Toll-like receptors and their role in experimental models of microbial infection

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Effective host defense against microbial infection depends upon prompt recognition of pathogens, activation of immediate containment measures, and ultimately the generation of a specific and definitive adaptive immune response. The innate immune system of the host is responsible for providing constant surveillance against infection; when confronted by pathogens it deploys a series of rapidly acting antimicrobial effectors while simultaneously instructing the adaptive immune system as to the nature and context of the infectious threat. Pathogen recognition and activation of innate immunity is mediated by members of the Toll-like receptor (TLR) family through detection of conserved microbial structures that are absent from the host. Experimental models of infection using TLR-deficient mice, as well as limited human studies, have clearly demonstrated the critical role of TLRs in host defense against most major groups of mammalian pathogens.
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Introduction

Multicellular eukaryotic hosts are constantly challenged by microorganisms that are ubiquitous in the environment or present as colonizers, mainly of the epithelial surfaces. While this microbial presence may be beneficial under certain conditions, even a slight disruption of host–microbe homeostasis can have devastating and potentially fatal consequences. Survival of the host is therefore critically dependent on efficient mechanisms for containment of normal flora as well as rapid elimination of invading pathogens. Such defenses are present in many forms, including physical barriers (keratinized surfaces, tight epithelial junctions), nonspecific secretory products (gastric acid, bile, lysozyme), and immunologic defenses. The primary function of the immune system is to identify and eliminate infection, and this is achieved through two major elements; an adaptive component consisting of exquisitely specific T- and B-lymphocytes that undergo clonal selection and expansion upon presentation of a foreign antigen, as well as an innate component that is characterized by its rapid mobilization and activation of effector mechanisms upon microbial challenge.¹ Of the two complementary subdivisions, the innate immune system has far more ancient evolutionary origins (being the only immune system of primitive multicellular organisms) and is transmitted via the germline without undergoing the somatic gene rearrangements that characterize antigen receptors of the adaptive immune system. Activation of innate immunity constitutes the first line of host defense

against infection, and provides instructive signals to the adaptive immune system by alerting it to the abnormal presence as well as the context of a microbial invader.^{2,3}

The principal cellular components of the innate immune system include polymorphonuclear leukocytes (PMNs), tissue macrophages, dendritic cells (DCs), mast cells, and natural killer (NK) cells. These diverse lineages are all characterized by their ability to rapidly respond to infection or a breach of normal tissue architecture and to recognize and remove microbes and/or damaged host cells. DCs play an especially critical role in bridging innate to adaptive immunity through presentation of microbial breakdown products to T-lymphocytes in the context of the major histocompatibility complex (MHC), thereby initiating specific adaptive immune responses. A variety of effector mechanisms of the innate immune system have been extensively characterized, including exocytosis of neutrophil granule contents, phagolysosomal fusion in the host cell cytoplasm, and production of reactive oxygen species and nitrogen intermediates. Despite this detailed understanding, the mechanisms of pathogen recognition by cells of the innate immune system that precede the induction of such effectors have remained elusive. Recently, a major molecular mechanism for innate immune recognition of pathogens was established through identification of the mammalian Toll-like receptor (TLR) gene family.^{4,5}

TLRs are one subset of a diverse group of molecules referred to as pattern recognition receptors (PRRs).^{1,3} Examples of previously identified PRRs include C-reactive protein, serum amyloid protein, and mannan-binding protein (all secreted into extracellular compartments), the mannose receptor and scavenger receptor (expressed on the macrophage cell surface), and the intracellular double-stranded RNA-activated protein kinase PKR. A major function of PRRs is to mobilize host

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defenses through several mechanisms including opsonization of foreign antigens and induction of host complement, coagulation, phagocytic, and proinflammatory signaling cascades. The microbial signatures recognized by PRRs have been termed pathogen-associated molecular patterns (PAMPs); these essential, invariant molecular structures are unique to microorganisms and shared among members of each class of infectious agents. To date, several of the 10 known members of the mammalian TLR family have clearly been shown to recognize these evolutionarily conserved microbial structures both *in vitro*, and in some cases *in vivo*, and to play an essential role in the maintenance of robust innate immunity. Identification of the TLR family represents a major advance in our understanding of host–pathogen interactions, and has stimulated tremendous interest in the field of innate immunity. In this article, we review selected aspects of TLR biology, and summarize studies that analyze the role of TLRs in experimental mouse models of infection. Brief reference will also be made, where possible, to work describing the role of TLRs in human host defense.

Drosophila Toll and the identification of mammalian TLRs

Identification of the first Toll receptor (and the origin of its nomenclature) has its origins in studies of embryonic development in *Drosophila melanogaster*.⁶ Through a mutagenesis screen, *Drosophila* Toll was identified as a gene controlling dorsoventral axis polarity in the developing fly embryo and determined to encode a transmembrane protein.⁷ Activation of Toll by its endogenous ligand, the cytokine-like peptide Spatzle, stimulates a signaling cascade culminating in the nuclear translocation of Dorsal, a transcription factor with homology to mammalian nuclear factor kappa-B (NFκB). This finding, combined with the recognition of the structural homology between the intracellular domains of *Drosophila* Toll and the mammalian interleukin-1 receptor (IL-1R) prompted investigation of the hypothesis that both molecules might also have functional similarities and play a role in innate immunity. Subsequent studies disclosed a critical role for *Drosophila* Toll in host defense; adult Toll mutant flies were unable to defend against experimental challenge with fungi and succumbed to overwhelming infection, yet retained normal responses to infection with Gram-negative bacteria.⁸ This susceptibility phenotype was shown to be due to a specific failure to synthesize the antifungal peptide Drosomycin in the *Drosophila* fat body (the equivalent of the mammalian liver) as a consequence of defective activation of dorsal immunity factor (DIF), another transactivator with homology to NFκB.⁹ Further investigations have shown that both fungal and Gram-positive infection activate the Toll pathway through the cleaved, activated form of Spatzle, and recruit members of an intracellular signaling cascade including the death-domain-containing adaptor proteins dMyD88 and Tube, as well as the serine–threonine kinase Pelle, leading to degradation of the inhibitor protein Cactus and nuclear translocation of DIF. Notably, host defense against Gram-negative bacterial infection in *Drosophila* is mediated by a distinct recognition and signaling pathway defined by

the *Imd* gene.¹⁰ The recently identified *Imd* gene product contains a death domain with closest sequence similarity to the mammalian tumor necrosis factor-α receptor-interacting protein (RIP).¹¹ *Imd* regulates the expression of all antibacterial peptides, particularly those that are most active against Gram-negative bacteria, and in response to certain stimuli may also participate in the apoptotic response. Pathogen recognition and specificity of these proteolytic cascades occurs upstream of the most proximal members described for each pathway, Spatzle and *Imd*. Recognition of Gram-positive bacteria (but not fungi) and activation of Toll is mediated by a circulating peptidoglycan-recognition protein (PGRP-SA) encoded by the gene *semmelweis*.¹² Another so-called PGRP (PGRP-LC) bearing a transmembrane domain was shown to mediate activation of the *Drosophila* *Imd* pathway in response to Gram-negative infection, indicating that members of the PGRP family represent critical microbial PRRs.¹³ Both soluble and membrane-bound mammalian PGRPs have been described,¹⁴ although their role in microbial host defense has not yet been defined. Based on structural homology, eight other Toll receptors have been identified in the *Drosophila* genome; all of these are expressed and presumably function during embryonic development, yet so far only the original member (Toll1) has been strongly implicated in host defense functions.¹⁵

Mammalian TLRs

Mammalian TLRs are a family of type 1 transmembrane receptors that share a common modular protein architecture consisting of a series of extracellular leucine-rich repeats flanked at the carboxy-terminal end by a cysteine-rich cluster that is connected by a single transmembrane span to a cytoplasmic Toll/Interleukin-1 receptor (TIR) homology domain. LRRs are found among proteins with diverse functions, many of which do not have an obvious role in immune defense, and presumably mediate protein–protein and protein–ligand interactions. Among mammalian TLRs, the length of the extracellular LRR differs, and this variation may contribute to the specificity for various microbial ligands (although direct binding of PAMPs to TLRs has not been formally demonstrated to date). The intracellular TIR domain is essential for transmitting a downstream signal in response to TLR activation. The cytoplasmic domain of human TLR4, the first mammalian TLR identified, exhibits a higher degree of sequence conservation to its mouse counterpart compared to the corresponding extracellular domains,¹⁶ suggesting that greater constraints on structural variation exist for the signaling function of the receptor compared to its role in ligand recognition. All mammalian TLRs are known to signal through MyD88, a cytoplasmic adaptor protein that consists of an N-terminal death domain and C-terminal TIR domain.¹⁷ MyD88 is recruited to the TIR domain of activated TLRs and through a homophilic association mediates a signaling cascade that results in the activation and nuclear translocation of NFκB as well as activation of MAP kinases.¹⁸ Other recently identified adaptor proteins that interact with mammalian TLRs include TIR containing adaptor protein (TIRAP)¹⁹ that functions downstream of TLR4 in a MyD88-independent signaling pathway and Toll interacting protein (TOLLIP)²⁰ that

interacts with TLR2, TLR4, and IL-1R and has been shown to downregulate both IL-1 β ²⁰ and TLR-mediated signaling *in vitro*.^{21,22}

Genetic mapping and regulation of mammalian TLR expression

The chromosomal locations of the known members of the mammalian TLR family are shown in Table 1. With the exception of human TLR10, all of the human TLRs identified to date have been cytogenetically localized. Three pairs of TLRs (TLR1/6, TLR2/3, TLR7/8) map to the same cytogenetic location, suggesting that they may have arisen via gene duplication events. The genomic location of several of the mouse TLR orthologues has not yet been published; however their chromosomal assignment may be predicted using comparative maps of the mouse and human genomes. The distribution of human TLR transcripts has been studied by real-time PCR analysis of normalized cDNA libraries and has revealed the most diverse expression pattern in spleen, peripheral blood leukocytes, lung, and gastrointestinal tract, consistent with the role of these tissues in immune defense and exposure to microbes.²³ In the THP-1 leukemia cell line, several factors including cell differentiation, exposure to PAMPs, live bacteria and several different cytokines altered the levels of TLR mRNA. Incubation of whole human blood with *Escherichia coli* upregulated leukocyte expression of TLR3, TLR7, and MyD88 while TLR1, TLR6, and TLR9 expressions were downregulated. In this study, TLR8 and TLR10 were differentially regulated, with increased expression in monocytes and decreased levels in granulocytes. Analysis of the human TLR4 promoter has identified a functional interaction between the PU.1 and interferon consensus sequence binding protein (ICSBP) transcription factors that regulates the basal expression of this TLR family member in myeloid cells.²⁴ The minimal promoter for human TLR2 has also been defined and contains binding sites for several transcription factors including Sp1 and Ets family members.²⁵ Notably, the human and murine TLR2 promoters lack sequence homology and exhibit differential activation in response to several PAMPs including LPS and mycoplasma macrophage activating lipopeptide 2 kD (MALP 2).

Numerous studies, conducted mainly *in vitro*, have led to the identification of a large number of different ligands

Table 2 Host defense defects conferred by defective TLR function

Microbial class	Pathogen	Mouse mutation
Gram-negative bacteria	<i>Salmonella typhimurium</i>	TLR4
	<i>Neisseria meningitidis</i>	TLR4
	<i>Haemophilus influenza</i>	TLR4
Gram-positive bacteria	<i>Staphylococcus aureus</i>	TLR4, MyD88
Fungus	<i>Candida albicans</i>	TLR4
Parasite	<i>Plasmodium berghei</i>	MyD88
	<i>Toxoplasma gondii</i>	MyD88
Virus	Respiratory syncytial virus	TLR4
Spirochete	<i>Borrelia burgdorferi</i>	TLR1, TLR2

for members of the TLR family.²⁶ Recognition of a single ligand has been demonstrated for TLR3 (polyI:C, representative of viral double-stranded RNA²⁷), TLR5 (bacterial flagellin²⁸), and TLR9 (hypomethylated CpG/bacterial DNA²⁹) while other TLRs exhibit broader specificities. TLR2 is capable of recognizing the widest repertoire of PAMPs including several associated with Gram-positive bacteria (peptidoglycan, lipoteichoic acid³⁰), mycobacteria (lipoarabinomannan,³¹ mycobacterial cell walls³²), protozoan parasites (*Trypanosoma cruzii* GPI anchors,³³ *T. cruzii* secreted protein Tc52³⁴), as well as microbial lipoproteins,^{35,36} glycoproteins,³⁷ glycolipids,³⁸ and nonenteric LPS.³⁹ TLR6 associates with TLR2 to recognize several other PAMPs including the dipalmitoylated mycoplasma lipoprotein (MALP 2),⁴⁰ phenol soluble modulin from *Staphylococcus epidermidis*,⁴¹ and fungal zymosan,⁴² while TLR1 associates with TLR2 to recognize triacylated lipoproteins such as *Borrelia burgdorferi* OspA.⁴³ TLR4 recognizes LPS,^{44,45} the LPS mimetic drug Taxol,⁴⁶ the fusion protein of respiratory syncytial virus (RSV),⁴⁷ as well as fungal ligands.^{48,49} Recently, TLR7 and TLR8 were reported to recognize small imidazoquinoline compounds that have antiviral properties,^{50,51} while a ligand for TLR10 has not yet been identified. Experimental confirmation of the role of the TLR family in host defense against microbes bearing one or more of these PAMPs has been obtained through the use of mice bearing spontaneous or genetically engineered defects of various TLR signaling components; these studies are summarized in the following section (see Table 2).

Gram-negative bacteria

Profound differences in susceptibility of certain inbred strains of mice to experimental challenge with Gram-negative bacteria were demonstrated long before the assignment of this phenotype to mutations of mammalian TLR4. Several studies using naturally mutant mice have implicated wild-type *TLR4* in successful host defense against a variety of Gram-negative bacteria. The prototypic example is the C3H/HeJ mouse strain that is exquisitely sensitive to progressive, overwhelming infection by *Salmonella typhimurium* (LD₅₀ < 2 colony forming units (CFU)) compared to closely related strains (LD₅₀ > 2 × 10³ CFU).⁵² This strain was also shown to be hyporesponsive to the proinflammatory properties of purified LPS, and both phenotypes were mapped to a single genetic locus on chromosome 4 initially termed

Table 1 Chromosomal location of TLR family members

Member	Human	Mouse
TLR1	4p14	Chr. 5 37.0 cM
TLR2	4q32	Chr. 3 ^a
TLR3	4q35	Chr. 8 ^a
TLR4	9q32–33	Chr. 4 33.0 cM
TLR5	1q41–42	Chr. 1 98.0 cM
TLR6	4p14	Chr. 5 37.0 cM
TLR7	Xp22.3	Chr. X ^a
TLR8	Xp22	Chr. X ^a
TLR9	3p21.3	Chr. 9 ^a
TLR10	?	?

^aDenotes predicted location based on human–mouse comparative mapping data.

Lps.^{53,54} Extensive studies led to the identification of *TLR4* as the gene encoded by *Lps*, with C3H/HeJ mice harboring a spontaneous point mutation (Pro⁷¹²→His⁷¹²) in the cytoplasmic signaling domain.^{55,16} Two other mouse strains bear mutations of *TLR4*; C57BL10/ScN and its subline C57BL10/ScNCr carry a genomic deletion and do not express *TLR4* while motor neuron degeneration (*mnd*) mutant mice have a large insertion within exon 2 that results in an aberrantly spliced transcript predicting a truncated protein.⁵⁶ Both of these latter strains are hyporesponsive to LPS and therefore would also be predicted to exhibit profound susceptibility to overwhelming Gram-negative infection.

Relative to mice bearing a wild-type *TLR4*, C3H/HeJ and C57BL10/Sc mutant mice have also been shown to develop prolonged bacteremia after experimental infection with *Neisseria meningitidis*, another Gram-negative bacteria recognized for its exuberant production of LPS.⁵⁷ Bacterial proliferation after intraperitoneal inoculation of *N. meningitidis* was several hundred times higher in *TLR4* mutants compared to control mice of the same genetic background, and the median time to bacterial clearance from the blood was 6 days compared to <1 day for wild-type animals. Finally, in a recent study of respiratory tract infection due to the Gram-negative organism *Haemophilus influenzae*, *TLR4* mutant mice exhibited reduced bacterial clearance from the lung associated with an impaired innate immune response characterized by reduced recruitment of neutrophils to the lung, impaired proinflammatory cytokine production in bronchoalveolar lavage (BAL), and reduced production of TNF- α , MIP-1 α and activated NF κ B in the airways.⁵⁸ Survival or dissemination of infection from the respiratory tract infection was not reported in these studies.

Human hyporesponsiveness to inhaled endotoxin has been associated with a common, naturally occurring missense mutations (Asp299Gly and Thr399Ile) of the extracellular domain of *TLR4*.⁵⁹ In this study, a strict individual correlation between the presence of this mutation and LPS hyporesponsiveness was not observed; however, comparison of groups bearing a wild-type *vs* mutant *TLR4* demonstrated a significant reduction in airway responsiveness to progressively increasing doses of inhaled LPS. This phenotype was associated with reduced *TLR4* protein expression on the airway epithelium. Transfection of wild-type *TLR4* into airway epithelia or alveolar macrophages obtained from subjects bearing the missense mutation restored LPS responsiveness (as measured by IL-1 α production and TNF- α production, respectively). Although these data support the relevance of *TLR4* to human LPS responsiveness, a retrospective analysis of the Asp299Gly mutation among 1047 patients in the United Kingdom failed to demonstrate an association of this mutation with the frequency or severity of documented meningococcal disease.⁶⁰ This contrasts with the experimental data described for inbred mice bearing *Tlr4* mutations and suggests that factors other than *TLR4* are responsible for human host defense against meningococcal bacteremia. Most recently, a genotypic analysis of 91 patients admitted to French intensive-care units demonstrated a similar frequency of Asp299Gly or Thr399Ile *TLR4* mutations compared to healthy blood donor controls, yet identified a significant association of the Asp299Gly mutation alone with septic shock.⁶¹

Gram-positive bacteria

A critical role for TLRs in host defense against Gram-positive bacteria was strongly suggested by gene disruption experiments demonstrating that *TLR2*-deficient mice were unable to respond to peptidoglycan derived from these organisms.⁶² Confirmation of this phenotype was obtained by infecting *TLR2*-deficient mice with *Staphylococcus aureus*.⁶³ In these studies, *TLR2*^{-/-} mice were significantly more susceptible to lethal infection after a high-dose challenge (1×10^7 CFU) relative to wild-type controls and this was associated with significantly high bacterial counts in the blood and kidney (but not in the spleen). No significant differences in survival or bacterial proliferation *in vivo* were observed in wild-type or *TLR2*^{-/-} mice after a low-dose (1×10^6 CFU) challenge. Relative to *TLR2*^{-/-} mice, *MyD88*^{-/-} mice challenged with 1×10^7 CFU *S. aureus* died more rapidly and exhibited higher bacterial counts in the liver and kidney after both low- and high-dose challenge. Production of TNF- α and IL-6 by peritoneal macrophages in response to heat-killed *S. aureus* was completely absent in *MyD88*^{-/-} mice and only reduced in *TLR2*^{-/-} macrophages, indicating a more definitive proinflammatory signaling defect conferred by *MyD88* deficiency. The observed difference in host susceptibility between these two knockout mice suggests that additional TLRs or possibly other receptors that signal through *MyD88* are also involved in host defense against Gram-positive bacteria.

A study examining polymorphisms in *TLR2* in a population of patients with septic shock has been published.⁶⁴ A mutation (Arg753Gln) within a group of highly conserved amino acids at the C-terminus of *TLR2* was identified in 3% of healthy blood donor controls; transfection of this mutant into human epithelial kidney (HEK 293T) cells revealed decreased cellular activation in response to bacterial lipoproteins but not LPS. The Arg753Gln *TLR2* variant was identified in two of 91 patients with septic shock; while this association was not statistically significant, both patients bearing the polymorphism were infected with *S. aureus*, suggesting that it may be a predisposing factor for severe Gram-positive infection.

Fungal infection

As described above, adult *Drosophila* Toll mutants were first shown to be highly susceptible to overwhelming fungal infection. In the light of this observation, it is somewhat surprising that there is relatively little evidence to implicate mammalian TLRs in antifungal host defense. *In vitro*, *TLR2* has been shown to recognize zymosan, a yeast cell wall particle, and *TLR4* plays a role in the induction of cytokines and intracellular signaling in response to stimulation with *Aspergillus fumigatus* and Cryptococcal polysaccharide capsule, respectively. A recent study compared the host response of C3H/HeJ mice to the genetically similar C3H/HeN (wild-type *TLR4*) strain after a nonlethal systemic challenge with the opportunistic fungus *Candida albicans*. At both day 1 and day 7 after experimental challenge, the C3H/HeJ strain was found to have a significantly (10-fold) higher fungal load in the kidneys compared to C3H/HeN control mice; however, later time points evaluating the clearance or progression of infection and its impact on mortality were

not reported. It may be concluded from this study that *TLR4* plays a role in restricting fungal replication in certain organs, although whether this ultimately translates into a deleterious outcome is unclear. The mechanism of impaired host defense in C3H/HeJ mice during *Candida* infection appears to be reduced synthesis of the chemokines KC and MIP-2 by macrophages, resulting in reduced neutrophil recruitment. Phagocytosis and killing of *C. albicans* by neutrophils was similar between C3H/HeJ and C3H/HeN strains, and no difference in proinflammatory cytokine production (TNF- α , IL-1 α , IL-1 β) was observed.

Parasitic infection

Participation of TLRs in host defense against experimental malaria was studied using *MyD88*^{-/-} mice infected with a virulent strain of *Plasmodium berghei*.⁶⁵ In this experimental model, lymphocyte-mediated liver injury is dependent on an elevated serum level of IL-12 and independent of IL-18. Interestingly, endogenous or exogenous IL-12 is essential for clearance of attenuated strains of malaria. *MyD88*^{-/-} mice infected with *P. berghei* showed similar mortality and parasitemia compared to wild-type controls: however they were resistant to liver injury and had significantly lower levels of serum IL-12, implicating the TLR pathway in this aspect of the host response. Infection of C3H/HeJ (*TLR4*-mutant) mice, *TLR6*-deficient or *TLR2/TLR4* doubly deficient mice did not reveal significant reductions in IL-12, suggesting that other TLR family member(s) may be responsible for mediating malarial recognition, or alternatively that a TLR-independent, *MyD88*-dependent signaling pathway is activated by *P. berghei*. The genetic control of host defense against experimental malaria is complex; independent studies using *Plasmodium chabaudi* have identified quantitative trait loci (QTLs) conferring host susceptibility on mouse chromosomes 9 (*Char1*), 8 (*Char2*), 17 (*Char3*) and 3 (*Char4*).⁶⁶ Through *in vivo* studies, *MyD88* has been shown to play a critical role in resistance to *Toxoplasma gondii*, an apicomplexan parasite.⁶⁷ In this study, *MyD88*^{-/-} mice failed to control acute *T. gondii* infection, succumbing 13–16 days after intraperitoneal parasite inoculation, while control mice survived for >60 days; a similar pattern of early mortality was observed for IL-12p40-deficient mice. This susceptibility phenotype was associated with a dramatically increased parasite burden in both knockout strains, indicating that both IL-12 and *MyD88* are essential for efficient control of *T. gondii* replication by the host. *MyD88*^{-/-} mice exhibited a defect in parasite-induced IL-12-dependent IFN- γ production *in vivo*, a pathway that was previously shown to mediate host resistance to *T. gondii*. *In vitro* stimulation of bone-marrow-derived neutrophils, splenic DC, and thioglycollate-elicited peritoneal macrophages from these knockout mice with soluble tachyzoite antigen (STAg) or live tachyzoites confirmed that parasite-induced IL-12 production is dependent on *MyD88*, although no TLR candidates (including *TLR2* and *TLR4*) have yet been formally implicated in this pathway.

Viral infection

Evidence for the participation of TLRs in host defense against viral infection was reported using RSV infection

of *TLR4*-deficient C57BL/10ScNcr mice.⁶⁸ These studies were based on earlier findings that implicated *TLR4* and *CD14* in the recognition of RSV fusion (F) protein.⁴⁷ Secretion of IL-6, IL-8, TNF- α , and IL-1 β was induced by incubation of human monocytes or thioglycollate-induced mouse peritoneal macrophages with purified RSV F protein or UV-inactivated whole RSV virus. In contrast, macrophages from mice deficient in either *CD14* or *TLR4* did not secrete IL-6 when incubated with RSV F protein. Following intranasal infection of *TLR4*-deficient mice with infectious RSV, delayed clearance was observed, with 100- to 1000-fold higher viral titers in the lungs of the C57BL/10ScNcr strain relative to control mice. Characterization of the immune response following RSV infection revealed decreased pulmonary infiltration by NK cells and CD14⁺ cells, diminished IL-12 expression by BAL cells, and impaired perforin-dependent cytotoxicity of BAL NK cells. These differences in innate immune response were not observed in parallel studies using a mouse-adapted influenza virus, indicating that *TLR4*-mediated viral recognition and host defense may be specific to RSV. It has been suggested that the RSV F protein may have specifically evolved to recognize and exploit *TLR4*, whose primary function is LPS recognition. Evidence for viral strategies to evade recognition by TLRs has also been derived from sequence analysis of two genes of the vaccinia virus, A46R and A52R, that have sequence similarity to TIR domains of mammalian TLRs.⁶⁹ Through *in vitro* studies, these gene products were shown to inhibit IL-1 and *TLR4*-mediated NF κ B activation, suggesting that they may function as antagonists of the innate immune response during intracellular viral replication. *TLR3* is another recently identified candidate for viral recognition and host defense, arising from studies using poly(I:C), a synthetic double-stranded RNA (dsRNA) analogue that is a molecular pattern associated with viral infection.²⁷ Cell transfection studies with dsRNA triggered NF κ B activation and production of type I interferons that are known for their antiviral properties. Macrophages from mice deficient in *TLR3* exhibited specific defects in the secretion of IL-6 and IL-12, activation of NF κ B, and induction of IFN- α or IFN- β , in response to poly(I:C), while B-cells from these mice did not upregulate surface expression of CD69, CD80, or CD86 in response to incubation with poly(I:C) or viral genomic dsRNA. *TLR3*-deficient mice were resistant to poly(I:C)-induced shock associated with reduced serum levels of IL-12. Although live virus infection of these mice has not yet been reported, based on the current data it is likely that *TLR3* plays a crucial role in antiviral host defense. Recent reports also suggest that mouse *TLR7* and human *TLR7* and *TLR8* may participate in antiviral host defense, based on the observation that small antiviral imidazoquinoline compounds are capable of stimulating cellular activation through these receptors.^{50,51}

Spirochetal infection

Innate host defense against the spirochete *B. burgdorferi* has been studied using *TLR1*- and *TLR2*-deficient mice.^{43,70} While initial *in vitro* studies showed that cellular recognition of lipoproteins from a variety of microorganisms including *Mycobacteria*, *Mycoplasma* sp. and the spirochetes *Treponema pallidum* and *B. burgdorferi*

is mediated by TLR2,^{35,36,71} more recent work has revealed a functional interaction between TLR2 and TLR1 that enhances cellular signaling in response to *B. burgdorferi* lipoprotein OspA.⁴³ Macrophages from both TLR1- or TLR2-deficient mice incubated with OspA exhibit impaired cytokine and nitric oxide (NO) production compared to wild-type controls. Sonicated preparations of whole *B. burgdorferi* stimulate IL-6 or NO production by TLR2^{-/-} macrophages, but only at 10–100-fold higher concentrations than that required for wild-type cells, suggesting that alternative (though less potent) TLR2-independent stimulatory pathways exist. Infection of TLR1^{-/-} and TLR2^{-/-} mice with *B. burgdorferi* produced a mild arthritis and carditis at 2 weeks post-infection with disease resolution by 8 weeks, a pattern that was similar to wild-type controls. Quantification of spirochetal load in various tissues (skin, ankle joints, ears, and hearts) at 2 weeks post-infection using PCR demonstrated elevated numbers of spirochetes at all sites in TLR1^{-/-} and TLR2^{-/-} mice compared to wild-type controls. In one study, TLR2^{-/-} mice failed to clear these high concentrations of spirochetes from their tissues at 4 weeks after infection, harboring 100-fold more bacteria than littermate controls in the ears and 10-fold more bacteria in the heart at both time points compared to wild-type or heterozygous controls.⁷⁰ These observations convincingly demonstrate a critical role for both TLR1 and TLR2 in host defense during the first several weeks following infection with *B. burgdorferi*. In contrast, neither TLR1 nor TLR2 deficiency markedly influenced the overall course of disease or the acquired humoral immune response as determined by ELISA and immunoblotting, suggesting that the presence of either genetic defect did not prevent the generation of an effective adaptive immune response associated with preserved B-lymphocyte function. Selective humoral hyporesponsiveness to vaccination with recombinant OspA was demonstrated in a small proportion of healthy human volunteers. Impaired production of TNF- α and IL-6 was observed following *ex vivo* stimulation of macrophages from these individuals with lipidated OspA. No significant mutations of either the TLR1 or TLR2 gene were detected in the low responders. Flow cytometric analysis of peripheral blood mononuclear cells from each of these individuals did reveal markedly reduced cell surface expression of TLR1 (but not TLR2), suggesting that their hyporesponsive phenotype is due to altered TLR1 function.

Mycobacterial infection

Experimental models specifically analyzing the contribution of TLRs to host defense against live mycobacteria have not yet been published. Nevertheless, extensive *in vitro* data implicating TLR2 in the recognition of mycobacterial lipoarabinomannan,³¹ a soluble tuberculosis factor,²² whole *Mycobacterium avium*,⁷¹ and *Mycobacterium tuberculosis*^{72,73} strongly suggest that one or more TLRs participate in mycobacterial host defense. Published genetic analyses of experimental mycobacterial infection among different strains of inbred mice have identified significant QTL for tuberculosis susceptibility (*ss11*) on mouse chromosome 1⁷⁴ as well as resistance (*Tr11–3*) on mouse chromosomes 1, 3, and 7.⁷⁵ TLR5 is located on chromosome 1 but is unlikely to be a

candidate gene for either the susceptibility or resistance phenotypes since it maps distal to both candidate intervals. Alternatively, TLR2 may be a potential candidate locus for resistance to infection by *M. tuberculosis* since it has been localized to chromosome 3 (though not precisely mapped).

Sequence analysis of the intracellular domain of TLR2 was performed in a group of 86 human subjects infected with *Mycobacterium leprae*, the causative agent of leprosy, in Korea.⁷⁶ In this study, patients were categorized as having one of the two major disease patterns, tuberculoid (characterized by few bacteria and a pronounced cell-mediated host response) and lepromatous (characterized by a large bacillary load and relatively weak host immune response). Ten of 45 (22%) of the lepromatous patients exhibited a C–T transition at nucleotide 2029, resulting in replacement of a conserved arginine residue with tryptophan at amino acid 677, while none of the tuberculoid patients nor control group carried this mutation. Functional studies were not performed in these patients; however, the authors postulate that this TLR2 mutation might result in defective mycobacterial phagocytosis by macrophages, resulting in an impaired Th1 response characteristic of patients with lepromatous leprosy.

Future directions

Although tremendous progress has been made towards understanding the biology of the TLRs and their essential role in innate immune recognition, many areas remain to be explored. Among the most vexing issues is the mechanism by which TLRs specifically recognize their ligands. In the absence of definitive experimental data, several alternative models have been proposed, including direct physical contact between the microbial ligand and TLR,⁷⁷ formation of a macromolecular complex including ligand, adaptor molecule(s) and TLR(s), or generation of endogenous ligands in response to infection (analogous to the system in *Drosophila*) that bind and activate TLRs. Recognition of endogenous ligands by TLRs is also an area of active investigation due to its potential relevance to autoimmune diseases, noninfectious inflammatory disorders, and elimination of neoplastic or damaged cells. A number of putative endogenous TLR ligands have already been studied *in vitro*, including heat shock protein 60 (Hsp60),⁷⁸ fibronectin,⁷⁹ and the extra domain A of fibrinogen.⁸⁰ Recent data have also demonstrated that low-molecular weight soluble hyaluronan (sHA) fragments derived from the extracellular matrix at sites of inflammation are able to induce DC maturation *in vitro* as well as *in vivo* through TLR4.⁸¹ Finally, identification of all relevant TLR ligands, combined with a comprehensive understanding of the different intracellular signaling pathways and repertoire of downstream targets activated by individual TLRs and their various combinations will significantly advance our understanding of host–pathogen interactions.

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