are evident: Tube and MyD88, Pelle and IRAKs, Cactus and IκB, Dorsal and NF-κB, respectively. The *Toll* ligand Spätzle is likely to mediate both host defense and embryogenesis (11), whereas LPS activates TLR4 in mammals. LPS also interacts with extracellular CD14 and LPS-binding protein (LBP), and MD-2 is additionally required for activation of TLR4 (12–14). In mammals, TLR-4, other TLRs (not shown), IL-1RI, and IL-18 receptor (not shown) share a similar intracellular domain, called Toll/IL-1R domain or TIR domain (Fig.1). The TNF receptor (TNFR) is not a member of the TLR/IL-1R superfamily, and TNFR additionally signals apoptosis. Some of the downstream components in the inflammatory cascade are shown. Intracellular MyD88 interacts with TIR domains of TLR and IL-1R *via* its own TIR domain. MyD88 recruits IRAKs *via* their death domains (gray areas). Activation of TLR, IL-1, and TNFRs results in release into the nucleus of NF-κB and the NFs of the activator protein-1 (AP-1) family. They are the immediate activators of the inflammatory cascade that includes inflammatory cytokines, inducible NO synthase (NOS-2), and other mediators.

main involved in signal transduction. This cytoplasmic domain is referred to as the TIR domain on the basis of its homology with IL-1RI.

From DNA sequence comparisons of TLR genes among *Drosophila*, reptiles, birds, and mammals, the genes are exceptionally well conserved. A common ancestor gene may have been present >350 million years ago. Compared with the cytoplasmic domain, the extracellular LRR is less well conserved between, for instance, man and mouse.

HOW THE HOST USES TLRS

The host response to pathogenic microorganisms depends on recognition of the antigenic determinants of the pathogen and activation of specific effector mechanisms. These include the appropriate postreceptor events for the elimination of the pathogen, signaling as to the route of entry, and localization of the pathogen in the host. Adaptive immunity in vertebrates uses the innate system in the selection and presentation of antigens to clonotypic recognition systems.

In mammals, TLR transmits the signal from the ectodomain to the cytoplasm, leading ultimately to the activation of NF-κB [Fig. 2; (11–14)]. This process has been best described between LPS and TLR4. The LPS-induced activation of TLR4 is mediated *via* the TIR domain that uses a conserved signal transduction pathway. This pathway requires MyD88, IRAKs, and other downstream intermediate proteins, not shown in Figure 2 (15, 16). MyD88 is a protein that interacts with the TLRs through its own carboxy-terminal TIR domain. Through its amino-terminal death domain, MyD88 recruits IRAK to propagate the proinflammatory signal that ultimately phosphorylates the IκB kinase complex consisting of IκB kinase α and β and a scaffold protein NEMO/IKKγ (17–19). This phosphorylation event liberates bound NF-κB from the cytoplasm into the nucleus. Both NF-kB and the activator protein-1 family (c-Jun, fos, ATF2, ELK1, and others) serve as nuclear transcription factors (some factors affect the stability of mRNA) in this chain of events. By binding cognate *cis* elements in many genes that control inflammatory events, NF-κB induces IL-1, IL-6, TNF-α, inducible NO synthase, and others. Secondary activation of cytokine receptors, which are widely distributed among immune and nonimmune cells, induces further cascades of cytokines, cytokine antagonists, chemokines, adhesion molecules, vasoactive agents, oxidants, antioxidants, proteases, antiproteases, and stress hormones. They are involved in various facets of the host defense or up-regulate the stress resistance of the host.

The *Drosophila* Toll pathway is critical for the regulation of drosomycin, an antifungal peptide that is synthesized in the fat body of the fly in response to a fungal challenge (11). Activation of *Toll* results in nuclear translocation of Dorsal, which is homologous to NF-κB in mammals. This then permits transcriptional up-regulation of the *Drosophila* drosomycin gene. *Toll* in *Drosophila* also serves as a dorsoventral embryonic regulatory signal, which is triggered by the Spätzle protein that is cleaved by a protease from its precursors (Fig. 2).

TOLL AGONISTS

The initial description of human TLR4 did not specify a physiologic ligand, although it was postulated that this would be a microbial cell wall product (6). The clue to this puzzle came from the long-standing observation that two mouse strains, C3H/HeJ and C57BL/10ScCr, were resistant to LPS (20, 21). Using a positional cloning approach, the locus was mapped to murine chromosome 4, the locus of the *TLR4* gene that proved to be what was later called the *LPS* gene. A histidine-to-proline mutation was found in C3H/HeJ mice, and the entire *TLR4* gene was deleted in C57BL/10ScCr mice (22). Animals with deleted *TLR4* gene expression fail to respond to LPS. These observations and those on Chinese hamster cells support the assumption that TLR4 is an essential element of the LPS receptor complex that directs subcellular responses to LPS in mice and, presumably, in humans (12, 22, 23).

Whether microbial products bind directly to the macrophage-associated TLR remains uncertain. LPS first interacts with the serum LPS-binding protein, and activation of monocyte-macrophages takes place after LPS (or peptidoglycan) binds to macrophage CD14. The latter is a glycosylphosphatidylinositol-linked cell surface glycoprotein and a TLR homologue with a leucine-rich extracellular domain. However, CD14 lacks the cytoplasmic domain and thus does not transduce a signal into the cell. The absence of CD14 reduces rather than abolishes the LPS or peptidoglycan response (12, 13). It may be that CD14-microbe complexes bind to or interact with TLRs, which are present in low numbers [1000 or fewer molecules per macrophage; (24)]. Another novel molecule, MD-2, binds to LPS and confers the response of TLR4 to LPS. MD-2 may be the link between LPS and the signaling of TLR4 (14).

The density of TLR influences the degree of inflammatory response to a microbial toxin (12). Considering the similarities in pathogens among vertebrates, the large interspecies difference in the TLR4 extracellular domain is remarkable. This is consistent with the possibility that the variable LPS structures from different Gram-negative bacteria exert a selection pressure on the genetic structure of the molecule. In addition, differences among human individuals in the LPS responsiveness of macrophages have also been observed, and a patient refractory to LPS effects was reportedly predisposed to recurrent infections (25). According to preliminary evidence, a common human genetic variation affecting a single amino acid in the extracellular domain of TLR4 altered the responsiveness of the airways to LPS challenge (26).

On the basis of expression in heterologous cells *in vitro*, LPS was deemed to also interact with *TLR2* (27, 28). However, mice lacking *TLR2* expression normally respond to LPS (29), but had a very low response to toxins from Gram-positive bacteria (30). Furthermore, *TLR2* was recruited to macrophage phagosomes containing specifically Gram-positive bacteria or yeast, whereas TLR4 was recruited to phagosomes containing Gram-negative bacteria (12, 30, 31). TLR2 seems to be a receptor for components of Gram-positive bacteria, mycobacteria, and yeast. According to Ozinsky *et al.* (32), the cytoplasmic TIR domains of individual TLRs are not functionally equivalent. TLR2 and TLR6 were both recruited to the macrophage phagosome, where they recognized peptidoglycan from Gram-positive bacteria, whereas only TLR2 and another unidentified TLR were required to identify Gram-positive bacterial lipopeptide. When constitutionally activated, the TLR4 homodimer became functionally active. However, TLR2 required TLR6 or TLR1 pairs in order to be functionally equivalent with TLR4, as judged on the basis of TNF induction.

Nucleotide sequences containing unmethylated CpG are found in prokaryotic DNA at much higher frequencies than in vertebrate DNA. CpG induced production of immune-stimulating cytokines in macrophages, dendritic cells and natural killer cells (33). Hemmi *et al.* (34) recently demonstrated that TLR9-deficient mice showed no immune response to CpG DNA. This led the investigators to postulate that TLR9 is involved in immunogenic identification of bacterial DNA.

It has been suggested that the expressions of TLR2 and TLR4 are restricted to hematopoietic cells, although studies show wide tissue distribution [Table 1; (7–10, 35, 36)]. *TLR4* was expressed in cardiac myocytes, in which ischemia induced its expression (35). The roles of the different members in the TLR family, identification of their respective ligands, and detailed characterization of the proteins recruited to their respective signalsomes remain to be determined.

Does the human TLR serve functions other than signaling a response to microbes? The first reports on possible endogenous ligands for TLRs are being published. According to Ohasi *et al.* (37), the potent proinflammatory response by the human heat shock protein 60 was absent in macrophages from mutant C3H/HeJ TLR4 mice, whereas they responded to CpG DNA. Macrophages from normal mice had a proinflammatory response to the heat shock protein 60, suggesting activation of TLR4. Besides this protein, other endogenous mediators are candidates as activators of TLR. The fact that recruitment of TLR2, TLR1, and TLR6 was induced by phagosomes containing IgG-coated erythrocytes suggests the role of immunocomplexes as activators of TLR (32). Inasmuch as mice with deleted *MyD88* gene have apparently normal embryonic development, it is unlikely that a TLR response controls embryonic development.

CLINICAL IMPLICATIONS

There are remarkable differences in the tolerance of mucosal surfaces for microbes. The number of bacteria in the gastrointestinal tract normally exceeds the number of somatic cells, but the nonpathogenic microbes do not induce the cytokine response. Other mucous membranes (peripheral airways, alveoli, and urinary tract) are generally sterile, despite their intermittent exposure to microbes. The differences in responsiveness remain largely unexplained. Apart from the cytokine cascade apparently controlled by TLRs, other microbial clearance mechanisms include specific antibodies, mucociliary escalator, phagocytosis, intracellular killing by phagocytes, and production of proteins involved in host defense (collectins, defensins, lysozyme, lactoferrin, transferrin, and others). MBL, SP-A, and SP-D of the collectin family bind to CD14, LPS, and other toxins (38, 39). However, a collectin may promote microbial

Table 1. *Tissue specificity of the expression of human TLRs by Northern blot analysis**

Organ	TLR1†‡	TLR2†‡§	TLR3†‡	TLR4†‡§	TLR5†‡§	TLR6‖	TLR7**	TLR8**	TLR9**
Brain	−	+	−	−	−ND	+	++	−	
Colon	−	−	−	−	−	ND	−	−	ND
Dendritic cells	+	+	+	+	+	ND	ND	ND	ND
Heart	−	+	−	+	−	−	−	++	−
Kidney	−	−	−	−	−	−	−	−	−
Liver	−	+	+	−	++	−	−	++	+
Lung	−	+	−	+	+	+	+	++	+
Lymphocytes	+	−	−	−	+	ND	ND	ND	ND
Monocytes	+	++	−	+	+	ND	ND	ND	ND
Muscle	−	+	−	−	−	ND	ND	ND	ND
Ovary	+	−	−	−	++	+	ND	ND	ND
Pancreas	−	−	++	−	−	ND	ND	ND	ND
PBL	−	++	−	+	++	ND	ND	ND	ND
Placenta	−	−	++	−	−	ND	ND	ND	ND
PMN	++	++	−	++	−	ND	ND	ND	ND
Prostate	−	−	−	−	+	ND	ND	ND	ND
Small intestine	−	−	−	−	−	ND	+	−	ND
Spleen	+	−	−	−	−	+	+	−	ND
Testis	−	−	−	−	+	ND	ND	ND	ND
Thymus	−	−	−	−	−	+	−	−	ND

Abbreviations: ND, not done; PBL, peripheral blood lymphocytes; PMN, polymorphonuclear leukocytes.
* ++ strong expression, + visible expression, − no visible expression.
† Rock *et al.* 7.
‡ Muzio *et al.* 36.
§ Chaudhary *et al.* 8.
¶ Frantz *et al.* 35.
‖ Takeuchi *et al.* 9.
** Du *et al.* 10.

clearance without activation of the NF-κB–based inflammatory cascade (40).

Most TLRs are expressed in several tissues, although often at very low levels. Besides the contribution to innate immunity, the potential roles of TLRs in signaling tissue destruction, chronic organ injury, differentiation, and neoplastic disease remain to be studied. A defect or abnormality in one component of the innate immune system may predispose to disease first in the presence of exceptional stress (41).

The innate immune system has critical roles in host defense during periods of transition, particularly during the early development of clonal immunity, when maternal immunoglobulins are present at low levels. In intrauterine infections, the unprepared host is affected by microbes or proinflammatory cytokines, with serious consequences (fetal death owing to shock, susceptibility to long-term organ injury) (42). The expression levels of the germ line–encoded components of host defense depend on the stage of differentiation. This deficient or abnormal responsiveness of the innate immune system in immature lung is in line with the variably low expression levels of SP-A (43), SP-D (44), TLR2, and TLR4 (45). The responsiveness of lung to cytokines and microbial components is complex and age-dependent, suggesting effective developmental regulation of the responsiveness of innate immunity (46, 47). Small premature infants with immature lungs may exhibit bronchopulmonary dysplasia, characterized by a lack of growth and alveolarization and deficient gas exchange (48). Increased chemokines and proinflammatory cytokines and low antiinflammatory IL-10 (49) and SP-A (43) associate with chronic lung disease, possibly as a sign of maladaptation of the immune system.

Susceptibility to recurrent infections is influenced by heredity and may, to some extent, involve microbial-binding proteins. A lack of *TLR4* expression predisposes mice to Gram-negative bacterial infection (50), and an absence of the scavenger receptor, to *Staphylococcus aureus* infection (51). Furthermore, defects in complement enhance bacterial infections both in mice (52) and in humans (53). A lack of SP-A in mice enhances infections originating from the respiratory tract, most notably Group B *Streptococcus* and *Pseudomonas* (41). In humans, deficient expression of MBL because of allelic variation has been associated with meningococcal disease (54, 55) and severe cystic fibrosis complicated with *Pseudomonas aeruginosa* infection (56). A particular SP-A haplotype, on the other hand, associates with susceptibility to recurrent respiratory infections in infants (57). Allelic variation of human TLR4 has been shown to influence airway responses in LPS challenge (26).

Whether any defect in the pattern recognition proteins influences the incidence or severity of allergies and autoimmune diseases remains to be studied (58, 59).

CONCLUSIONS

Allelic variation in the genes involved in innate immunity may account for individual differences in the responses to corresponding pathogens. For example, a single nucleotide polymorphism in the TLRs could explain some of the clinical heterogeneity of the responses to an infectious challenge (26). Another example of how allelic gene variation modifies the balance between host and pathogen is the MBL gene that correlates with differences in life expectancy among patients

with identical cystic fibrosis genotypes (56). The role of different TLRs in such diseases as cystic fibrosis as well as the efforts to ascertain whether polymorphisms in TLRs play a role in modifying the innate responses in this and other childhood diseases will inevitably attract a great deal of attention. Deficient or altered proteins involved in microbial binding or subsequent signaling potentially lead to an inappropriate acquired immune response (54, 55, 59). The role of innate immunity is critical during early childhood, when the clonal development of antibodies and other host defenses is inadequate. It would seem that premature infants are particularly vulnerable to subtle dysregulation of innate immunity. Understanding the role of each TLR is the first step toward determining the effects that variations in TLR expression and primary sequence have on modifying the host response to an infectious challenge. This knowledge may help us develop novel preventive and therapeutic strategies.

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