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Toll-like Receptors in Inflammation



Luke A.J. O'Neill
Elizabeth Brint

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Progress in Inflammation Research

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Toll-like Receptors in Inflammation

Luke A. J. O'Neill
Elizabeth Brint

Editors

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Preface

The field of Toll-like receptors (TLRs) emerged in the late 1990s from three separate fields, only two of which were concerned with inflammation research [1, 2]. These fields were interleukin-1 (IL-1) signal transduction, the search for the receptor of the Gram negative bacterial product lipopolysaccharide (LPS) and curiously, the basis for establishment of dorsoventral polarity in the developing *Drosophila melanogaster* embryo. Several investigators were exploring the molecular basis of the inflammatory effects of the cytokine IL-1, the gene encoding the Type I IL-1 receptor having been cloned in 1988. In 1991, it was realised that the cytosolic domain of this protein was highly homologous to the cytosolic domain of the *D. melanogaster* protein Toll (a name coined by Nusslein-Volhard and colleagues to describe the gene which when mutated makes developing flies look Toll, German for ‘cool’ or ‘weird’). At first this seemed odd, until it was realised that Toll activates a transcription factor called dorsal, which is a homologue of NF- κ B, a well-known inflammatory signal activated by IL-1. The link with Toll was further strengthened with the demonstration that similar to IL-1, Toll also participates in host defence, being required for antifungal immunity in the fly. The first human Toll was reported in 1997, with four others appearing in 1998. One of these, termed Toll-like receptor (TLR)-4, was the long sought for receptor for LPS, answering a key question in the innate immune response to bacteria that leads to sepsis. By 2002, many microbial products were shown to be ligands for various TLRs, with TLR1, 2, 4, 5, 6 and 9 sensing various bacterial, mycobacterial and fungal products, and TLR3, 7, 8 and 9 sensing viral nucleic acids. These will be detailed in various chapters in this book. Complexities in signal transduction were then revealed, with different adapter proteins being recruited to different TLRs, leading to the activation of specific sets of genes appropriate for the elimination of the invading pathogen. A whole new system had therefore been found, which mediated the initial response to infection, with TLRs sensing microbial products leading to activation of signalling pathways culminating in the enhanced expression of immune and inflammatory genes.

Because of their profound proinflammatory effects, researchers interested in the molecular basis of inflammatory diseases began examining TLRs as possible play-

ers. Clear roles for TLRs in the pathogenesis of such conditions as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), respiratory disease, vascular disease, inflammatory bowel disease and liver disease have emerged. Either an over-activation of TLRs, or a dysregulation in negative signalling mechanisms (many of which have been described [3]), might lead to disease via enhanced production of proinflammatory cytokines. Variation in the sequences of TLR genes have also been reported in the human population, with certain variants increasing the risk of disease, further validating the role of TLRs in the inflammatory process that leads to pathology. These findings have lead to an interest in targeting TLRs therapeutically to limit disease.

This book brings together experts in the field of TLRs, all of whom have made key discoveries. Basic information on how TLRs signal will be presented as will the role of TLRs in various pathologies. The ultimate hope is that the rapid accumulation of information on the biomedical importance of TLRs will lead to greater insights into disease processes and from there to the manipulation of the TLR system therapeutically in order to develop better treatments for inflammatory diseases where there is still a clear unmet medical need.

September 2005

Luke A.J. O'Neill
Elizabeth Brint

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TLRs as bacterial sensors

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Introduction

About a century ago, Metchnikoff was one of the first to realize that we are endowed with an inherent ability to recognize foreign matter, and moreover, that a similar cell-based defensive system operates in eukaryotic organisms as diverse as humans and “water fleas” (*Daphniae*). By studying lower organisms, i.e., starfish or water fleas, he observed “phagocytes” cells (e.g., macrophages) could attack foreign objects, ranging from rose thorns to fungal spores, engulfing the latter. The phagocytosis and the subsequent intracellular killing of bacteria were recognized as an essential part of immunity, protecting the host against pathogens that would otherwise destroy it. The principle that the dichotomous relationship between innate or natural immunity and adaptive or acquired immunity, which develops only in vertebrates, depends upon lymphoid cells, and grows stronger with recurrent exposure to antigen, grew from that time. Today we know that the innate immune response is an essential precursor to the adaptive immune response. The primary recognition of microbes therefore falls within the purview of the innate immune system, and for decades, the goal of innate immunologists has been to understand how microbes are recognized; i.e., what receptors are involved, how microbial killing is mediated and how the innate immune cells are able to sound an alarm via systemic mediators.

The discovery of TLRs as sensors of microbial molecules

It was recognized early on that the inflammatory responses induced by pathogens could be mimicked by specific molecules of microbial origin [1]. By 1955, it was known that Pfeiffer’s “endotoxin” was lipopolysaccharide (LPS), and it was clear that LPS was capable of augmenting the adaptive immune response to a protein antigen; i.e., it served an immunoadjuvant effect. In 1975, it was shown that the immunoadjuvant effect of LPS, a particularly inflammatory structural component of Gram-negative bacteria, depended upon the integrity of a single locus known as *Lps* [2, 3].

The existence of a master control locus for LPS responses had been revealed by two allelic mutations [4, 5] which were found to abolish all responses to endotoxin, and at the same time, to cause hypersusceptibility to Gram-negative infection. TLR4 was a homologue of the protein Toll, first identified in *Drosophila melanogaster* as a regulator of embryonic dorso-ventral polarity [6], and later shown to be an essential component of the *Drosophila* innate immune system [7]. Based on the fact that Toll serves an immune function in *Drosophila*, it was proposed that TLR4 might “activate adaptive immunity” in mammals [8]. However, the precise function of TLR4 was first disclosed by the positional cloning of the *Lps* locus, which revealed TLR4 as the membrane spanning component of the LPS receptor [9].

The identification of mammalian TLR4 as the principal receptor mediating the immunoadjuvant effect of LPS greatly enhanced our understanding of how microbial recognition occurs. Additional TLRs were cloned and currently a total of 10 human TLRs (1–10) and 12 mouse TLRs (1–9; 11–13) have been identified based on genomic analysis. It is believed that each TLR recognizes a limited repertoire of broadly conserved molecules of microbial origin. For example, unmethylated DNA (common to all microbes) is recognized by TLR9; bacterial lipopeptides are recognized by TLR2, acting in conjunction with TLRs 1 and 6; etc. (Fig. 1). All TLRs have a common Toll/interleukin-1 receptor (TIR) motif in the cytoplasmic domain that is believed to bind to cytoplasmic adaptor proteins that also contain TIR motifs. In addition to TLRs, the TIR motif is shared by the IL-1 receptor and IL-1 receptor-related family of signaling adaptor molecules. To date a total of five TIR domain-containing proteins have been identified that include MyD88, Mal (also known as Tirap), Trif (also known as TICAM-1), Tram (also known as TICAM-2) and Sarm [10]. Although most TLRs activate a common signaling pathway involving the adaptor molecule MyD88, other adaptor molecules are specifically involved in the signaling pathways of only a subset of TLRs. To date, many of the kinases involved in the MyD88-dependent pathways have been described and include IRAK-1, IRAK-4 and transforming growth factor- β -activated kinase-1 (TAK1) [11]. MyD88 is involved in the signaling pathways downstream of all TLRs except TLR3 and it is thus conceivable that activation of different TLRs results in the activation of rather conserved signaling cascades.

Although the discovery of TLRs as sensors of microbial components has been an important step in our understanding as to how the innate immune system is able to recognize and eliminate pathogens; important questions remain. First of all, since we are challenged daily by an indefinite number of pathogens that are as diverse as protozoa, bacteria, fungi or viruses, how are we able to recognize these pathogens with such a limited number of recognition receptors? Furthermore, it might be supposed that each microbe is best contained as the result of a tailor-made response both in terms of innate as well as adaptive immune immunity (i.e., parasites require a different response than bacteria or viruses), and the nature of an

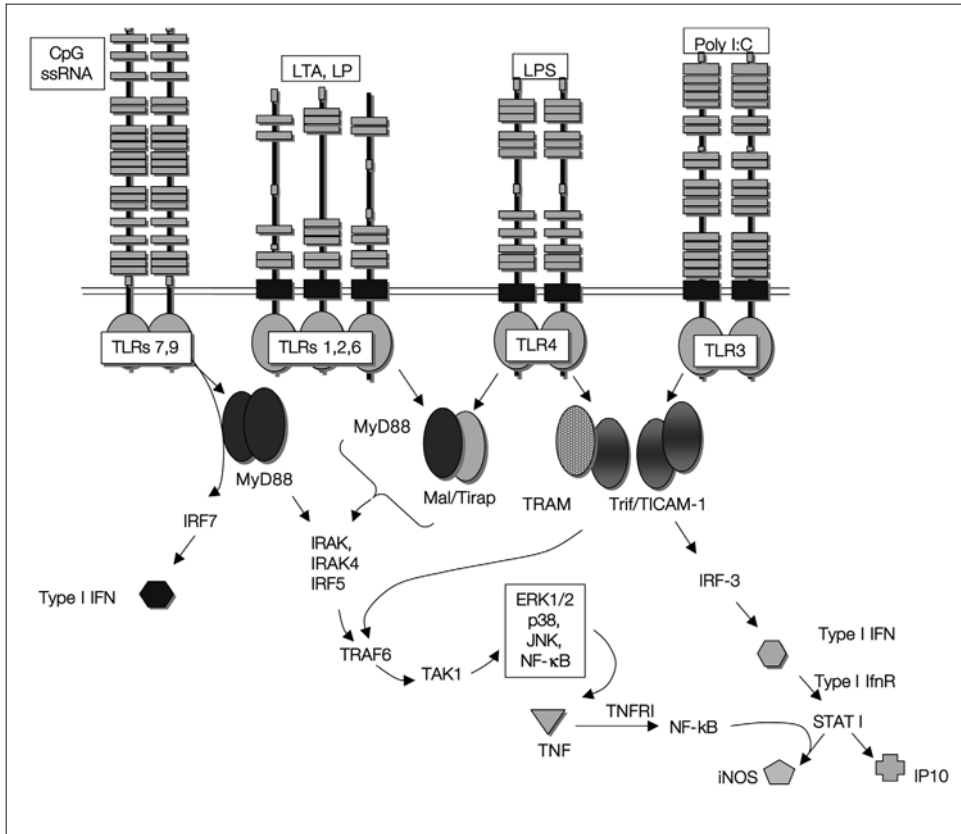


Figure 1

Activation of TLRs by various pathogen-derived molecular components and activation of downstream signaling pathways (see text for further explanation).

adaptive response is influenced by events that occur upstream of T and/or B cell activation. Since the number of TLRs we possess is limited and a striking convergence of signals occurs downstream of the TLR receptors, how can such a unique signature exist?

One hypothesis that would allow for a broad recognition and still show specificity is that each TLR senses a distinct repertoire of conserved microbial molecules, and collectively, TLRs would operate as a bar code reader [12]. This hypothesis assumes that activation of different TLRs results in the induction of TLR-specific signaling pathways; a concept that is not evident for all TLRs. Alternatively, individual TLRs may signal in different ways depending upon whether co-receptors are present and upon the ligands that activate them.

TLRs indeed recognize molecular components that are conserved throughout pathogens. For bacteria, the surface is a key element in host–pathogen interaction; it not only provides sensing ability for bacteria with its environment, but also provides a first opportunity of the host to sense bacterial presence. The surface properties and molecular composition of bacterial membranes are well defined and are rather conserved among various bacterial species. Bacteria consist of different architectural regions: (1) appendages (or proteins such as Flagellin that are attached to the bacterium); (2) a cell envelop that consist of capsule, cell wall and plasma membrane; and (3) a cytoplasmic region, containing bacterial DNA and ribosomes. All of these compartments have molecular components that are able to activate TLRs. So far, besides LPS, peptidoglycan [13], lipoteichoic acid [14, 15], di-acylated [16] and tri-acylated [17] bacterial lipopeptides, mannans [18], inosine:cytosine polymers (dsRNA) [19], unmethylated DNA (CpG) [20], flagellin [21], and single-stranded RNA [22, 23], have been reported to result in activation of TLRs.

Genetic studies have shown that TLR2 and TLR4 are essential for the response to bacterial infections [17, 24, 25]. Although TLR9 has been reported to recognize bacterial DNA, susceptibility of TLR9 knockout/mutant mice have been primarily described for viral infections [26–28]. Whereas the importance of TLRs in the recognition of pathogen-derived molecular components is now widely accepted, the complexity of TLR-ligand interactions or how different TLRs can activate unique signaling pathways is still the subject of intensive research.

Sensing of Gram-negative bacteria by the TLR4/MD2 complex

Gram-positive bacteria possess a thick cell wall consisting of multi-laminar peptidoglycan polymers (disaccharides cross-linked by short chains of amino acids) complexed with lipoteichoic acid molecules. On the other hand, Gram-negative bacteria have a relative thin cell wall that consists of a single layer of peptidoglycan encased within an outer membrane. The outer membrane of Gram-negative bacteria contains LPS, which can induce strong inflammatory responses in mammals. The LPS-TLR4 pathway has been studied extensively and provides us with some surprising clues of how TLR-ligand interaction might occur at the supramolecular level and how specificity within this receptor complex can result in unique signaling responses.

LPS is exceptional in its ability to induce strong immune responses, marked by the production of proinflammatory cytokines such as tumor necrosis factor- α (TNF- α) and interleukin-1 (IL-1), which in high concentrations can be detrimental to the host, but can also result in protective immune responses. The LPS molecule consists of three characteristic regions: (1) a lipid A moiety, (2) a core oligosaccharide and (3) an O-oligosaccharide side chain that can vary in length. Bacterial variants with

LPS exhibiting long O-oligosaccharide chains form “smooth” colonies while those with LPS lacking O-oligosaccharides chains form “rough” colonies.

The LPS receptor is multipartite. In 1990, Wright et al. [29] showed that the lysosylphosphatidylinositol (GPI)-anchored plasma membrane protein CD14 is an important part of the LPS receptor complex, though incapable of signaling by itself. Furthermore, a plasma protein known as LPS binding protein (LBP) was known to engage LPS and to facilitate its interaction with CD14 [29]. CD14 has been found to exist as a soluble protein (sCD14) in the serum and as a GPI-anchored protein (mCD14) of myeloid lineage cells. Pursuant to the identification of TLR4 as the membrane spanning component of the receptor, a small exteriorized protein known as MD-2 was also found to be an essential component of the complex [30, 31]. The TLR4–MD-2 complex is able to physically bind LPS, but the LPS-receptor interaction is greatly enhanced by CD14 without direct physical interaction of CD14 with this complex [32]. Until recently (see below), it was believed that all LPS molecules were sequentially engaged by LBP and CD14, ultimately leading to the formation of a complex consisting of LPS, CD14, myeloid differentiating factor 2 (MD-2) and TLR4. Genetic evidence of direct interaction between TLR4 and LPS [33] and MD-2 and LPS [32] has been presented in support of this model. Binding of LPS to the TLR4/MD-2 complex is thought to lead to multimerization or conformational change of the receptor and the recruitment and activation of intracellular adaptor molecules.

Activation of TLR4 initiates a unique set of intracellular signaling events. Unlike all other TLRs, TLR4 activates both MyD88-dependent and MyD88-independent pathways, which first became apparent when MyD88 knockout macrophages displayed delayed (but not absent) activation of Mitogen-Activated Protein Kinases (MAPK) and nuclear factor κ B (NF- κ B) when exposed to LPS [34]. Studies using Trif mutant (*Trif^{L-ps2}*) or Trif and Tram knockout mice subsequently showed that the LPS signaling pathway is bifurcated, depending upon MyD88 and Mal/Tirap on the one hand and on Trif and Tram on the other (Fig. 1) [35–37].

The adaptor molecule Trif is of particular interest in that it is able to induce a signaling pathway downstream of TLR3 and TLR4 [35, 36, 38, 39]. Trif acts, in large part, to activate IRF-3 resulting in the subsequent production of type I interferons (IFN), which are required to link innate and adaptive immune responses [40–42]. Type I IFN is now well recognized for its involvement in the maturation of antigen presenting cells (APCs), causing upregulation of cell surface expression of Major Histocompatibility Complex (MHC) class I and class II molecules, and co-stimulatory molecules such as CD40, CD80 (B7-1) and CD86 (B7-2). This allows for optimal interaction between APCs and naïve CD4⁺ and CD8⁺ T-cells through binding of MHC class I or II associated peptides and the T-cell receptor (TCR) [43]. The Trif/Tram pathway thus efficiently drives T and B cell responses.

Of all the TLRs, the signaling pathways downstream of TLR4 appear to be the most complex. At present, it is believed that the TIR-domain of TLR4 directly asso-

ciates with Tram and subsequently forms a heterodimer with Trif resulting in initiation of the signaling cascade [44]. The adaptor molecule Mal was found to mediate signaling cascades downstream of TLR2 and TLR4 receptors [45–47]. Originally, Mal was thought to possess no additional functional domains and to serve as a bridge between the cytoplasmic domain of TLR2, TLR4 and the adaptor molecule MyD88. Yet, recent results suggest Mal to have a putative binding domain and directly interacts with TNF receptor associated factor-6 (TRAF-6) [48]. It remains unclear, however, whether Mal is able to provide specificity that differs from the common MyD88 signaling events.

CD14: More than an LPS-binding molecule?

Since CD14 lacks signaling potential by itself, it was long assumed to serve primarily as a facilitator of LPS responses; concentrating LPS towards the receptor. However, recent genetic studies provide evidence for a more elaborate function of CD14 in the recognition and signaling pathways downstream of TLR4.

Studies using CD14 mutant and CD14 null mice show activation of the MyD88-dependent pathway to be normal in response to rough LPS and lipid A, whereas smooth LPS cannot signal via MyD88 in such mice [49]. These findings suggest that the TLR4/MD2 complex can distinguish between rough and smooth LPS, but this distinction is nullified in the presence of CD14 (Fig. 2). More importantly, CD14 was shown to be required for activation of the MyD88-independent signaling pathway in response to all LPS chemotypes [49]. These observations have transformed our view of the role of CD14 in LPS-signaling and leave open an important question regarding the extent to which CD14 is able to “fine-tune” LPS signaling. The TLR4/MD-2 receptor complex is clearly able to function in two distinct modes: one in the presence of CD14, in which full signaling occurs and one in the absence of CD14, which is limited to MyD88-dependent signaling. Presumably, different cell types respond to LPS in a different way depending upon whether they express CD14 or not. Moreover, soluble CD14 would be expected to influence signaling, permitting the “full” signaling mode to occur even in cells that cannot express CD14. Indeed there are a number of cell types that express TLR4 but lack CD14; these include epithelial cells, endothelial cells, and some hematopoietic cells (lymphoid cells and mast cells, for example).

The MyD88-independent (Trif/Tram) pathway is responsible for LPS-induced type I IFN production. Besides the important role of type I IFN in the onset of an adaptive immune response, much of the toxicity caused by widespread LPS activation is mediated by type I IFN [50]. For the host it would thus be beneficial to limit the production of type I IFN, and restrict its exposure to cells that function as professional antigen presenting cells. It remains to be determined how CD14 is able to

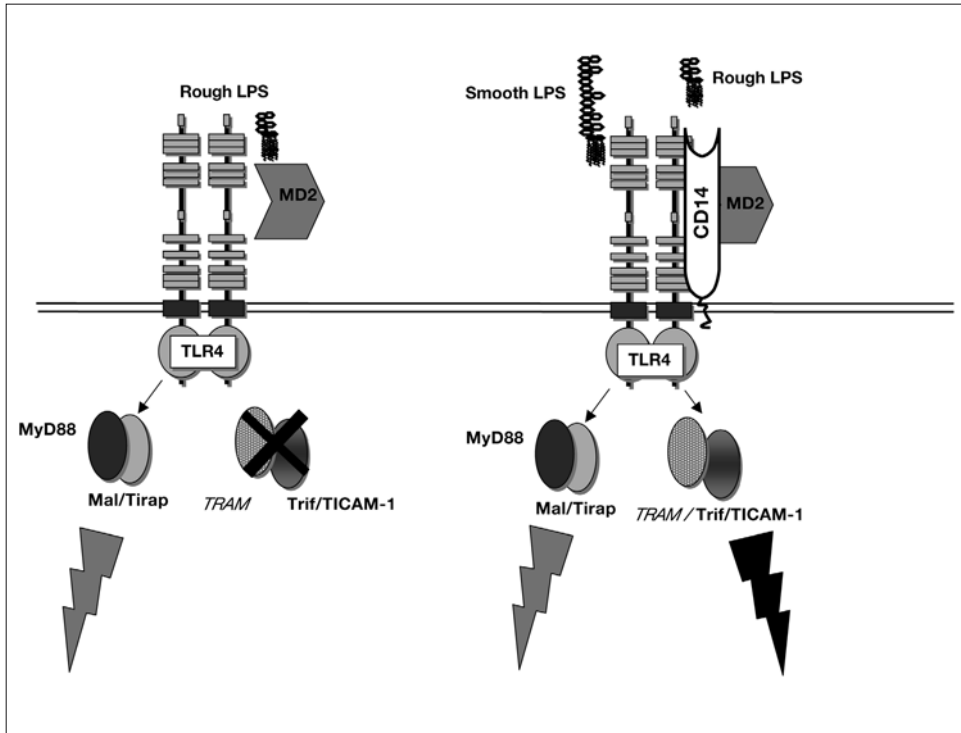


Figure 2

The role of CD14 in Myd88-dependent and independent signaling in response to rough and/or smooth LPS. In absence of CD14, rough but not smooth LPS is able to directly activate TLR4 resulting in activation of the Myd88-dependent but not-independent pathway [49]. In the presence of CD14, both rough and smooth LPS result in activation of the MyD88 and Trif dependent signaling pathway.

modify the LPS responses. One model holds that CD14 affects the supramolecular structure of the TLR4/MD-2 complex. Alternatively, the binding of CD14 to the TLR4/MD-2 complex might lead to conformational changes that permit MyD88-independent signaling.

It is also becoming clear that the role of CD14 in TLR-signaling is far more diverse than was previously believed. Besides its regulatory role in LPS signaling, CD14 mediates signaling by TLR2/6 ligands and plays an essential role in type I IFN production induced by vesicular stomatitis virus [49]. In its dual role as a facilitator of TLR2/TLR6 and TLR4 stimuli, CD14 transduces signals from structurally disparate ligand molecules. It may be inferred that the TLR2/TLR6 complex interacts with CD14 much as the TLR4/MD-2 complex does.

Recognition of Gram-positive bacteria

Peptidoglycan was once believed to be the major component of Gram-positive bacteria involved in the onset of inflammatory responses mediated by TLRs. It was thought to activate TLR2, initiating a signaling cascade via the MyD88/Mal pathway, and independently, to activate the intracellular Nod1 and Nod2 pathways, leading to activation of NF- κ B [51]. The TLR2 activating potential of peptidoglycan, however, has been under scrutiny since recent evidence has shown that the bioactivity for TLR2, present in crude preparations of *Staphylococcus aureus*-derived peptidoglycan, was due to contaminating lipoteichoic acid rather than peptidoglycan [52, 53]. Though it evidently does not recognize peptidoglycan, TLR2 has been shown to be essential for recognition of Gram-positive bacteria and is known to have exceptionally broad ligand specificity. Besides lipoteichoic acid, TLR2 recognizes diacylated as well as triacylated lipopeptides [13, 14, 16–18], and also molecular components derived from fungi [54, 55] and protozoa [56]. Given the limitations of informational content present within a single protein, the broad specificity observed for TLR2 seems unusual. One explanation for the wide range of ligands that bind TLR2 is that it effectively forms heterodimers with TLRs 1 and 6 [57]. In addition, TLR2 interacts with multiple proteins that serve as adaptors, including CD14 and Dectin-1, thereby facilitating the response towards TLR2 and acquiring the ability to bind a broad repertoire of structurally diverse ligands [49, 54, 55, 58]. More recently, it was found that the scavenger receptor CD36 functions as a co-receptor, acting in conjunction with TLR2, and specifically recognizes lipoteichoic acid as well as mycoplasma-derived lipopeptide (R-MALP-2) [52] (Fig. 3). Mice lacking functional CD36 were shown to be hyper-susceptible to Gram-positive infections such as *Staphylococcus aureus*. However, these mice responded normal to triacyl-lipopeptides and diacyl-lipopeptides containing a peptide moiety distinct from that observed in MALP-2. These findings suggest that molecular recognition properties of CD36 are highly specific and represent a novel and critical function for this class of scavenger receptors.

CD36: Its function as scavenger receptor revisited

CD36 is a member of the scavenger receptor type B family, which encompasses three paralogous proteins encoded by both the mouse and human genomes [59]. Each of these proteins has two membrane-spanning domains, one at the N-terminus and the other near the C-terminus of the polypeptide chain. In the case of CD36, the protein is anchored in caveolae or lipid rafts, and most of the amino acid chain projects into the extracellular space (few residues, if any, project into the cytoplasm). Several functions have been assigned to CD36 (also known as platelet glycoprotein IV (GPIV) [60], glycoprotein IIIB [61], PAS IV [62] on the basis of bio-

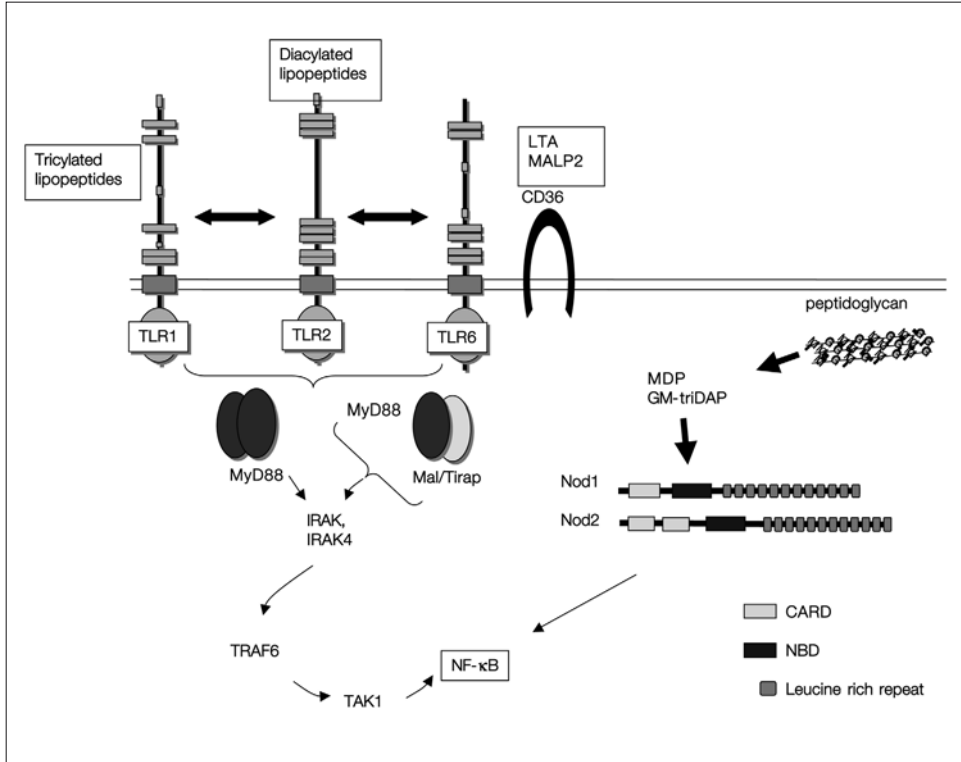


Figure 3

Recognition of bacterial-derived lipopeptides and/or peptidoglycan through TLRs and/or cytosolic Nod proteins. Bacterial lipopeptides are able to activate TLR2 directly, or heterodimers consisting of TLR1/2 or TLR2/6, resulting in the activation of the MyD88-dependent pathway with activation of NF-κB. The scavenger receptor CD36 acts as a sensor of diacylglycerides such as lipoteichoic acid (LTA) and MALP-2 in concert with the TLR2/6 heterodimer. Alternatively, peptidoglycan-derivatives such as muramyl dipeptide (MDP) and GM-triDAP activate NF-κB through activation of Nod1 or Nod2 proteins.

chemical and genetic data), and specific molecular domains – mostly within the N-terminal quarter of the protein – have been identified as important for several of these functions (reviewed in [63]). CD36 has been implicated as a receptor for endogenous molecules, including thrombospondin A and oxidized LDL [64]. Furthermore, it has been implicated in the cytoadhesion of plasmodium-infected erythrocytes [65], through non-opsonic phagocytosis triggered by PfEMP-1, a protein encoded by *Plasmodium falciparum* [66, 67]. In addition, CD36 was shown to be involved in the removal of outer segments by retinal pigmented epithelium [68].

However, the recognition of defined molecules of eubacterial origin and the direct link to receptors of the innate immune system appears to be a major (and until recently unrecognized) role for CD36. At present, it is possible, though uncertain, that the two remaining mammalian CD36 family members operate in an analogous fashion.

LTA is characteristic of nearly all Gram-positive bacteria, and di-acylated lipopeptides are made by a great number of Gram-positive and Gram-negative organisms. The sensing function of CD36 is therefore likely to be exercised quite broadly. In human populations, CD36 deficiency caused by structural mutations of the *CD36* gene [69–71] may affect platelets and monocytes (type I) or platelets alone (type II). The latter defect occurs at population frequency between 3% and 11% in Japan; the former is somewhat less common, but is by no means rare. A high frequency of nonsense and missense mutations of CD36 has been observed in African populations as well [72]. It may be assumed that CD36 deficiency is likely to cause some degree of selective immunocompromise in humans as it clearly does in mice, likely balanced by a phenotypic advantage yet unknown.

Bacterial sensing via TLR-independent pathways

Although there is no doubt that TLRs are essential to efficient recognition and eradication of pathogens, it is worth mentioning that the innate immune system has evolved TLR-independent pathways that, in parallel, provide immune-specific protection for the host. Some of these pathways are rather well established and involve, for instance, the complement system, or NK cell receptors (reviewed by [73, 74]). Other pathways are just starting to emerge and thus far limited information is available as to how they contribute to bacterial sensing.

While TLRs do not sense peptidoglycan, derivatives of peptidoglycan are detected through a pathway requiring nucleotide-binding oligomerization domain (Nod) proteins. The family of Nod proteins include Nod1 and Nod2 (Fig. 3). These proteins are expressed in the cytosol and whether directly or indirectly, mediate recognition of structurally distinct types of peptidoglycan. Peptidoglycan consists of glycan chains containing alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) sugars that are coupled by short peptides. Whereas Nod1 is activated by the PGN degradation product GlcNAc-MurNAc-L-Ala- γ -D-Glu-*meso*-diaminopimelic acid and primarily senses Gram-negative bacteria, Nod2 is able to recognize muramyl dipeptide, a minimal structure present in Gram-positive and Gram-negative bacteria. Both Nod1 and Nod2 contain caspase-activating and recruitment domains (CARD), which are thought to interact with the CARD domain of a serine/threonine kinase called RIP2 (also known as RICK or CARDIAK) [75]. Ultimately, this leads to the phosphorylation of I κ B α and subsequent

translocation of NF- κ B. Genetic variation in the genes encoding NOD proteins in humans has been associated with inflammatory diseases such as Crohn's disease [76, 77], and recently, an infectious phenotype (susceptibility to enteric *L. monocytogenes* infection) has been observed in Nod2 knockout mice [75]. This subject is discussed further in the chapter by Fukata et al. in this book.

Another TLR-independent pathway involved in the recognition of bacteria is mediated by the CD1d-dependent activation of NKT cells. NKT cells recognize endogenous or bacterially derived glycosphingolipids that are presented by DCs via the CD1d major histocompatibility complex-like molecules that have evolved to specifically capture lipid antigens. The CD1d activated NKT cells thus comprises an alternative innate pathway that leads to early recognition and production of inflammatory cytokines such as IFN- γ . Certain Gram-negative, LPS-negative bacterial species such as *Ehrlichia muris* and *Sphingomonas* "escape" TLR-recognition and the host response to infection by these bacterial species depends exclusively upon recognition via the CD1d pathway [78, 79]. Mice lacking NKT cells have been shown to be defective in efficient clearing of *Sphingomonas* from the liver, confirming the importance of this pathway for eradication of this specific bacterial strain.

Although it is clear that TLRs are a major group of receptors involved in the recognition of bacteria, other innate immune pathways have shown to contribute to an efficient eradication of bacteria. Whereas some pathways act primarily in synergy with TLRs, other pathways can act independently with the host relying exclusively on these pathways to eliminate pathogens. The relative contribution of these pathways will be a major focus for future studies, and can be expected to complement the unique inflammatory "signature" that is required for different pathogens.

Concluding remarks

Metchnikoff was aware that the innate immune system evolved to efficiently detect essentially all pathogens. Later it became apparent that it does so with a remarkable economy of genes. The TLRs do so by detecting conserved pathogen-derived molecules, triggering both an innate and an adaptive immune response. The relatively loose specificity of the receptors is at least partly explained by cooperation between TLRs and scavenger receptor molecules such as CD14 and CD36, which also enhance the sensitivity of detection. In addition, these co-receptors may help to enforce the cell specificity of the innate response. Moreover, it must not be forgotten that the host has evolved numerous TLR-independent pathways that contribute to the eradication of pathogens, usually acting in concert with the TLR system. Together these pathways perform the almost impossible task of resisting infection by a nearly limitless variety of pathogens.

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Toll-like receptors and rheumatoid arthritis: is there a connection?

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Rheumatoid arthritis

Rheumatoid arthritis (RA) is one of the most common autoimmune diseases, affecting ~0.5–1% of the adult population, with three times more females than males effected. RA is associated with a persistent inflammatory polyarticular synovitis mainly affecting the peripheral joints. In the early stages of the disease, proliferation and oedema of synovial lining cells occurs. The RA joint is characterised by the synovial tissue being infiltrated by a large number of mononuclear cells, recruited into the joint via the upregulation of chemokines and expression of adhesion molecules on endothelial cells. RA is associated with increased angiogenesis [1]. The normally thin synovium becomes thicker resulting in a joint that is swollen, puffy to the touch and often tender. As the disease progresses, the synovium invades the joint cartilage and bone to form an area known as the ‘pannus’ (Fig. 1). This structure is involved in the irreversible joint destruction through bone resorption and the breakdown of cartilage. The release of enzymes like metalloproteases, aggrecanases and cathepsins leads to the degradation of proteoglycans in the cartilage. The damage to the bone mediated by osteoclasts eventually leads to deformities and the possibility of tendon and ligaments becoming damaged. The resulting unstable inflamed joints cause pain and major disability for the patient (Fig. 2).

There is strong evidence for a key role of activated inflammatory cells in the initiation and progression of RA. Infiltrating, activated CD4⁺ T cells are thought to activate macrophages by cell–cell mediated contact to release cytokines and chemokines that in turn activate and recruit other inflammatory cells to the joint, creating a continuous cycle of inflammation (for a review see [2]). Activated CD4⁺ T cells also stimulate B cells to produce immunoglobulins, including rheumatoid factor (RF) which can be helpful in the diagnosis and prognosis of RA.

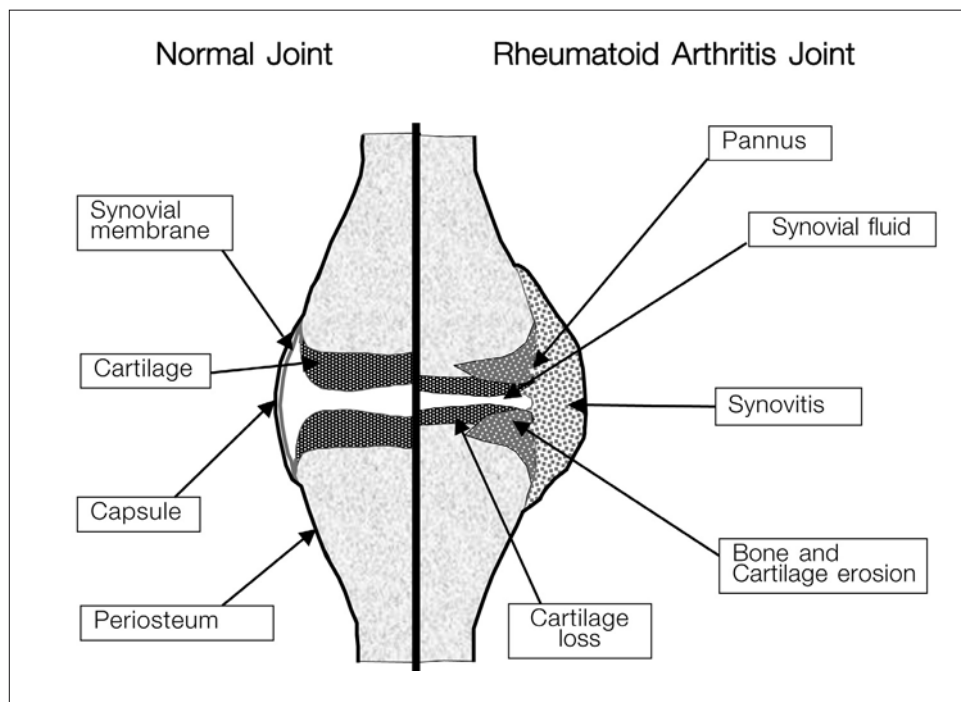


Figure 1

Diagram of a normal and rheumatoid arthritis joint

Left hand shows normal joint with intact cartilage and no bone damage. Right side shows thickened synovial membrane, cartilage thinning and pannus consisting of T lymphocytes, macrophages, fibroblasts, plasma cells and dendritic cells.

Cytokines and MMPs in RA

The chronic release of cytokines and matrix metalloproteinases (MMPs) is important in the pathogenesis of many chronic inflammatory diseases, especially RA. Since the discovery of cytokines in the 1960s [3], the important role of cytokines in modulating the immune system and disease has been gradually uncovered. Cytokines have multiple pleiotrophic activities, acting at low concentrations usually over small distances for short periods of time. Their actions include modifying the expression of membrane proteins, altering proliferation, and secretion of effector molecules. With the cloning of cytokine cDNAs in the 1980s and 1990s and the generation of monoclonal antibodies to cytokines and their receptors has transformed cytokine research [4].



Figure 2

Deformity of the hands in a rheumatoid arthritis (RA) patient

This disease causes synovial proliferation and joint destruction, usually in a symmetrical pattern. It is most visible in the small joints of the hands and feet.

In the RA joint/synovium increased levels of many cytokines have been documented, tumour necrosis factor (TNF)- α [5], IL (interleukin)-1 [6, 7], IL-6 [8], IL-8 [9], IL-10 [10], IL-12 [11], IL-15 [12] and IL-18 [13]. This is not a complete list, other factors are increased in RA but will not be discussed in this review, for example vascular endothelial growth factor (VEGF) which has a central role in the angiogenic process in RA [14]. TNF- α is predominantly produced by macrophages but also by lymphocytes, natural killer cells and mast cells. It can activate macrophages, endothelial cells, synovial fibroblasts, chondrocytes and osteophytes and can stimulate osteoclast differentiation, release of MMP, prostaglandins and cytokines and induce endothelial adhesion molecules expression.

Like TNF- α , IL-1 is considered a key proinflammatory cytokine in RA, with biological properties very similar to TNF- α . The effects of the two cytokines are often

additive or synergistic, but at the local level IL-1 is a more potent inducer of MMPs. It also stimulates expression of eicosanoids, inducible nitric oxide synthase (iNOS), and receptor activator of NF- κ B ligand (RANKL) among many other factors suggesting it a significant proinflammatory mediator in RA. IL-1 has two isoforms, IL-1 α and IL-1 β , a 17-kd protein mainly produced by monocytes and macrophages [15] which was the first cytokine to be identified in the synovial fluid of RA patients [16] suggesting it contributes to the pathogenesis of RA. Blockade of IL-1 in RA has modest anti-inflammatory effects and gives moderate joint protection [17], less profound than TNF- α blockade [18].

IL-6 is a cytokine produced by T cells, monocytes, macrophages, and synovial fibroblasts. It has pleiotrophic activity from differentiation of B cells into plasma cells, to activation of auto reactive T cells leading to the generation of autoantibodies [19]. It also upregulates intercellular adhesion molecule (ICAM) -1 expression [20], causes proliferation of synovial fibroblasts, activation of osteoclasts involved in bone resorption [21] and recruitment of immunocompetent cells into the synovium among other effects [22]. Blockade of IL-6 using anti-receptor monoclonal antibodies has a therapeutic effect in RA [23].

IL-1, TNF- α and IL-6 have been the major therapeutic targets in RA, but many other factors that play a role in the disease process leading to the chronic inflammation in RA are now being investigated. Although, IL-8 upregulates β 2 integrins and induces neutrophil migration to inflamed tissue, use of anti-IL-8 antibodies has proved effective in animal models [24], but showed disappointing efficacy in RA trials and is no longer being investigated by Abgenix (<http://www.abgenix.com/productdevelopment/?view=DevelopmentStrategy>). IL-12 is a proinflammatory cytokine that helps regulate the equilibrium between T helper cell (Th) 1 and Th2 cells, and enhances cytotoxic T cell-mediated lysis and natural killer (NK) cell activity. IL-12 synergises with a variety of cytokines and induces the production of interferon (IFN)- γ and proinflammatory cytokines [25]. Blockade of IL-12 p40 is beneficial in animal models [26], but as this subunit has more recently shown to be shared with IL-23, it is not clear whether IL-12 or IL-23 blockade produced this result. Another cytokine that could be a therapeutic target is IL-15. This activates T cells, in turn promoting the release of inflammatory cytokines including more IL-15, and stimulation of macrophages via a cell contact dependent manner to release TNF- α . IL-15 blockade is effective in animal models [27] and is presently in Phase II clinical studies [28].

IL-18 is produced by macrophages, articular chondrocytes, and osteoblasts [29, 30] and was originally identified as an IFN- γ inducing factor [13]. The pro-form of IL-18 is cleaved by IL-1 β -converting enzyme (caspase 1) to yield an active 18-kDa glycoprotein that is closely related to IL-1 α and IL-1 β [31]. It is capable of enhancing production of IL-1 and TNF- α and works in synergy with IL-12 and IL-15, inducing cell proliferation and increasing the production of other cytokines such as IFN- γ , TNF- α , and granulocyte-macrophage colony-stimulating factor (GM-CSF)

by Th1 clones [29, 32]. In the murine collagen type II induced arthritis (CIA) model, mice expressing the murine gene encoding IL-18bp showed less severe inflammation or bone destruction in joints than mice not expressing IL-18bp [33]. IL-18bp (Tadekinig- α) is currently in a Phase IIa trial for RA (<http://www.serono.com/products/areas.jsp?major=1&minor=4>).

The factors described so far are all proinflammatory mediators. Several anti-inflammatory mediators are also present in significant quantities in the synovium. These include IL-1 receptor antagonist (RA), IL-18 binding protein (bp), transforming growth factor (TGF)- β , soluble TNF-R, IL-10 and IL-4. IL-1RA specifically blocks the effects of IL-1 by binding the cell surface receptor IL-1R1 and preventing activation by IL-1 β . IL-18bp is a naturally occurring molecule which binds IL-18 in the fluid phase preventing it from binding to cells. It is similar to the receptor, but is a secreted protein [34]. IL-10 produced by monocytes, macrophages, B cells and T cells and IL-4 produced by CD4⁺ Th2 cells, both act *in vitro* to inhibit T cell proliferation and decrease the production of cytokines including TNF- α , IL-1, IL-6, IL-8 and can increase the production IL-1RA [35–37]. However, in the disease state these inhibitory factors do not appear to be produced at adequate levels to neutralise the upregulated inflammatory molecules, as has been shown for IL-10 which on addition to rheumatoid cultures shows a decrease in the production of TNF- α and IL-1 β [10].

Current therapies

Advances in the understanding of the role of cytokines in RA have lead to new developments in the treatments available to patients. Current approaches have focused on targeting cytokines for therapeutic intervention. The anti-TNF- α approved treatments in the clinic are infliximab (Remicade®) [18, 38], etanercept (Enbrel®) [39] and adalimumab (Humira®) [40] and the IL-1 inhibitor is anakinra (Kineret®) [41, 42]. The key inflammatory cytokines are TNF- α and IL-1 and targeting of these factors has been the most beneficial although current therapies targeting other inflammatory factors in the RA joint are under development as discussed briefly above. Targeting inflammatory cytokines has the inherent complication that these factors are important in immune defence, thus compromising immune regulation, with some infections occurring. Patients receiving anti-TNF- α therapies have experienced a lupus like syndrome, demyelination syndrome and serious infections including bacterial sepsis and *Mycobacterium tuberculosis*, although these complications are rare (less than 1/1000). The poor understanding of the signalling pathways involved in the release of inflammatory and protease molecules in RA has been a limiting factor in the production of more specific treatments which would aim to increase efficacy and reduced toxicity.

Aetiology of RA

The defining cause of RA still remains elusive. Environmental factors like mechanical stress and cigarette smoking have been demonstrated to have a role in disease susceptibility [43]. There have also been suggestions that RA may be initiated by bacterial or viral infections [44], but this has been a contentious issue. Attempts to identify which infectious pathogen is present in the inflamed joints of RA patients have continued to be unsuccessful and irreproducible. An alternative hypothesis that has become more favourable is disease initiation by endogenous antigens via loss of peripheral tolerance [45]. They are equally as capable as exogenous infectious agents at eliciting an immune response and may prove to be a more likely stimulus to generate the inflammation of the early stages of RA (Fig. 3).

There is a genetic influence in RA, but the concordance in identical twins (15%) [46] is the lowest for all autoimmune diseases, suggesting that there is a role for other stimuli. Antigen presenting cells use major histocompatibility complex (MHC) class II antigens to present antigenic peptides to CD4⁺ T cells. In RA CD4⁺ T cells infiltrate the joint. MHC class II antigens have been associated with RA [47]. In particular, the human leukocyte antigen (HLA)-DR molecule chains HLA-DRB1 *0404, *0401, *0405, *0101 and *1402 which all share a common epitope [48]. This suggests that there may be antigen presentation of a viral/bacterial pathogen or endogenous proteins such as a citrullinated protein [49] or human cartilage glycoprotein 39 [50], in the initial immune response of RA.

Toll-like receptors

Toll-like receptors are a family of receptors that can respond to pathogens. They are ubiquitously expressed and have also been detected on cells found in the RA synovial joint in particular antigen presenting cells (APCs) and synovial fibroblast like cells [51–53]. TLRs recognise pathogen associated molecular patterns (PAMPs) (e.g., lipoproteins, lipopolysaccharide, unmethylated CpG, flagellin, dsRNA, etc.), but also have the capacity to recognise endogenous proteins and other molecules released during inflammation and cell death, such as HSP (heat shock protein) 70 and fibronectin.

TLRs signal by a similar signalling cascade to the IL-1R involving the adaptor protein myeloid differentiation protein 88 (MyD88) [54–56], IL-1-R-associated kinases (IRAK) 1, 2 and 4 [54, 55, 57] and TNF receptor-associated factor (TRAF) 6 [58] which activate inhibitor of NF- κ B kinase (IKK) and the transcription factor nuclear factor κ B (NF- κ B). Many NF- κ B-dependent genes are important in the inflammation associated with RA, and increased NF- κ B activation has been observed in the rheumatoid synovium [59]. Evidence from MyD88 knockout mice indicated the existence of other adaptor molecules, as although some lipopolysac-

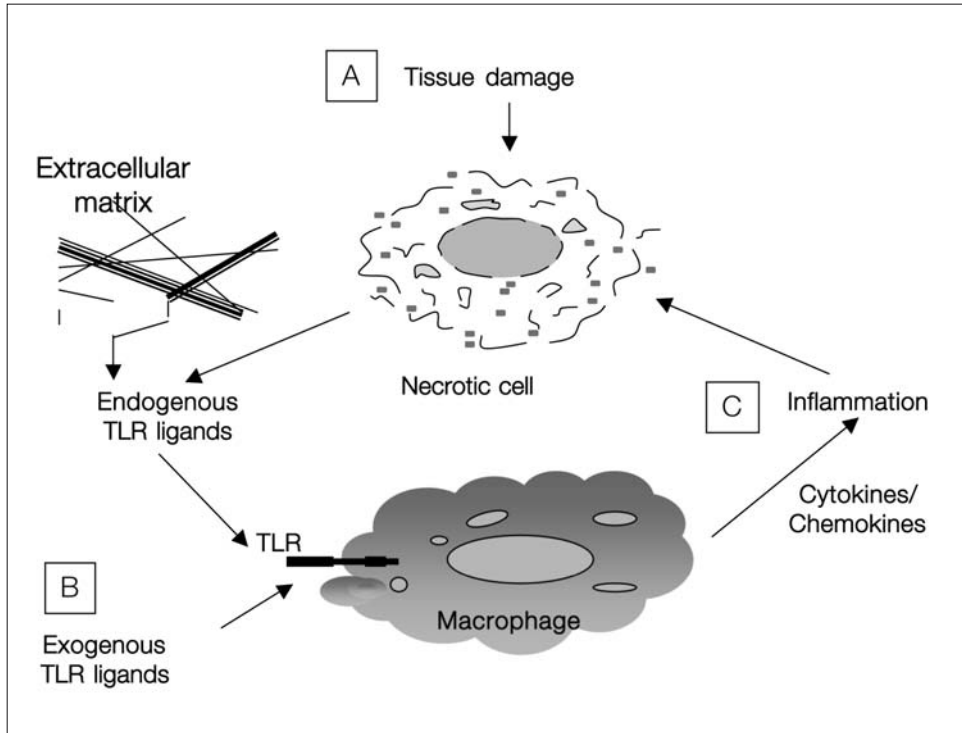


Figure 3

Hypothesis for TLR participation in inflammatory conditions such as rheumatoid arthritis

a) Cells undergoing necrotic cell death due to tissue damage, releasing endogenous TLR ligands (e.g., heat shock proteins 60, 70 and gp 96) and/or breakdown of extracellular matrix (fibronectin fragments). Subsequently, macrophages are activated through TLR signalling and produce proinflammatory cytokines and chemokines which induce inflammation. b) Exogenous TLR ligands from bacterial or viral infection active macrophages through TLRs resulting in inflammation. c) The inflammation generated by endogenous and exogenous ligands cause cells to further tissue damage and release of endogenous TLR ligands.

charide (LPS) responses were decreased in knockout mice, NF- κ B activation was still observed [60]. The next TLR adaptor protein identified was MyD88 adaptor-like (Mal) protein [61] also termed TIR domain-containing adaptor protein (TIRAP) [62]. Two other adaptors Toll-interleukin-1 receptor domain containing adaptor inducing interferon- β (TRIF) [63] also referred to as TICAM-1 [64], TRIF related adaptor molecule (TRAM) [65] also known as TIRP [66] or TICAM-2 [67] have been identified. They also activate NF- κ B [63, 65, 67], along with interferon regulatory factor (IRF)-3 and IRF-7 [65, 68].

Endogenous TLR ligands and products of tissue destruction

It is now recognised that in addition to binding PAMPs, TLR's can also recognise endogenous signals released from injured tissue and cells undergoing necrotic cell death, such as HSP60. There are already examples of endogenous molecules signalling through TLR2, TLR3, TLR4 and TLR9. HSPs are recognised by TLR2 and TLR4, in particular HSP60 [69], HSP70 [70] and gp96 [71], which are released by cells undergoing necrosis. HSP-peptide complexes are able to elicit peptide specific CD8⁺ T cell responses without adjuvants [72–74] as well as delivering an endogenous maturation signal to antigen presenting dendritic cells (DC) [69–71].

DCs stimulated with heat shock protein gp96 produce similar levels of proinflammatory cytokines, such as TNF- α , as DCs stimulated with bacterial LPS [71]. Similar results were obtained with macrophages treated with hsp60 and hsp70 [69, 70]. In murine DCs and macrophages HSPs signal in a MyD88 and TRAF6 dependent manner. Signalling through TLR2 is dependent on endocytosis of HSP60/70 and gp96 where as signalling via TLR4 is dependent on the co-receptor MD-2, similar to LPS signalling via TLR4. Studies showed that gp96 signals specifically through TLR2 and TLR4 while TLR3, TLR7, TLR8 and TLR9 showed no responsiveness implying specificity (in gain of function) [69–71]. However in studies of human macrophage-colony stimulating factor (M-CSF) derived macrophages, TLR4 signalling does not appear to employ MyD88 or Mal [75].

However, other studies have suggested that highly purified HSP70 does not induce TNF- α release from murine macrophages. The observed TNF- α inducing activity of HSP70 previously reported [70] has been suggested to be due to contamination of samples with LPS [76].

Another endogenous ligand that can activate TLR4 is cellular fibronectin, produced in response to tissue damage [77–80]. Okamura et al. were able to demonstrate that extra domain A (EDA)-fibronectin uses TLR4 as a receptor for the activation of a transfected TLR4 human embryonic kidney (HEK) 293 cell line [81]. Fibronectin contains alternatively spliced exons encoding type III repeat EDA and EDB. In response to exposure to EDA or EDB-containing fibronectin, synovial cells start producing proinflammatory cytokines and MMPs [79].

Infected cells are a source of endogenous TLR ligands. They release cytokines and chemokines as well as antimicrobial peptides, such as defensins [82–84]. One family member, β -defensin, functions as a chemoattractant of immature DCs through the CC chemokine receptor (CCR) 6 [85, 86]. In addition to this function it was shown that murine β -defensin2 acts directly on immature DCs through TLR4, inducing upregulation of co-stimulatory molecules and maturation of DCs [87]. As a consequence the immune response shifts to a Th1 dominated response, suggesting that β -defensin plays important role in the surveillance of pathogens. β -defensin may also act as a counter measure to suppressive microbial factors by generating a full scale Th1 immune response in the form of positive feedback.

Pre-packaged signals, which induce APC activation through TLR's, have been identified. These pre-packaged signals are released by cell necrosis, which could be induced by tissue damage, stress factors or infection, resulting in the release of cell components and the induction of an inflammatory response [88]. Apoptotic cells however retain their membrane integrity and are rapidly cleared by macrophages before lysis and therefore do not induce an inflammatory response [89]. Hence, DCs are activated by exposure to necrotic cells but not apoptotic cells [90, 91]. Recently the receptor and signalling molecules responsible were discovered. NF- κ B activation in the RAW macrophage cell line through exposure to necrotic cells requires MyD88 and TRAF6 signalling from TLR2, while TLR4 and TLR6 were not required [92]. mRNA [93] and dsDNA [94] which can activate TLRs have been identified from necrotic cells. Additionally, IgG-chromatin complexes [95] were also shown to activate B cells via TLR's.

HEK293 cells stably expressing TLR3 can be stimulated by dsRNA leading to activation of NF- κ B and the secretion of IL-12, IL-8 and IFN- α . DCs also respond to dsRNA by expressing activation markers, which can be inhibited, by a TLR3 specific antibody. DCs treated with endogenous dsRNA released from necrotic cells have been shown to produce IFN- α , this can be inhibited by pre-treating necrotic cells with RNase [93].

Similar results are obtained using dsDNA. Genomic dsDNA triggers the maturation of DCs and macrophages as measured by upregulation of MHC class I and II. Unmethylated CpG motifs do not appear to be responsible for the immune response induced by genomic dsDNA. The nature of this response also differs between bacterial CpG DNA and double-stranded murine DNA, the latter does not induce cytokine production. It has been hypothesised that genomic DNA may promote host survival by improving immune recognition of pathogens at sites of tissue damage or infection. However it is still unclear through which receptor this signal is transmitted. One candidate is TLR9, which recognises CpG DNA. It is a strong candidate as it is capable of recognising self-DNA (IgG-chromatin) complexes in B cells [95]. Endogenous DNA on its own is normally inert [96]. However, activation of the antigen receptor on B cells primes the cells so that TLR9 is able to be stimulated by endogenous DNA. The defining difference between bacterial and endogenous DNA is bacterial DNA is unmethylated whilst endogenous DNA has 70–80% of its CpGs methylated. Interestingly cells from autoimmune mice and humans show a decrease in this methylation [97] but elimination of methylation from murine DNA does not enable it to stimulate B cells [98]. So the mechanism by which bacterial and endogenous DNA activates TLR9 appears to be more complex than simply methylation.

Other endogenous TLR ligands can be generated from the remodelling or destruction of the extracellular space. Polysaccharide fragments of heparan sulfate as well as oligosaccharides of hyaluronic acid (HA) activate DCs as measured by co-stimulatory protein expression, morphology and T cell stimulation. Heparan sulfate

is part of the cell membrane as well as of the extracellular matrix (ECM) [99]. While soluble heparan sulfate is not found in healthy tissues in significant amounts, its level rises in the extracellular fluid around wounds [100], the synovium of arthritic joints [101] and the urine of patients with infections [102]. HA is the major component of the ECM, at sites of inflammation it is rapidly degraded [103, 104]. Inhibition of TLR4 or use of mutated TLR4 in DCs inhibited this response to soluble heparan sulfate [105] as well as to HA [106] indicating a role for TLR4 in the recognition of these molecules. The molecular mechanism of this recognition is not known.

The clotting factor fibrinogen is another example of an endogenous TLR4 ligand. Fibrinogen is usually found in the vasculature, however due to inflammation and the resulting increased permeability of endothelial cells, fibrinogen diffuses into the extravascular space [107]. Macrophage cell lines treated with fibrinogen secrete chemokines such as macrophage inflammatory protein 1 α (MIP1 α), MIP1 β and MIP2, which requires functional TLR4 [108]. Fibrinogen can therefore enhance an already ongoing immune response by activating macrophages, and the recruitment of further leucocytes (Fig. 3).

TLRs can bind many endogenous ligands in addition to their better characterised pathogenic ligands (see Tab. 1). Most have been identified for TLR4 which binds chemically unrelated and structurally different endogenous ligands. Heparan sulfate, HA, HSP60, 70 and gp 96, fibrinogen and fibronectin share no major homology with LPS. Added to this list are also saturated fatty acids, but not unsaturated acids. These were shown to induce NF- κ B in a macrophage cell line via TLR4 [109]. It is likely that the list of endogenous TLR ligands is far from complete and will grow in the years to come.

Toll-like receptors in RA

Reports of bacterial components and endogenous TLR ligands in the synovium of RA patients have supported the idea of TLRs having a role in the initiation or progression of the disease. Peptidoglycan and bacterial DNA, recognised by TLR2 and TLR9 have been reported in the human RA synovium [110], although the presence of DNA is debatable. Interestingly bacterial components have also been reported in normal synovial tissue without any excessive inflammation [111] as observed in RA. Endogenous TLR ligands such as hyaluronan oligosaccharides, fibronectin fragments, HSPs, necrotic cells and antibody-DNA complexes are present in the RA joint [112–114].

Bacterial components have been used to induce experimental arthritis in animal models. Rats injected with a streptococcal cell wall preparation develop a chronic arthritis similar to human RA [115]. Mice given an intra-articular injection with bacterial peptidoglycan develop severe destructive arthritis [116]. Another example

Table 1 Exogenous and endogenous Toll-like receptor (TLR) ligands

TLR	Exogenous ligands	Endogenous ligands
TLR2	Lipoproteins/lipopeptides	HSP60 [69]
	Peptidoglycan	HSP70 [70]
	Lipoteichoic acid	Gp96 [71]
	Lipoarabinomannan	
	A phenol-soluble modulin	
	Glycoinositolphospholipids	
	Glycolipids	
	Porins	
	Zymosan	
	Atypical LPS	
TLR3	Double-stranded RNA	mRNA [93]
TLR4	LPS	HSP60 [69]
	Taxol	HSP70 [70]
	Fusion protein (RSV)	Gp96 [71]
	Envelope proteins (MMTV)	Fibronectin [81]
	Hsp60 (<i>Chlamydia pneumoniae</i>)	Oligosaccharides of hyaluronic acid [106]
		Heparan sulphate [105]
		Fibrinogen [108]
TLR9	Unmethylated CpG DNA	Chromatin-IgG complexes [95]

TLR2 binds the exogenous ligands lipoproteins/lipopeptides (various pathogens), peptidoglycan (Gram-positive bacteria), lipoteichoic acid (gram-positive bacteria), lipoarabinomannan (mycobacteria), A phenol-soluble modulin (Staphylococcus epidermidis), glycoinositolphospholipids (Trypanosoma cruzi), glycolipids (Treponema maltophilum), porins (Neisseria), zymosan (fungi) and atypical LPS (Leptospira interrogans) as well as the endogenous ligands heat shock protein (HSP) 60, hsp70 and hsp96. TLR3 binds double stranded RNA from viruses as well as self mRNA. TLR4 binds the exogenous ligands LPS (gram-negative bacteria), taxol (plant), fusion protein (RSV), envelope proteins (MMTV) and hsp60 (Chlamydia pneumoniae) as well as the endogenous ligands such as mRNA, hsp60, hsp70, hsp96, fibronectin, oligosaccharides of hyaluronic acid, polysaccharide fragments of heparin sulphate and fibrinogen. TLR9 binds unmethylated CpG DNA from bacteria as well as chromatin-IgG complexes.

of the use of bacterial components to stimulate disease is adjuvant induced arthritis [117]. Streptococcal cell wall-induced arthritis has recently been shown to require both TLR2 and MyD88 for the induction of disease [118]. In another model of serum-transferred inflammatory arthritis, TLR4 was shown to play an important role [119].

Animal models suggest that TLRs may play a part in disease pathogenesis, although their direct relevance to human disease is unclear. Another approach to investigating the role of TLRs in RA has been through examination of naturally occurring TLR polymorphisms. Recent studies of gene polymorphisms of TLR2 and TLR4 have shown no association with susceptibility to RA. A single nucleotide polymorphism (+896A→G) resulting in the amino acid substitution Asp299Gly within the TLR4 gene disrupting TLR4 signalling, was not associated with susceptibility to RA [120, 121]. Another group investigated two polymorphisms of TLR2 (Arg677Trp and Arg753Gln) and two of TLR4 (Asp299Gly and Thr399Ile) and also found no association of these polymorphisms with the disease [122].

TLR2 was the first TLR identified in human RA, expressed at the mRNA level in fibroblast-like synoviocytes at sites of attachment and invasion into cartilage and bone and then at the protein level in fibroblast-like synoviocytes, CD16⁺ monocytes and CD16⁺ macrophages in the synovial lining layer [52, 53, 123]. Fibroblasts-like synoviocytes cultured from synovial membrane cells express TLR2 and TLR9, but only respond to bacterial peptidoglycans not to CpG DNA (a TLR9 ligand), and also show upregulation of TLR2 in response to peptidoglycans [123]. TLR2 and TLR4 expression in the synovial lining layer has been reported to be increased in RA in moderately inflamed tissue when compared to uninfamed tissue. Surprisingly, when comparing severely inflamed tissue with uninfamed tissue this increase is not as impressive for TLR4, and no longer present for TLR2 [51].

B cells can produce RF independently of T cells, with effective activation of these cells being produced by co-activation of the antigen receptor and TLR9 [95], which detect bacterial DNA [124]. This data suggests a link between the innate immune system and the initiation of RA and autoimmunity. Although some groups have reported bacterial DNA in the synovium of RA patients [110, 125, 126], this has been disputed by a more sensitive study where they could detect muramic acid, a chemical marker for bacteria, in a small number of patients but could not identify any bacterial DNA [127]. Bacterial components may be transported into the joint by macrophages from the gut [128] which are recruited during inflammation by increased endothelial permeability, chemokines and upregulated adhesion molecules. Pathogen associated molecular patterns from intracellular pathogens may persist in macrophages, potentially stimulating chronic cytokine production [129].

Components of the TLR signalling pathways have been shown to play a role in the inflammatory signalling cascades constitutively active in RA. NF- κ B and its upstream activator IKK2 but not IKK1 regulate the expression of cytokines and MMPs [130, 131] spontaneously released in synovial tissue cultures. However, TLRs are not the only route that results in IKK2 and NF- κ B activation; other stimuli include cytokines, oxidative stress, UV light can also activate these molecules. The goal now is to determine whether there is a direct functional connection between TLRs and the pathogenesis of RA, and/or whether other key molecules are involved.

Conclusion

TLRs have been implicated in the pathogenesis of autoimmune diseases and may have a common role in the initiation of chronic inflammatory conditions. The manifold activities of TLRs allows them to serve a bridging function between the innate and the adaptive immune system, thus making them attractive candidates for a role in RA. The activation of TLRs might be the earliest event in the pathogenic cascade of RA and then through TLR9 autoantibody production may sustain the inflammatory response. The ligand for the initial stimulus that initiates RA still remains elusive. If TLRs are the initial signalling receptors it could be a pathogen or an endogenous TLR ligand released during inflammation or tissue damage, but is likely to be a complex mixture of stimuli.

In the last few years data providing evidence of TLR expression and/or upregulation in the synovium of patients with RA has been published, although no functional consequences of their presence in the synovium have yet been demonstrated. More encouraging evidence comes from animal models that demonstrate pathogen initiated models of arthritis, but these data are in contrast with the absence of significant polymorphisms in patients. Overall, TLRs are attractive candidates for the receptors involved in early inflammatory mechanisms of RA, but much more work needs to be done to determine if there is a functional link, and to evaluate the exact role and extent to which they influence disease.

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Toll-like receptor 9 and systemic autoimmune diseases

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Introduction

Coley's toxin

More than a hundred years have passed since the surgeon William B. Coley discovered that advanced cancer patients suffering from sarcoma could be successfully treated with repeated bacterial infections or injections of bacterial extracts in the vicinity of their tumor [1, 2]. Despite many setbacks and inconsistent data this pioneering work with bacterial extracts led to the approval of the attenuated mycobacterium bacillus Calmette-Guerin (BCG) as a pharmaceutical agent, which is now widely used for the local treatment of bladder cancer [3, 4].

The recent discovery of germline-encoded so-called pattern recognition receptors (PRR) of the innate immune system that recognize conserved pathogen-associated molecular patterns (PAMP) to sense the presence of microbes now allows us to understand the mechanisms by which bacterial extracts elicit anti-tumor responses by activating both innate and adaptive immune responses. To allow the discrimination between self and invading pathogens, PAMPs must be specific for microbes and absent in the host or at least confined to compartments inaccessible for the correlating receptor. The recognition of these molecular signatures initiates a host of immediate innate immune effector mechanisms and via the activation of dendritic cells and the induction of co-stimulatory signals controls and shapes the induction of an adequate adaptive immune response [5]. So far the best-characterized PRRs are the Toll-like receptors (TLR). In humans ten members of this family have been identified and these receptors recognize a wide spectrum of microbial structures that comprise lipids (e.g., lipopolysaccharide [LPS] recognized by TLR4), lipopeptides (recognized by TLR2), proteins (e.g., flagellin recognized by TLR5) and nucleic acids (recognized via TLR3, 7, 8 and 9) [6]. While PRRs have evolved to detect the presence of microbes and induce an adequate protective immune response, Coley's studies already demonstrated two further aspects of these receptors: their ability to induce auto-reactive immune responses (in his case against the self-tumor tissue) and

the powerful possibility to use the manipulation of pattern recognition receptors in therapeutic strategies against disease.

Toll-like receptors are involved in the recognition of non-self and altered self

In multicellular organisms about 5% of the genome is devoted to proteins acting in the defense against infections [7, 8]. Loss or dysfunction of certain defense genes results in increased vulnerability to infection as exemplified by over 80 known primary human immunodeficiencies [9]. Often, these genetic defects lead to susceptibility to a precise range of microorganisms.

The TLR protein family is fundamental for the induction of innate immunity to a wide variety of pathogenic microorganisms. Mutations of *Drosophila toll* or orthologous mammalian TLRs illustrate that the inability to sense the presence of microorganisms in otherwise sterile compartments can have dramatic consequences for the host [10–13]. Yet, there is little doubt that in many inflammatory syndromes, such as the sepsis syndrome, excessive activation of the same receptors is the basis for the pathological state. This trade-off between resistance to immune pathology and susceptibility to infection is explicitly observable in mice lacking TLRs or downstream adaptor proteins [12, 14, 15]. The importance of TLRs in the detection of microbial pathogens and their crucial role in the induction of mechanisms to clear the infection is well documented. However, recently it became increasingly apparent that some, if not all, members of the TLR family are also essentially involved in the recognition of molecules that appear in non-infectious alterations of the normal physiological state.

Janeway formulated the theory of ‘extended self and non-self’ which hypothesizes that microorganisms are distinguished from host ‘self’ molecules by virtue of germ-line encoded receptors that recognize molecular signatures that are present in microorganisms but are absent from the host [16, 17]. In addition, endogenous host-derived molecular signatures that are not present in a healthy state may appear under certain conditions associated with disease or tissue repair. These conditions include infections, cancer, and traumatic tissue damage. Matzinger has famously described the potential range of immune stimuli that can trigger the innate arm of the immune system in her ‘danger theory’. According to this theory, certain diseases are caused by endogenously released danger signals that directly activate an innate immune response [18–21].

Many groups have reported endogenous TLR ligands. Endogenous TLR activator molecules include members of the heat-shock protein family [22–26], HMGB1 [27], fibrinogen [28], surfactant protein A [29, 30], extracellular matrix molecules [31, 32], modified lipoprotein particles [33], and nucleic acids [34–38]. With the exception of the endogenous nucleic acids as endogenous TLR ligands all other reported self-molecules were shown to utilize TLRs 2 and 4. These two TLRs have

evolved to sense minute amounts of microbial substances. Hence small amounts of contaminants could potentially account for or contribute to TLR stimulatory activities found in preparations of endogenous material. For some of the reported endogenous ligands controversial reports exist [39, 40], and LPS or other bacterial lipoproteins have been postulated to at least contribute to the TLR2 and TLR4 stimulatory activities found in preparations of different endogenous materials [41–45]. The question to what extent and under which circumstances TLRs are activated by endogenous material is of highest importance as TLR activation by self or altered self could form the basis for several sterile chronic inflammatory diseases. The scope of prospective pharmacological interference of TLR activation would be greatly extended to several highly prevalent diseases to which current therapies are unsatisfactory.

Unmethylated CpG-rich sequences in DNA activate immune cells via TLR9

Two primarily independent tracks of investigations converged in the mid 1990s leading to the identification of CpG (cytidine-phosphate-guanosine) motifs in microbial DNA as a defined immunostimulatory molecular signature. Initially, it came as a surprise when a series of elegant studies performed by Tokunaga et al. showed the first evidence for immunostimulatory activity of bacterial DNA and demonstrated that most of the anti-tumor activity of BCG lysates was mediated by its content of bacterial DNA [46–48]. DNA had been considered an immunological inert structure due to its similarity in all species. In parallel, a number of studies with antisense nucleotides indicated that synthetic single stranded oligodeoxynucleotides (ODN) can have immunostimulatory properties [49–51].

It was the achievement of A.M. Krieg to combine these two fields and to identify unmethylated CG dinucleotides within certain sequence contexts, so-called CpG motifs, to be responsible for the immunostimulatory properties of microbial DNA and synthetic ODN [52, 53]. The general minimal consensus for an immunostimulatory motif is an unmethylated XCGY, where X is any base but C, and Y is any base but G [52]. However as discussed below, the flanking bases, modifications and the larger sequence contexts can greatly influence the potency and kind of immune stimulation that is induced. Vertebrate and microbial DNAs (derived from bacteria as well as DNA viruses) differ markedly in their CpG content due to a phenomenon termed CG suppression: CG dinucleotides in vertebrate genomes occur only about a quarter as frequently as a random utilization would predict while they occur at the statistically expected frequency of 1 in 16 dinucleotides in microbial DNA. Furthermore, the bases flanking CpGs in vertebrate genomes are not random: The most common base preceding a CpG is a C and the most common base following a CpG is a G [54]. As noted above, these types of CpG motifs do not support strong immune stimulation. In addition to the differences in CG content, CpG dinucleotides are not methylated in microbial DNA but are routinely methylated at the

5-position of more than 70% of the cytosines in vertebrate DNAs [55, 56]. Taken together, these subtle structural differences between microbial and vertebrate DNA are recognized as foreign by immune cells.

Hemmi et al. identified TLR9 as the signaling receptor that mediates the immunostimulatory activity of CpG motif-containing microbial DNA and synthetic CpG oligonucleotides: Mice deficient in *tlr9* lacked all aspects of CpG induced cellular activation [10]. The signaling cascade induced by TLR9 activation involves the adapter Myd88 and a signaling complex that contains Irak1, Irak4 and Traf6 and can activate IRF7, IRF5, NF- κ B and the MAP kinase pathway [57].

TLR9 expression and sequence specificity differs between humans and mice

In humans the expression of TLR9 is restricted to certain cell types. Considerable levels of TLR9 mRNA are detected only in B cells and a small subset of dendritic cells, the plasmacytoid dendritic cells (pDCs) [58]. Another study reported that human neutrophils also express mRNA for TLR9 and respond to CpG DNA after treatment with GM-CSF [59]. pDCs are known for their ability to secrete high amounts of type I IFN during viral infections, making them responsible for most of the systemically detectable interferon- α/β seen early on during viral infections [60]. These cell types seem to be the primary target cells for CpG DNA in humans, while other cell types like monocytes, myeloid DCs, T cells or NK cells do not express considerable amounts of TLR9 and therefore cannot react directly to CpG DNA. However, these cells can be indirectly activated by cytokines produced by pDCs and/or B cells in response to CpG DNA [61]. The situation is different in mice where cells from the myeloid lineage such as monocytes/macrophages and myeloid dendritic cells also express TLR9 and can respond directly to CpG DNA [62]. This difference in the primary target cells between the two species is likely to be responsible for the more vigorous CpG induced immune responses in mice compared to humans and one has to be cautious to predict results in humans based on results in mice.

In addition to the difference in their expression pattern the TLR9 receptors themselves have diverged throughout evolution between species (e.g., 24% difference at the amino acid level between humans and mice). The precise sequence motifs, i.e., the flanking regions around the central CG that are optimal for activating mouse and human B cells (CpG-B ODN; see below) differ [63]. The optimal CpG flanking region in mice consists of two 5' purines and two 3' pyrimidines with GACGTT being a potent example while human TLR9 is optimally triggered by the motif GTCGTT and/or TCGTA [52]. It should be noted that most studies that compared the sequence specificity of TLR9 from different species have utilized phosphorothioated ODNs of the B-class. A recent report indicates that the sequence specific CpG DNA recognition of mouse or human TLR9 cannot be observed with ODN containing the natural phosphodiester backbone [64].

Three different classes of CpG ODN

The work with CpG DNA is greatly facilitated by the ability of synthetic, single stranded ODN of 6–30 bases in length that contain one or more CpG motifs to mimic the effects of microbial DNA and trigger or block TLR9 activation. Nowadays, ODNs are easy to synthesize which avoids the problem of contamination that haunts the isolation of natural microbial DNA. Highly pure synthetic DNA can be used as pharmaceutical agents. As the natural phosphodiester backbone of DNA is sensitive to nucleases, applications with CpG ODN generally use (at least for part of the molecule) the nuclease-resistant phosphorothioate (PTO) backbone, which markedly prolongs the *in vivo* half-life of modified ODNs. These modified CpG ODN have proven to be effective vaccine adjuvants and are highly effective immunotherapeutics in numerous animal models of infectious disease, cancer, allergy and asthma, and are now tested in clinical studies [52, 65].

On the basis of structural and functional characteristics three distinct classes of synthetic ODN are described in the literature denoted CpG-A (also known as D-type), CpG-B (also known as K-type) and CpG-C [66–70] (Fig. 1). All three ODN types mediate their effects via TLR9 and the original functional definition of these classes is based on their effects on the primary human target cells, the B cell and the pDC. CpG-A ODN are potent inducers of IFN- α in pDC, but are weak in their ability to directly activate B cells. CpG-B ODN are potent activators of B cells and induce the maturation and secretion of chemokines and cytokines in pDC, but do not induce the production of IFN- α in pDC. CpG-C class ODN combine functional characteristics of CpG-A and CpG-B and are potent activators of B cells and induce IFN- α in pDC even though to a lesser degree than CpG-A ODN. The mechanisms behind the differential pharmacological effects observed with the different CpG DNA classes are not completely understood. It appears that certain structural features especially the ability to form secondary and higher molecular structures influence the functional characteristics of the three CpG types. CpG-B ODN normally contain multiple CpG motifs on a phosphorothioate modified backbone and do not form secondary structures *in vitro*. CpG-A ODN contain a central palindromic sequence with at least one CpG motif on a phosphodiester backbone that is flanked by poly G motifs that carry the phosphorothioate modification. The combination of a self-complementary sequence and poly G motifs that can associate to G tetrads leads to the spontaneous formation of large nanoparticle-like complexes by CpG-A [69, 71]. These supramolecular structures influence the high induction of IFN- α in pDCs. Consistent with this notion is the fact that CpG-B ODN coated artificially on beads of similar size to the spontaneous particles formed by CpG-A acquire the ability to induce IFN- α in pDC [71]. CpG-C ODN have a uniform phosphorothioate backbone modification and combine a 5' non-palindromic segment like in a CpG-B ODN with a 3' palindromic segment as in a CpG-A ODN, but lack poly G motifs. Their self-complementary part allows the formation of sec-

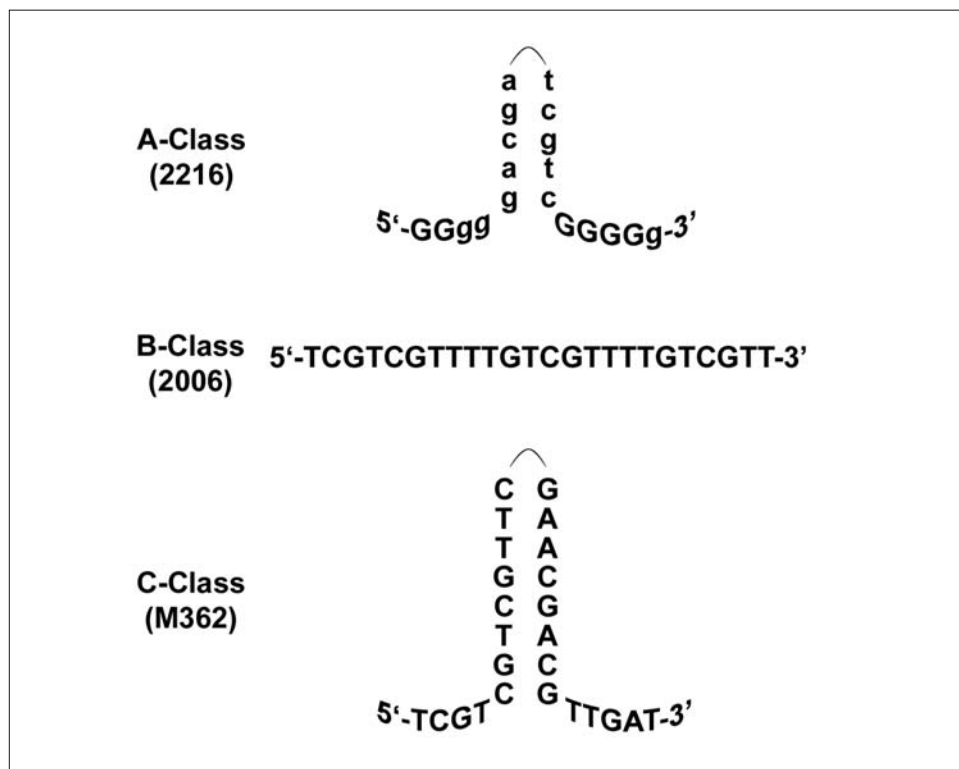


Figure 1

Example sequences and potential structural characteristics of stimulatory CpG-containing oligonucleotides (ODN) are shown. Phosphorothioate backbone is indicated by capital letters and phosphodiester backbone is indicated by non-capitalized letters. The A-Class ODN has a chimeric backbone of phosphorothioate and phosphodiester linkages and poly G ends and forms a central hairpin structure. The A-class ODN can also associate to higher order structures via interactions at the poly G ends. The B-Class and C-Class ODN have phosphorothioate backbones and the C-Class can form hairpins via palindromic sequences.

ondary structures like dimers or hairpin loops but they do not form large particles that can be observed with CpG-A [69, 72]. It is still ill defined how the formation of secondary structures in CpG ODN influences the signal induced in TLR9 expressing cells. It has been proposed that the duration and compartmentalization of ligand/receptor interaction can influence the ability of murine dendritic cell subtypes to produce interferons. It was demonstrated that the A-class ODNs reside for extended periods in endosomal structures in murine pDCs, while B-class ODNs are faster trafficked into lysosomal compartments in these cells [73]. Whether the dif-

ferential trafficking behavior of the A- or B-class ODN is responsible for the different functional cytokine output in human dendritic cells, remains to be elucidated. Natural microbial and plasmid DNA cannot be clearly categorized into one of these classes of CpG ODN but their downstream effects and cytokine profile resemble weak CpG-C ODN.

Nucleic acid recognizing TLRs are expressed in intracellular compartments

Bacterial cell walls contain a plethora of TLR-stimulating molecules but molecular structures including DNA and RNA present inside microorganisms are also capable of stimulating certain TLRs. Thus, some TLR ligands become accessible only after microbial disruption. It is plausible that most internal microbial products will be released after phagocytosis and enzymatic digestion.

Studies using antibodies to detect TLRs indicate that both TLRs 2 and 4 are expressed on the plasma membrane. Other TLRs, such as, e.g., TLR9 and the other the nucleic acid recognizing TLRs 3, 7, 8, cannot be detected on the surface of immune cells, even when epitope-tagged proteins are heterologously expressed in cell lines [74] (and unpublished results).

In resting cell lines and resting pDCs endogenous and heterologously expressed TLR9 was highly expressed in the endoplasmic reticulum. Trafficking studies of fluorescently tagged synthetic B-class ODN in human plasmacytoid dendritic cells revealed that the trafficking pattern of CpG-DNA within pDCs dramatically changed over time. After 5 min incubation, CpG-DNA was observed in early endosomes; these vesicles concentrated in a paranuclear area after 15 min. When cells were incubated with CpG-DNA for 30 min, most of the fluorescent signal was observed in tubular lysosomal compartments [74]. As stated above, CpG-DNA oligonucleotides do not represent all features of microbial DNA, and might be processed differently than natural sources of exogenous DNA such as bacterial DNA. In order to investigate the trafficking behavior of authentic bacterial DNA released after phagocytic uptake, bacterial DNA of *Escherichia coli* was *in situ* labeled with the photolyzable DNA intercalating dye ethidium monoazide bromide. When these fluorescent bacteria were fed to pDCs, DNA was observed to be released from the bacteria and trafficked to tubular lysosomal structures similarly to fluorescent CpG-DNA oligonucleotides [75].

In stimulated cells TLR9 relocates to the early endosome where signaling is initiated. The translocation of TLR9 to the endosome is an essential initial step in TLR9 signaling, which is indicated by the fact that cells recruit a fluorescent version of MyD88 to CpG-DNA containing vesicles shortly after CpG-DNA enters the cells [74, 76].

The mechanisms by which ER-resident TLRs access the early endosome are not well understood and are the subject of current studies. Furthermore, it remains

unsolved what exactly happens to DNA within the endosome. It is conceivable that DNA is modified in the environment of endo-lysosomal compartments and that such modified DNA may act as neo-antigens that influence the stimulatory activity of DNA. TLRs 7 and 8 are closely related to TLR9 and share many structural features. These TLRs recognize the small synthetic imidazoquinoline-like molecules imiquimod (R-837) and resiquimod (R-848), and guanosine analogs such as loxoribine [77–80]. Recently it has been reported that TLRs 7 and 8 can be activated by single stranded RNA sequences [81–84]. TLRs 7 and 8 are expressed similarly to TLR9 (unpublished observations). This common subcellular segregation suggests a general mechanism how TLRs involved in the recognition of nucleic acids are activated. Elucidation of the steps involved in the activation of TLRs that are expressed in internal cellular compartments will be critical for the successful development of pharmacological modulators of these receptors.

Endogenous nucleic acids can promote autoimmune disease

Clearly, most systemic inflammatory diseases have a polygenic, multifactorial nature and the susceptibility to develop disease is dependent on complex interactions of multiple genes [85, 86]. Systemic autoimmune diseases are characterized by the production of antibodies against self-antigens such as chromatin or small ribonucleoproteins. These macromolecular structures are normally localized in intracellular compartments and not directly accessible to immune detection. However, under circumstances, such as tissue damage, infection or under the influence of environmental factors endogenous antigens may be released from cells and become exposed to immune cells. It is thus not surprising that defective genes that are normally involved in the clearance of cell debris are associated with the development of autoimmune diseases. Many of these genes are operative in the ongoing process of ‘silent’ cell removal; a process termed apoptosis or programmed cell death. Cells undergoing apoptosis express molecules on the surface that tag them as apoptotic cells and lead to the recognition and engulfment by scavenger cells, such as macrophages. Several genes that contribute to the removal of apoptotic cells were directly shown to promote auto-immune pathologies. Deficiency in various soluble factors that normally bind apoptotic cells and enhance their clearance have been implicated in auto immune disease development [87–89]. For example, deficiency in C1q a complement component that binds to apoptotic cells and enhances the clearance by phagocytes leads to destructive autoimmune disease and mice deficient in C1q cannot effectively clear apoptotic bodies [90]. Lack of certain macrophage receptors, that identify and bind apoptotic cells, leads to autoimmune disease [91, 92]. Furthermore, lack of factors that are involved in the clearance of immunogenic material from the extracellular milieu, such as serum amyloid P, which binds chromatin, or DNase I results in auto immunity [93, 94]. Together, these facts indicate

that a combination of an accelerated apoptosis with a defect in the clearance of apoptotic cells results in increased release of auto antigens which in the context of a certain genetic background provides enough immunogenic material that can drive and sustain autoimmune disease states.

Circulating nucleic acid containing immune complexes and systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a common autoimmune disease that is characterized by the loss of tolerance to self-antigens and the consequent production of autoantibodies [95, 96]. The predominant auto-antigenic targets in SLE are nucleic acid containing macromolecular complexes such as chromatin or ribonucleoprotein particles. Sera of SLE patient contain high levels of autoantibodies against nuclear antigens, such as antibodies against DNA. Tissue deposition of antibodies and immune complexes cause inflammation and injury of multiple organs leading to diverse clinical manifestations that include glomerulonephritis, dermatitis, vasculitis, thrombosis, seizures, and arthritis [97, 98]. For most people, lupus is a mild disease affecting only a few organs. For others, it may cause serious and even life-threatening problems. More than 16,000 Americans develop lupus each year. It is estimated that 500,000 to 1.5 million Americans have been diagnosed with lupus.

Several lines of evidence have long suggested that anti-DNA antibodies are fundamentally involved in the pathogenesis of SLE: (i) The levels of anti-DNA antibodies in SLE patients are positively correlated with disease activity and are predictive for the development of disease [99–101]; (ii) Fc receptors are necessary for the activation of immune cells by SLE sera and certain mutations in Fc receptors are associated with SLE [102, 103]; (iii) Immune complexes isolated from SLE sera trigger the activation of pDCs and stimulate the release of interferon- α [104–107]. It is now well established that sustained elevated levels of interferon- α is another serological hallmark of SLE [108]. Interferon- α correlates with clinical and serological manifestations of SLE and comprehensive gene expression data point out that many interferon-responsive genes are hyperactivated in SLE patients [105, 109]. Lupus prone NZB mice crossbred into the IFN receptor knockout mouse have lower levels of circulating anti-DNA antibodies and diminished disease which indicates the pathogenetic importance of the induction of the interferon axis by nucleic acid containing immune complexes [110].

Cumulating evidence furthermore suggests that the DNA component of DNA immune complexes is essential for the stimulation of immune cells. As outlined previously in this chapter, TLR9 recognizes DNA that contains non-methylated CpG dinucleotides, which are more abundant in microbial than in mammalian DNA. However, the TLR9 stimulating activity of self-DNA may represent the basis of the development of inappropriate immune activation in susceptible individuals. Sano et

al. have analyzed DNA purified from SLE DNA containing immune complexes as early as 1989. The DNA was found to be rich in guanine and cytosine content and showed 5–6 times higher than the expected frequency of CpG dinucleotides [111]. As mentioned above, in the mammalian genome, CpG dinucleotides are underrepresented and most cytosine bases are methylated at the carbon-5 position. However, around 50% of human genes are associated with so-called ‘CpG-rich islands’ that contain the statistically expected CpG frequency and the cytosines in these areas are typically unmethylated [112, 113]. Cytosine methylation in gene promoter regions regulates transcriptional activity and elsewhere is important in numerous mechanisms of regulating gene expression [114–118]. Thus, mammalian or self-DNA contains structural features that activate TLR9 to produce interferons and other cytokines. Indeed, human PBMCs were stimulated to secrete cytokines by synthetic oligonucleotides that contained DNA sequences that were found in DNA cloned from SLE immune complexes [119].

A variety of enzymes, such as DNA methyltransferases, control the methylation of cytosine. The well-known phenomenon of drug-induced lupus erythematosus appears to be related to DNA hypomethylation. Procainamide is a competitive DNA methyltransferase inhibitor and hydralazine can indirectly inhibit DNA methylation by blocking the upregulation of DNA methyltransferases Dnmt1 and Dnmt3a [120, 121]. Both of these drugs are major causes of drug-induced lupus. Over 90% of patients undergoing treatment with procainamide for 1–2 years mount antinuclear antibodies and ~20% of them develop lupus-like symptoms. Therefore, factors that disturb the closely controlled state of cytosine methylation of mammalian DNA may induce SLE in susceptible individuals.

TLR9 is a receptor for self-DNA in SLE immune complexes

Perhaps the most exciting evidence that TLR9 may be involved in the pathogenesis of SLE comes from studies done in mice, and now followed up in humans. Autoreactive B cells that express an antigen receptor for IgG autoantibodies (rheumatoid factor) were efficiently activated by engaging the antigen receptor and TLR9 [35]. It was further shown that also non-rheumatoid factor autoreactive B cells could be activated by IgG/DNA immune complexes and that the cellular activation required hypomethylated CpG motifs in the DNA [36]. Chromatin-containing immune complexes could activate not only murine B cells but also murine dendritic cells. In the latter cell type the activation was dependent on the expression of Fc gamma receptors [37]. This study furthermore demonstrated that chromatin immune complexes activate myeloid DCs by a TLR9-dependent and by a TLR9-independent pathway. These data suggested that anti-DNA antibody/DNA complexes cause systemic inflammation via TLR9, and that SLE might, in fact, derive from the same pathophysiological processes.

These mouse studies were followed up with investigations of human cells. Immune complexes isolated from patients with SLE stimulated human PBMCs and purified pDCs to induce a variety of cytokines and chemokines. The immune stimulatory activity was found to be DNase sensitive and required intact anti-DNA antibody. These results clearly implicate human TLR9 as being essential for the recognition and stimulatory activity of isolated immune complexes in human patients. It was further shown that TLR9 is actively recruited to fluorescently labeled immune complexes as they enter cells whereupon ligand/receptor binding events take place [38]. Thus, TLR9 and potentially the RNA recognizing TLRs 7 and 8 can be viewed as a loophole leading to cellular over-activation in DNA or RNA immune complex mediated diseases and these receptors may therefore qualify as premier drug targets.

Activation of TLR9 proceeds in a multi-step process

Our current models of TLR ligand recognition suggest the possibility that CpG-rich DNA and potentially guanosine-rich RNA sequences from immune complexes present in SLE trigger inflammation by activation of TLR9 and/or TLRs 7/8. Pharmacological modulators of TLR7-9 could therefore prove to be of great therapeutic value. Although pharmacological modulation of these TLRs will remain a symptomatic therapy, it can be expected that targeting these TLRs will lead to a more specific and effective therapy compared to the current standard therapy of SLE. TLR9 specific pharmacotherapy for example would only affect a small subset of cells and would target two pathogenetically key cell types, the B cell and pDCs.

The mode of activation of TLRs implicated in nucleic acid recognition is complex as TLRs 7–9 are expressed in intracellular compartments [74, 76, 122] and recognition of CpG-DNA involves several essential steps (Fig. 2). Nucleic acids are thought to bind cells via, as of now, undefined surface receptors and are subsequently endocytosed and trafficked into endo-lysosomal compartments where signaling is initiated. Endosomal acidification appears to be a necessary subsequent step in signaling to nucleic acids as drugs that interfere with endosomal acidification also block signaling to nucleic acids or homologous molecules [123]. Chloroquine is one of the drugs that modified endosomal microenvironments and is a potent TLR9 inhibitor. In this context it is not surprising that chloroquine, which is also known as an anti-malarial agent, is one component of the standard therapy of SLE. There is experimental evidence that DNA binding to TLR9 has a pH optimum with optimal binding between pH 5 and 6. This implies that there are regulatory mechanisms of TLR9 activation in place that only allow TLR9 activation in certain subsets of endosomes and prevent activation of TLR9 in other or outside endosomes. DNA is like other endosomally targeted molecules most likely highly concentrated within the endosome and the concentration of DNA could thus influence how and which DNA sequence can activate TLR9. We found that TLR9 can bind many dif-

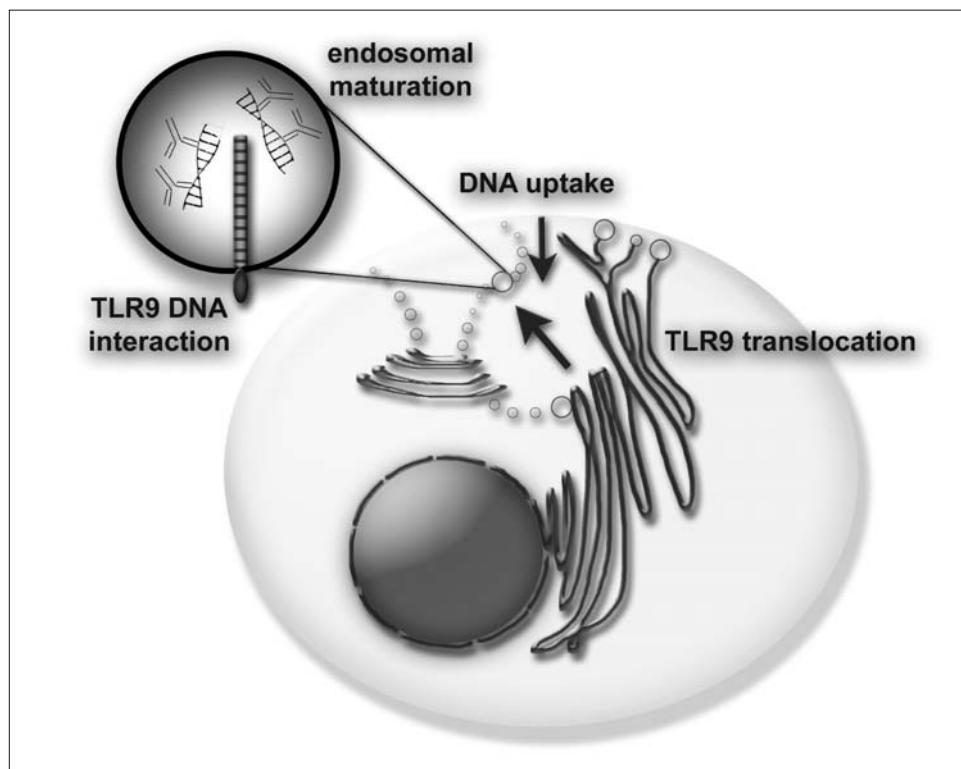


Figure 2

There are several essential steps that are required for TLR9 activation: 1) DNA has to be delivered into intracellular compartments. 2) TLR9 translocates to endo-lysosomal compartments. 3) TLR9 activation is optimal after endosomal maturation.

ferent DNA sequences and structures: Stimulatory and non-stimulatory sequences bind TLR9 [74] (and unpublished observations). This is in accordance with the notion that non-stimulatory DNA can competitively block TLR9 activation by stimulatory DNA sequences. In recent years potent TLR9 inhibiting sequences have been worked out. These inhibitory ODN are commonly about 15 bases long carry the phosphorothioate modification and contain a pyrimidine-rich triplet preferably CCT, which is positioned with a spacer 5' to a GGG sequence [124, 125]. Inhibitory ODN block the immunostimulatory effects of microbial DNA and activating CpG ODN. Most likely inhibitory ODN act as competitive antagonists at the level of TLR9, but this has to be formally proven.

Recent data suggest that the concentration of DNA in the endosome is an important determinant for TLR9 stimulatory activity of a given sequence. It was demon-

strated that when endosomal DNA concentration is enhanced (by forcing DNA into cells via polycationic lipid complexation) TLR9 could be activated by otherwise non-stimulatory sequences. Under these probably unphysiologically high endosomal DNA concentrations even DNA without the canonical CpG motifs became TLR9 stimulatory [126]. These data indicate that TLR9 binds DNA rather promiscuously and that the concentration of DNA can dictate TLR9 activation. TLR9 may thus have a dual role in recognition of DNA: Firstly, TLR9 can recognize small amounts of DNA that resembles microbial DNA (such as the CpG DNA) and secondly TLR9 can additionally recognize high concentrations of endosomal DNA without the same sequence stringency.

It is important to better understand the different steps involved in TLR9 activation and the mechanism of receptor activation as this has important implications for the development of specific TLR9 modulating therapeutic agents.

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Toll-like receptors and airway disease

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Introduction

The prevalence of asthma has doubled over the last 20 years and this disease now affects 7–11% of the population of the United States [1] and Europe [2, 3]. Although its incidence has recently stabilized in developed countries [4], asthma remains a major cause of morbidity and is the leading cause of hospitalization in children under 15 years of age [5]. Airway inflammation, hyper-responsiveness and remodeling are hallmarks of asthma, which is more properly termed a clinical syndrome rather than a specific disease. Most studies of the immunologic basis of asthma have focused on the adaptive immune response, but in recent years it has become increasingly evident that the innate immune system also plays an important role. Since the lung is continuously exposed to a wide variety of airborne antigens and toxins, it is essential that immune responses to aerosols are selective, rapid, and appropriate. Such responses require precise regulation of both proinflammatory and anti-inflammatory responses. Members of the Toll-like receptor (TLR) family initiate innate as well as adaptive immune responses following their binding of pathogen-associated molecular patterns (PAMPs). Different members of the TLR family recognize different PAMPs. For example, TLR4 recognizes endotoxin from Gram-negative bacteria [6, 7], TLR5 recognizes bacterial flagellin [8], and TLR9 recognizes DNA containing unmethylated CpG motifs present in bacterial DNA [9]. Although signaling from members of the TLR family generally lead to proinflammatory responses, several lines of epidemiological and experimental evidence suggest that TLRs can either exacerbate asthma or lower its prevalence, depending on the timing and dose of exposure to TLR ligands. Here, we review recent advances contributing to our understanding of how TLRs influence the incidence and severity of asthma.

Inhaled endotoxin and airway disease

Human exposure to inhaled endotoxin has been most extensively studied in the context of occupational lung disease where repeated exposures can lead to chronic

bronchitis, emphysema, and asthma. Experimental findings with humans [10, 11] and mice [12–14] are consistent with these epidemiological observations and further demonstrate that the clinical and biologic consequences of endotoxin inhalation depend on the length of the exposure. A single exposure to inhaled endotoxin, which activates signaling through TLR4, results in symptoms including fever, cough, dyspnea [12, 15–17], neutrophilic inflammation, production of proinflammatory cytokines and the development of airway hyper-responsiveness that commences within minutes of challenge and can persist for up to 48 h [18]. Prolonged exposure to either inhaled corn dust extract [19] or endotoxin [20] results in persistent changes to the airway that include sub-epithelial remodeling. The biologic response to inhaled endotoxin varies considerably among different individuals, suggesting that genetic background has a major influence on responses to endotoxin [18]. In support of this concept, a common polymorphism of TLR4 (D299G) is, in humans, associated with diminished airway response to short terms exposure to endotoxin [21], although murine studies suggest that genes other than TLR4 can also modify the airway response to this toxin [22].

TLR and neutrophil recruitment

TLR-dependent neutrophil recruitment to the lung is an important first line of defense against bacterial infections but the products of neutrophils are also toxic to the lung and their recruitment must be tightly regulated to maintain health [23–26]. For example, prolonged inhalation of endotoxin can cause airway remodeling that is dependent on the recruitment of neutrophils from the vascular space to the air-space [27]. Alveolar macrophages are also important in innate immune responses to inhaled endotoxin within the lung. For example, macrophage expression of TLR4 is required for neutrophil recruitment to the lung following inhalation of endotoxin [28–30], and it is becoming increasingly evident that TLR signaling by pulmonary monocytes leads to the production of factors that impact on both airway smooth muscle [31] and epithelial cells [32]. The importance of TLR4 in terms of endothelial cells' responses to inhaled endotoxin is less clear, although for systemic endotoxin, endothelia-derived TLR4 expression is required for neutrophil sequestration within pulmonary capillaries [33].

TLR and common inhaled environmental toxins

Ligands of TLRs are ubiquitous in the environment and are therefore frequently inhaled. For example, endotoxin, which is a component of the Gram-negative bacterial cell wall, is commonly found in dust from domestic and occupational environments [34], and contributes to the response to bioaerosols [13]. Detectable lev-

els of endotoxin have been found in dust from the home environment, tobacco smoke, indoor ventilation systems, particulate matter in air pollution and in a wide variety of workplace environments [15, 16, 35–40]. Although bacterial DNA is also ubiquitous in the environment and can be isolated from both home and farm dust [41], the presence of endotoxin is better documented, in part because the limulus amebocyte assay can detect very low levels of endotoxin. It is possible that other TLR ligands are as ubiquitous as endotoxin but have not been as widely detected as the assays used to measure their concentrations are generally less sensitive. In addition, the environmental levels at which individual TLR ligands can impact on asthma is currently not known.

Inhalation of ambient particulate matter (PM₁₀) has long been known to exacerbate airway disease. Epidemiological studies have shown that asthma-related hospital emergency room visits increase during periods of increased PM₁₀ levels [42–44], and in experimental studies, human volunteers exposed to PM₁₀ have elevated levels of pulmonary neutrophils (PMNs), protein and chemokines such as IL-8 [45]. In addition, exposure of human alveolar macrophages to increased PM₁₀ *in vitro* causes an increase in oxidant radical generation [46] and proinflammatory cytokines including TNF- α , IL-6, and IL-1 β [47]. Although there is no evidence that TLRs can directly bind particulate matter, particulate matter can contain biologically significant amounts of endotoxin [39, 48], suggesting that part of the inflammatory response to this pollutant might be due to this organic content. Low levels of microbial products within particulate matter contributes to the inflammatory response *in vitro*, in a manner which appears to be TLR-dependent [36, 49]. Thus, the primary mechanism of TLR-dependent signaling following exposure to inhaled particulate matter is likely related to contamination with biologic material.

Like particulate matter, ozone is a common urban air pollutant that contributes to increased morbidity in human populations and increased levels of ambient ozone are also associated with an increase in asthma-related emergency room visits [43, 50–52]. Humans exposed to ozone develop neutrophilic inflammation, increased expression of proinflammatory cytokines and decrements in pulmonary function [53–58]. The magnitude of these biologic responses varies considerably among different human subjects [57, 59] and among inbred strains of mice, suggesting a genetic basis for susceptibility to ozone [60, 61]. Several lines of evidence suggest that TLRs are involved in the biologic response to ozone. Endotoxin-resistant C3H/HeJ mice, which have a dysfunctional TLR4 receptor, show reduced neutrophilic inflammation [60], and airway permeability [61] following inhalation of ozone. Also, studies with recombinant inbred (RI) mice have revealed that a locus containing *tlr4* is important in ozone-induced hyperpermeability [61, 62] and genetically engineered TLR4-deficient mice have a diminished airway hyper-responsiveness following exposure to sub-chronic ozone [63]. There is no evidence, however, that ozone interacts directly with TLR4 and the mechanism by which this receptor might be acting is unclear. One possibility is that exposure to ozone increases the

availability of an endogenous ligand of TLR4 resulting in an increase in signaling. One candidate for such a ligand is fibronectin, which binds TLR4 [64] and is upregulated following exposure to ozone [65]. However, a role for fibronectin, or any other endogenous TLR ligand, in the airway response to ozone has not yet been demonstrated. Nonetheless, it is clear that TLR signaling can contribute to airway inflammation and hyper-responsiveness in response to both particulate matter and ozone.

TLR ligands during allergic sensitization

On the surface, findings related to the actions of TLRs in allergic asthma appear highly contradictory as endotoxin has been reported to both exacerbate asthma and diminish its incidence. Multiple epidemiologic studies have shown that exposure to TLR ligands in childhood is protective against developing asthma later in life. Examples of this include individuals living on farms who have a reduced risk of developing hay fever or asthma [66–68], the inverse relationship shown between prior measles infection and allergic disease [69], episodes of fever early in life affect the natural history of asthma by preventing the development of atopy [70], the risk of developing asthma is decreased with increased numbers of siblings [71] and levels of endotoxin in the bed linen of school-aged children are inversely proportional to the incidence of hay fever and atopic asthma [72]. This general epidemiological observation, that exposure to pathogens or their products early in life protects against the future development of asthma or atopy in adult life, is known as the ‘hygiene hypothesis’. However, in apparent contrast to these observations, exposure to endotoxin is also associated with exacerbations of asthma. In patients with documented dust mite allergy, the levels of endotoxin in the home environment are more closely related to exacerbations of asthma and wheeze than the levels of specific antigen [73–75].

Part of the apparent contradiction among these different epidemiologic findings might result from opposing actions of TLR ligands to the sensitization of allergens and responses to them. In principle, these two aspects of TLR function can be addressed experimentally in animal models of asthma. However, even when the effect of TLR ligands is restricted to sensitization observed results have varied, depending on experimental conditions. In the most common model of ovalbumin-induced allergic inflammation, mice are sensitized by an intraperitoneal (i.p) injection of ovalbumin complexed with the adjuvant aluminum hydroxide (alum), which promotes T_H2 responses. In rats and Balb/c mice, exposure to LPS at the time of ovalbumin sensitization diminishes subsequent allergic responses in the lung following antigen challenge [76, 77]. The strain of mice used might be relevant to this experiment because we have not observed an effect of i.p. injection of LPS at the time of sensitization in a different strain of mice, C57BL/6J (D. Cook, unpublished

observations). This difference in the response of Balb/c and C57BL/6J mice to endotoxin during the sensitization phase might be due to single nucleotide polymorphisms between these two strains in the *tlr4* gene [22]. While the functional significance of these polymorphisms remains unclear, it is possible that these single nucleotide polymorphisms or other host modifier genes could alter the phenotype to inhaled endotoxin and allergen co-exposures *in vivo*. A second confounding aspect of this model is that commercial preparations of ovalbumin contain various levels of endotoxin, which are likely to impact allergic responses in a dose-dependent fashion. Therefore, without knowing the amount of contaminating endotoxin in different preparations of ovalbumin, it is difficult to directly compare experimental results from different laboratories. Finally, the route of sensitization has a considerable impact on the ability of endotoxin to affect allergic responses. Eisenbarth and colleagues have recently used aerosolized ovalbumin to sensitize mice rather than the conventional i.p. injections of alum-complexed ovalbumin. These investigators have shown that, on its own, aerosolized, endotoxin-free ovalbumin cannot sensitize mice, but acquires immunogenic properties when mixed with small amounts of endotoxin. In addition, this method of sensitization requires TLR4, whereas conventional immunization with alum-complexed ovalbumin does not [78]. Interestingly, if higher concentrations of LPS are added to ovalbumin in this method of sensitization, increased numbers of neutrophils are seen in the inflammatory infiltrate following antigen challenge [78]. Although endotoxin is the most widely-studied TLR ligand in this regard, it is not the only one. Treatment with Pam3Cys, a synthetic ligand of TLR2, during immunization enhances T_H2 cytokine production, serum IgE and airway hyper-reactivity in allergen challenged mice. In contrast, administration of the TLR9 ligand, unmethylated CpG DNA, during sensitization attenuates the development of asthma [79].

TLR ligands during allergen challenge

In addition to their effects on sensitization, TLR ligands can also affect allergic responses when given during the challenge phase of the model [76, 78, 80, 81]. Generally, these ligands are proinflammatory and increase the allergic response when the period of challenge is relatively short [76, 78, 80, 82]. Recruitment of T_H2 cells into the lung associated with endotoxin exposure can occur independent of a specific allergic antigen [82]. In contrast, during prolonged challenges, low doses of endotoxin can suppress allergic inflammation in both mice [83–85] and rats [77, 86–88]. Similar suppression of allergic inflammation in murine models has been observed with ligands of TLR2 [89, 90], TLR9 [91], and with ligands of TLR4 other than endotoxin [92]. This suppression of experimental asthma by chronic low doses of TLR ligands is consistent with the hygiene hypothesis and provides a model to investigate mechanisms underlying this phenomenon. Unlike TLR2 and TLR4, sig-

naling through TLR9 effectively prevents the development of atopic airway disease and can reverse established eosinophilic inflammation [93]. Thus, unmethylated CpG, a ligand for TLR9, attenuates multiple components of the allergic phenotype including eosinophilic airway inflammation, serum IgE, T_H2 cytokines, airway hyper-responsiveness, sub-epithelial fibrosis and goblet cell metaplasia [91, 94–100]. Synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG, or immunostimulatory sequence-DNA (ISS-ODN) have a similar suppressive effect on murine models of allergic asthma [101]. For this reason, synthetic TLR agonists could have important therapeutic potential in human allergic disease [102]. In 2004, Coley Pharmaceutical in collaboration with Sanofi-Aventis initiated both clinical studies of a TLR therapeutic compound for asthma and a second compound currently is undergoing preclinical evaluation. Furthermore, immunostimulatory sequences (ISS-ODN), either alone or combination with allergens, are currently under investigation for therapeutic applications in allergic asthma by the biopharmaceutical company, Dynavax Technologies. Results from these clinical trials are unavailable at this time.

Exacerbations of allergic asthma

Exacerbations of asthma result in an acute decrement in lung function and can be caused by many inhaled environmental or infectious insults. Asthma exacerbations are an important clinical problem and contribute significantly to use of healthcare resources, quality of life and challenges in asthma management [103–105]. Both bacterial and viral infections are major factors associated with exacerbations of allergic asthma (review [106]), although the specific components of these pathogens which results in a worsening clinical status have not been extensively studied. Severe asthmatics can present with relatively high numbers of neutrophils in the airways [24]. Therefore, the mechanisms leading to exacerbations of asthma could be related to either enhanced allergic inflammation (T_H2) or recruitment of neutrophils (T_H1) into the airways, or both. Given the potential of TLR signaling to both enhance allergic responses and promote neutrophil recruitment, it is reasonable to propose an involvement of these receptors in exacerbations. Not surprisingly, studies with inhaled endotoxin have revealed that endotoxin, and its receptor TLR4, can be associated with exacerbations of allergic asthma [74, 107–109]. Similarly, administration of *Mycoplasma pneumoniae* to allergic mice during the challenge phase can exacerbate their allergic responses [110]. This effect is due at least in part, to lipopeptides of this organism that are recognized by both TLR2 and TLR6 [79, 111]. Streptococcus is another common respiratory pathogen associated with exacerbations of asthma [112]. Although the lipoteichoic acid (LTA) component of the cell wall of streptococcus is a known ligand for TLR2 [113], a specific role for TLR signaling in exacerbations of allergic asthma related to streptococcal infection has

not been established. In addition to these bacteria, common viral pathogens, including respiratory syncytial virus (RSV), are associated with exacerbations of childhood asthma. RSV has recently been shown to induce signaling through TLR4 [114]. It is therefore of interest that polymorphisms in TLR4 are associated with the severity of RSV-induced bronchiolitis in infants [115]. The links between susceptibility to commonly encountered pathogens, TLRs and exacerbations of existing airway disease are an area of active investigation.

TLR polymorphisms and association studies of allergic disease

Given the opposing effects of endotoxin, it is perhaps not unexpected that some studies have not revealed an effect of the common TLR4 polymorphism (D299G) on the overall incidence of asthma [116–118]. Individuals having this polymorphism have a blunted airway response [21] and reduced systemic inflammation [119] in response to inhaled endotoxin. Consistent with these observations, a study of asthma specifically associated with endotoxin in house dust showed that people with the TLR4 polymorphism (D299G) had a decreased risk of bronchoreactivity [117]. These observations are consistent with the hypothesis that endotoxin can exacerbate existing airway inflammation and that individuals with the D299G polymorphism have diminished pulmonary responses to endotoxin. However, other studies found that asthmatic individuals with the D299G polymorphism have an *increased* severity of atopy [120] and an increased incidence of atopic asthma [121]. Each of these associations with common polymorphisms of TLR4 is consistent with the ability of LPS to exacerbate existing asthma and to decrease atopy respectively, and suggest that the D299G polymorphism could be predictive of airway and atopic responses in a specific subset of the population.

Genetic approaches to identify associations between airway disease and activation of the innate immune system are not limited to TLR4. Polymorphisms of the TLR4 co-receptor CD14 are associated with increased levels of soluble CD14 and an enhanced biological response to endotoxin. Polymorphisms of CD14 have been associated with both a decrease in total serum IgE in asthmatic children [122] and a decrease in lung function among endotoxin exposed farmers [123]. The observation among children is consistent with the hygiene hypothesis concerning attenuation of allergic symptoms with enhanced response to endotoxin. The enhanced biologic response among farmers with exposure to high levels of occupational endotoxin would be expected to demonstrate decreased lung function, as was observed. In another study, common polymorphisms of TLR2 among European farmers were associated with protection from asthma, atopy and hay fever [124]. This observation would suggest that a blunted response to TLR2 agonists is protective against the subsequent development of allergic asthma. While this is inconsistent with the hygiene hypothesis, the exposure dose to ligands of TLR2 among farmers as well as the func-

tional significance of this polymorphism of human TLR2 remains poorly understood. Similarly, polymorphisms of TLR6 have been associated with a decreased risk of asthma in African Americans [125]. Finally, polymorphisms of TLR10 have been associated with an increase risk of asthma in two separate cohorts [126].

It has become clear that TLR-dependent signaling is critical for the activation of the adaptive immune response. Given the complexity and broad range of environmental challenges which lead to the clinical diagnosis of asthma, it is not surprising that apparent divergent phenotypes are observed with attenuation of TLR-dependent signaling associated with many polymorphisms. Little is known about the ligands of many TLRs, including TLR6 and TLR10. Furthermore, it remains unknown if polymorphisms of TLRs affect interactions between other accessory molecules required to maintain unaltered signaling. Despite limitations of genetic association studies, these observations provide insight into the role of TLRs in the development and progression of human airways disease.

Mechanisms of toll-dependent regulation of allergic asthma

TLR-mediated activation of the innate immune system can either diminish or exacerbate asthma, depending on the dose, timing, duration of the exposure to the TLR ligand, and the genetic background of the affected individual. TLR agonists can act as adjuvants during sensitization. Epidemiologic and murine data support the theory that chronic exposure to TLR agonists early in life protects against the future development of asthma. Activation of the innate immune system with acute exposures to TLR agonists can adversely affect airways disease in patients with existing lung disease by either promoting the recruitment of inflammatory cells into the lung or enhancing the proinflammatory response to inhaled allergen. Therefore, TLR-dependent signaling can modify allergic inflammation by at least four independent mechanisms depending on dose, duration of exposure and host factors. These four independent mechanisms of regulation of allergic inflammation include; as an adjuvant during sensitization to antigen, enhanced allergic inflammation with acute exposures at moderate dose, enhanced neutrophilic inflammation at high dose and suppression of allergic response with chronic low dose exposure. As shown in Figure 1, the role of toll-like receptors in airways disease appears to be a two-edged sword. Low dose exposure to TLR ligands early in life potentially protects against future development of asthma, while exposure later in life can contribute to the progression of asthma. Clear understanding of the TLR-dependent mechanisms, which regulate allergic inflammation, will be required to introduce novel therapeutic interventions.

As TLR signaling can either exacerbate or attenuate airway disease, it is likely that TLRs affect multiple cellular and molecular mechanisms that affect the asthma phenotype in different ways. The protective effect of endotoxin on asthma has been

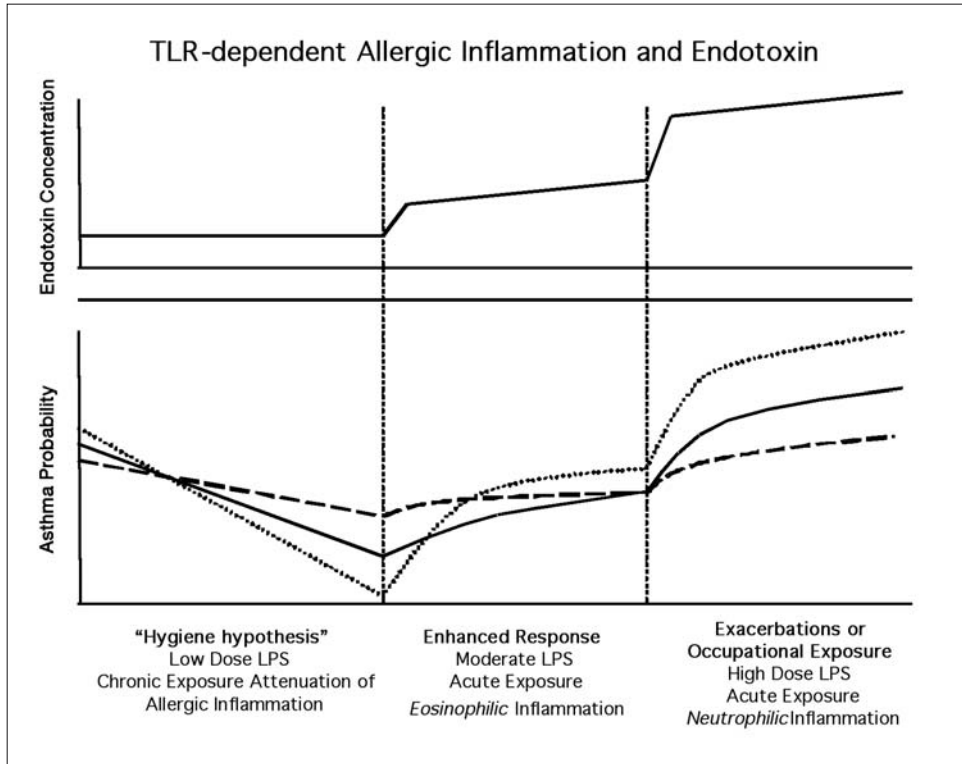


Figure 1

Context and dose of endotoxin impact the development of allergic inflammation

Furthermore, the biologic impact of endotoxin dose is determined by genetic background (normal solid, CD14 (-159T or -1619G) dotted, TLR4 (D299G) dashed). In normal individuals; low dose endotoxin can act as an adjuvant during sensitization, chronic exposure can attenuate allergic inflammation, acute exposure to moderate doses of LPS can enhance allergic inflammation, acute high dose exposures can cause neutrophilic inflammation (solid line). CD14 polymorphisms, associated with enhanced response to endotoxin, results in enhanced suppression with chronic low dose, enhanced allergic inflammation with moderate dose LPS, and enhanced neutrophilic inflammation with high dose LPS (dotted line). Conversely, TLR4 polymorphisms, associated with blunted response to endotoxin, results in reduced suppression with chronic low dose LPS, reduced response to moderate dose LPS, and reduced response to high dose LPS (dashed line).

widely ascribed to the stimulation by TLR signaling on T_H1 responses, which can, in some settings down-regulate T_H2 responses [127]. For example, the T_H1 cytokine IL-12 increases levels of IFN- γ and IL-18 and causes a reduction in allergen-induced

airways hyper-responsiveness [128]. However, several other lines of evidence call into question the notion that the ratio of T_H1 and T_H2 cytokines is directly linked to asthma. For example, transfer of antigen-specific T_H1 cells fail to diminish allergic inflammation in allergen-challenged mice [129, 130] and helminth-infected children, in some developing countries, have very high levels of T_H2 cytokines but have a very low incidence of allergies [131–133]. While TLR-ligands are associated with the production of T_H1 cytokines, it has become evident that T_H1 cells can either downregulate or augment the asthma phenotype [130, 134, 135]. Interestingly, in an antigen specific model of allergic inflammation, recruitment of T_H2 cells is preceded by infiltration of T_H1 cells, suggesting a role of T_H1 cells in allergic inflammation [130]. Adoptive transfer experiments support the cooperative role of both T_H1 and T_H2 cells to promote eosinophilic airway inflammation [135]. Similar cooperativity is observed in respiratory viral infections where T_H1 -prominent inflammation leads to enhanced T_H2 allergic inflammation [136]. These observations support the possibility that low to moderate doses of inhaled TLR ligands could promote early recruitment of T_H1 cells into the lung, which in turn, enhance both T_H2 cell recruitment into the lung and the asthma phenotype. TLR specific regulation of T_H1 and T_H2 cell recruitment into the lungs and the impact on subsequent asthma phenotype has not yet been reported. Therefore, it remains unclear whether TLR-dependent alteration in the T_H1/T_H2 balance directly contributes to the incidence or severity of allergic asthma and it is likely that other TLR-dependent mechanisms play an important role.

The effect of TLR ligands on asthma might be related to their impact on dendritic cells (DCs), the potent antigen presenting cells residing in the parenchyma of the lung and many other organs. TLR signaling in DCs results in increased levels of co-stimulatory molecules and proinflammatory cytokines [137], which facilitate adaptive immune responses to the antigen. Although most studies of this type have shown that TLR signaling leads to production of T_H1 cytokines and stimulate this type of immune response, some evidence suggests that TLR4 can also contribute to T_H2 immune responses. Thus, C3H/HeJ mice demonstrate reduced levels of allergic responses to ovalbumin than control mice [138]. In addition, low doses of aerosolized endotoxin together with ovalbumin can sensitize mice to this antigen, whereas ovalbumin on its own cannot [78]. The latter finding in particular suggests that although DCs have not been shown to produce T_H2 cytokines upon stimulation with TLR ligands, their capacity to promote T_H2 responses might nonetheless be enhanced. In contrast to these observations, however, bone marrow-derived DCs treated with both antigen and moderate doses of endotoxin suppress T_H2 airway response [139]. However, levels of endotoxin vary widely among different commercially-available preparations of ovalbumin, and results from different investigators are therefore difficult to interpret, even when apparently similar procedures are used. In addition to their apparent ability to act as an adjuvant during sensitization, TLR ligands can also enhance allergic inflammation when provided during the chal-

lenge phase of the response. This property might also be related to TLR signaling in DCs because these cells are necessary to maintain immune responses in the lung, even in previously sensitized mice [140]. Endotoxin-dependent recruitment of T_H2 cells into the lung can occur independently of a specific allergen challenge [82] and might also be related to TLR signaling in DCs.

Another mechanistic possibility to explain the actions of TLR ligands involves their impact on TH1-independent regulation of allergic responses. T regulatory cells (T_{reg}) have emerged in recent years as important negative modulators of immune responses [141]. These cells, which often display both CD4 and CD25 on their surface, regulate the actions of other T cells by both cell–cell interactions and production of inhibitory cytokines such as IL-10 or TGF- β [142–144]. The impact of TLR ligands on T_{reg} cells is only beginning to be understood, but it appears that these ligands can both enhance and inhibit Treg function. Thus, IL-6, a cytokine produced by DCs upon stimulation by either endotoxin or CpG, can block the suppressive activity of CD4⁺/CD25⁺ T_{reg} cells [145]. On the other hand, these T_{reg} cells express many types of TLRs and proliferate in response to stimulation with endotoxin [146]. Furthermore, direct stimulation of CD4⁺/CD25⁺ T_{reg} cells with endotoxin enhances their suppressor activity [146] and TLR3-dependent induction of IL-10 in CD4⁺/CD25⁺ T cells has been linked to suppression of immunity against *Candida albicans* [147]. These observations suggest that TLR signaling can either enhance or inhibit T_{reg} cells activity. Although the relationship between dose and duration of exposure to TLR ligands and T_{reg} activity has not been carefully studied, it is reasonable to suggest that these levels might differentially impact T_{reg} cell function.

Finally, the ability of unmethylated CpG DNA to attenuate allergic inflammation suggests that these molecules may have therapeutic utility. CpG DNA elicits a T_H1 -mediated immune response, which might explain its ability to reverse the T_H2 -mediated allergic phenotype. The cells responsible for this activity might be either DCs [148] or B cells, both of which express TLR9 [149]. While the molecular mechanism related to CpG DNA suppression of allergic responses remain unclear, recent work by Hayashi et al. reveal that unmethylated CpG DNA induce high levels of indoleamine 2,3-dioxygenase (IDO) [150], which can inhibit T cell reactivity [151] and also induce T_{reg} response [152]. Increased levels of IDO are associated with attenuation of eosinophilic inflammation and airway hyper-reactivity in a murine model of asthma [150]. Thus, induction of IDO might be a mechanism by which unmethylated CpG attenuates allergic phenotypes in models of asthma.

Conclusion

Multiple lines of investigation have revealed the importance of TLRs in the development and progression of asthma. By signaling in response to inhaled pathogens or endogenous ligands, TLRs regulate both innate and adaptive immune responses

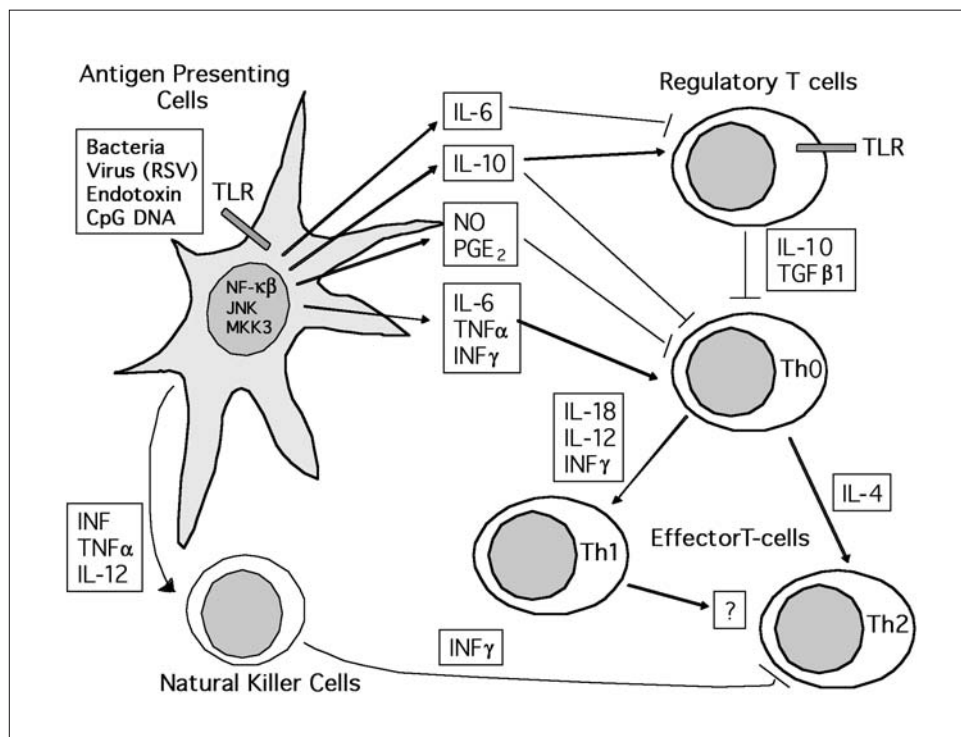


Figure 2

Mechanisms associated with TLR-dependent regulation of inflammation

TLR receptors are expressed on both antigen presenting cells as well as T-regulatory cells. Activation of TLRs can result in production of both proinflammatory or anti-inflammatory cytokines, which impact T_H1/T_H2 balance. IL-6 can block the suppressive activity of T_{regs} . Direct TLR stimulation of T_{regs} can both enhance suppressive activity and induces proliferation of these cells. IL-10 can enhance suppressive activity of T_{regs} .

in the lung and impact airway inflammation and hyper-reactivity. Mechanisms associated with TLR dependant regulation of inflammation in the context of Th1, Th2 and T reg function are shown in Figure 2. The effect of TLRs on asthma can be either beneficial or detrimental depending on many host factors, as well as, dose, duration, and intensity of exposure to TLR ligand. The ability of TLR agonists or antagonists to pharmacologically manipulate the immune system to prevent the development or progression of airway disease is an expanding area of investigation. Given the complexity of the asthma phenotype, it is likely that timing and dose of TLR receptor agonists/antagonists will greatly affect their impact on airway disease. While it is likely that pharmacologic TLR agonists/antagonists will alter biologic

responses to inhaled antigens, they may also affect susceptibility to common pathogens and this must be carefully monitored. TLR-agonists have already been studied as adjuvants to increase efficacy of vaccinations [153–155]. Pharmacologic manipulation of the innate immune system in attempts to control airways disease will require an intricate knowledge of the biological response to inhaled TLR agonists and antagonists. It is possible that with an improved understanding of the multiple means which TLRs modify airway phenotype, we will be able to target these receptors to modify the development and progression of airways disease. The timing and biologic context of stimulation of TLRs appears to determine the response of the airways.

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Toll-like receptors and vascular disease

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Introduction: Inflammation and atherosclerosis

Toll-like receptors (TLRs) have emerged as the primary proximal sensory apparatus that enables first-line innate immune surveillance systems to detect the presence of foreign pathogens and rapidly mount a vigorous defense. The role of TLRs in normal homeostatic processes and pathologic mechanisms of diseases that might at first glance seem far-removed from pathogen defense has already become apparent; in terms of the magnitude of the public health problem, vascular disease unquestionably constitutes the most important.

Vascular disease, particularly atherosclerotic disease, manifests a strong age dependence and plagues more affluent nations partly because of lifestyle, but also because of the sharp discordance in life expectancies and healthcare availability and delivery in developed *versus* emerging nations. Average global life expectancy is about 63 years, but ranges from a low in most of Africa of less than 55 to a high of 75–85 in countries such as the United States, Europe, and Japan. Infection remains the primary cause of death in the world, but deployment of improved healthcare into underdeveloped parts of the world are expected to markedly raise average global life expectancies to the point where prevalence of cardiovascular disease takes the lead as the world's number one health problem.

Atherosclerosis-based diseases such as coronary artery disease and cerebrovascular disease frequently strike without warning and account for 35% of all deaths in the United States [1–3]. In about one-third of cases, the first and last overt indication of the presence of the disease is a fatal event such as myocardial infarction or stroke. Fatal myocardial infarctions or strokes occur at the rate of about one every 40 seconds or so in the US – 120 times the rate of death from HIV. Few families will not be impacted directly or indirectly by atherosclerosis, and the vast majority of healthcare providers will encounter patients with preclinical or overt cardiovascular disease. Prevention is the first line of defense; once the disease becomes clinically

manifest, effective options are dramatically reduced. There is no cure, regression is very unusual even with intensive treatment, and therapies that unequivocally improve long-term outcomes are disappointingly few.

How does atherosclerosis develop?

A comprehensive description of the sequence of events in atherogenesis is beyond the focus of this review, but has been well described in several review papers [4–7]. The hallmark feature of atherosclerosis is focal and chronic inflammation in the arterial wall. Inflammation constitutes the first response of innate immunity after a threat is detected, indicating that atherosclerosis is first and foremost an immune-based disease [4]. Virtually all major and minor cellular effectors of the immune system have been shown to be present and in most cases instrumental in the development of plaque [4, 8–11]. Briefly, development of atheromata begins when lipoproteins are retained within the arterial subendothelial matrix [12] (Fig. 1). Why serum lipoproteins become trapped in the arterial subendothelium remains unclear. The Framingham Heart Study and many others unequivocally established that risk of myocardial infarction or stroke increases linearly with serum low-density lipoprotein (LDL) cholesterol levels [13, 14]. Nevertheless, subendothelial lipoprotein retention is probably not a simple function of serum LDL levels for a number of reasons: (1) kinetic analyses suggest that trapping of lipoproteins is explained more by selective retention rather than increased delivery [15, 16]; (2) selective mutations of apoB100 that alter its ability to interact with proteoglycans in the extracellular matrix does not change transendothelial flux of lipoproteins, but plaque development is inhibited [12]; (3) the propensity of the extracellular matrix to retain lipoproteins has a strong genetic component [17]; (4) atherosclerosis occurs rarely in veins and many arteries, implying that lipoproteins are not retained there either, despite presumably similar subendothelial extracellular matrix composition; (5) atherosclerotic plaque tends to be focal, unevenly distributed and does not develop linearly with time; (6) patients with extensive atherosclerosis can present with normal serum cholesterol levels and lipoprotein profiles and atherosclerosis develops in some individuals with quite low serum cholesterol; (7) the established ability of HMG Co-A reductase therapy to stabilize plaques and slow the progress of atherosclerosis does not necessarily involve significant changes in lipoprotein profiles [18, 19]. Interestingly however, smooth muscle cells (SMCs) treated with statins secrete an extracellular matrix that resists lipoprotein retention [20]. Once trapped in the subendothelial matrix, lipoproteins tend to undergo oxidative and covalent modifications that stimulate the next phase of plaque development, an inflammatory response [21].

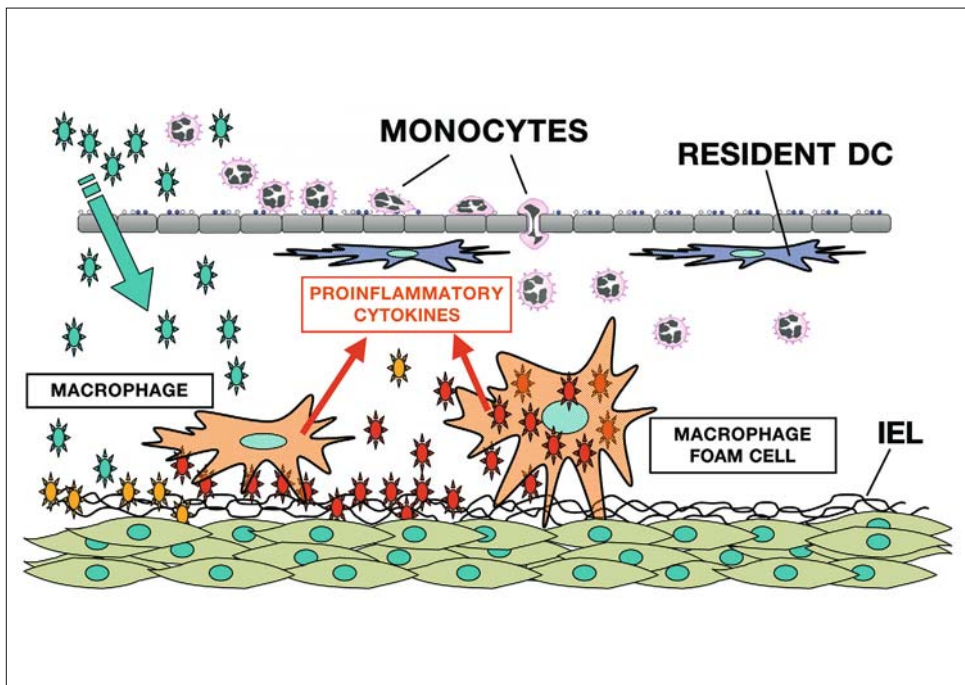
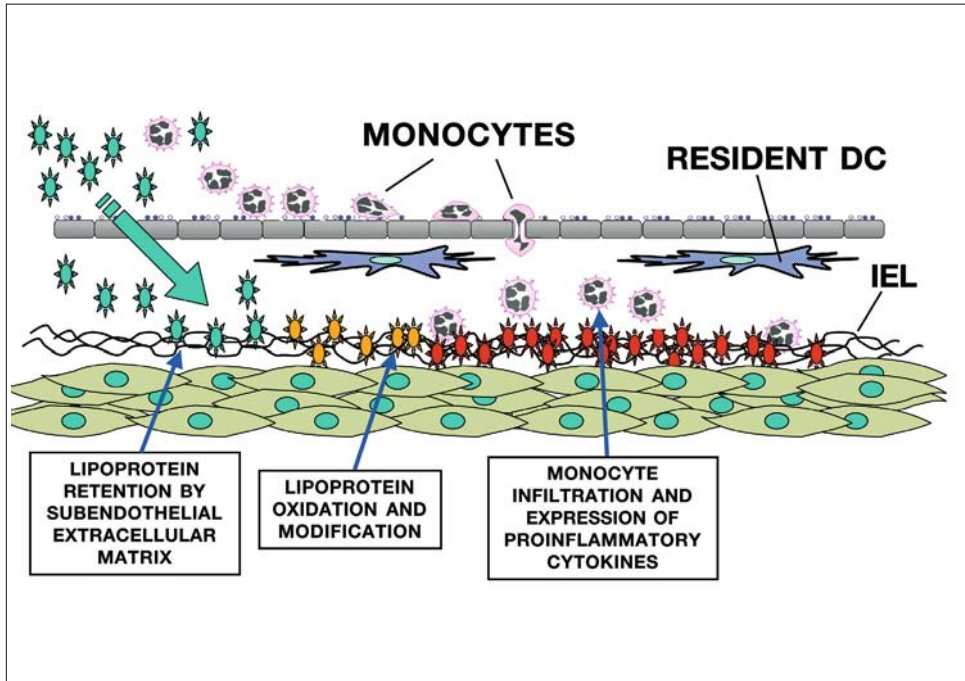
Lipoprotein modification in the subendothelium serves as an inflammatory nidus that generates numerous signals and in response, a diverse collection of mul-

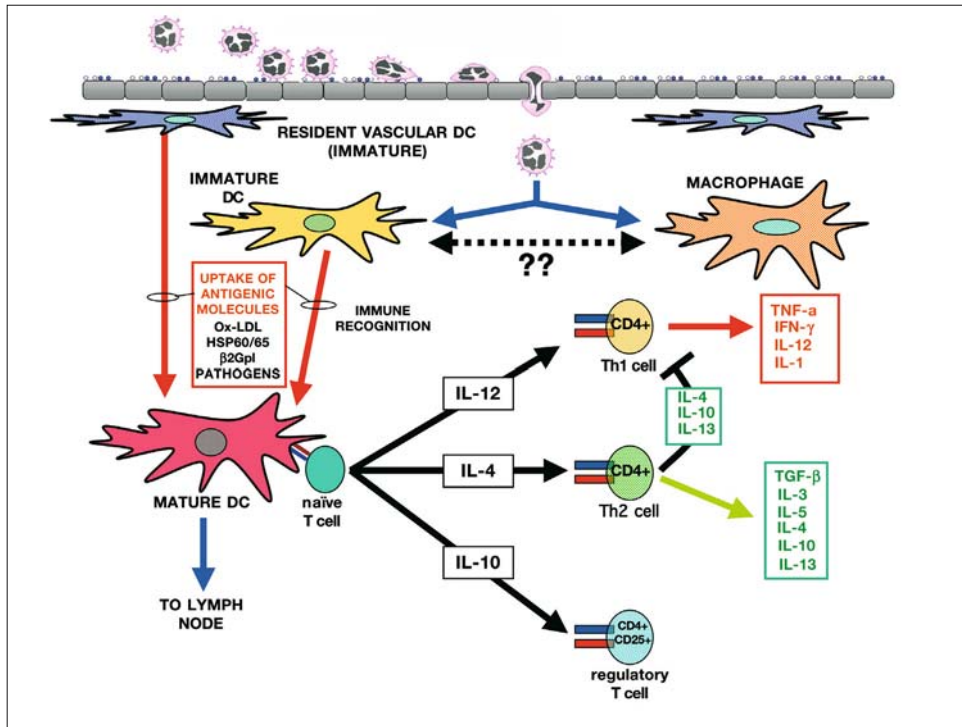
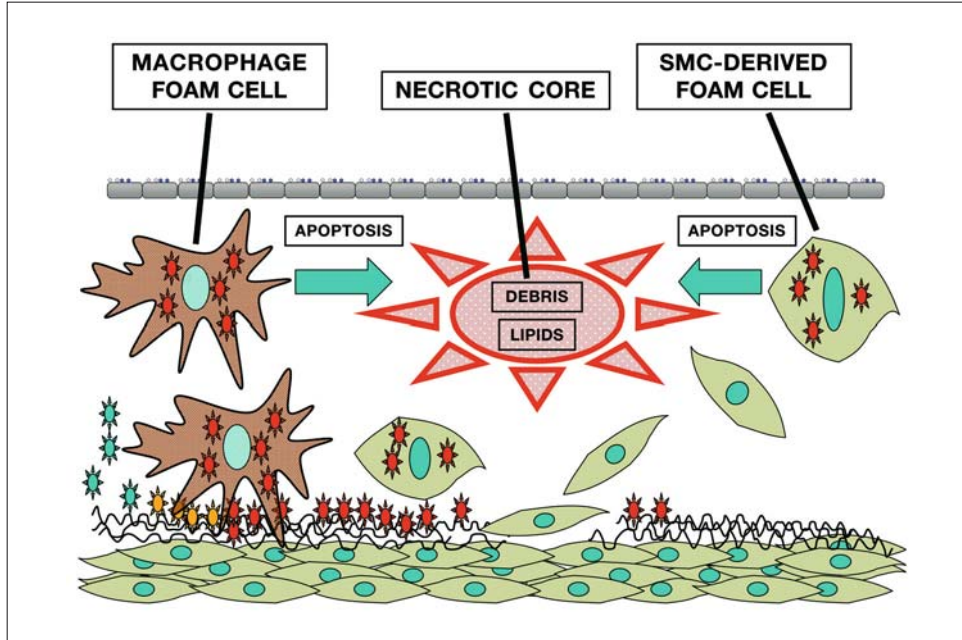
tifunctional leukocytes may assemble, including monocytes, T cells, B cells, mast cells, dendritic cells (DCs) and neutrophils. The resultant expression of cytokines, chemokines, growth factors, proteases and migratory stimuli constitutes a focal inflammation [5–7]. Monocytes develop into tissue macrophages, ingest modified lipids, and may promote further inflammation. SMCs from the medial layer of the

Figure 1 (see following double page)

Development of atherosclerosis

The focal, chronic inflammatory nidus in the arterial wall that characterizes atherosclerotic plaques begins when lipoproteins are deposited in the subendothelial tissues, where they interact with proteoglycans and other structural elements of the ECM (A). Oxidative and covalent modification of lipoproteins to variable degrees may ensue, which directly stimulates expression of adhesion molecules, cytokines, and chemokines by the overlying endothelium that in turn activate an innate immune response. As shown in B, a number of immune cell types may be recruited, notably cells of the mononuclear phagocytic lineage, but it is also noteworthy that DCs and T cells seem to be the very first immune cells present, have been seen in arteries of fetuses and young children, and may therefore precede development of plaque. DCs in particular are thought to perform sentinel functions in normal arteries; DCs appear to form a network analogous to that seen beneath the skin with Langerhans cells. Infiltrating mononuclear cells can differentiate into monocyte/macrophages or DCs. Macrophages in particular take up modified lipoproteins, but may be unable to process them fully, and may then become engorged with lipids and become foam cells (B). Abnormal breakdown of ECM components occurs, and is thought to facilitate migration of SMCs from the media into the developing plaque where they lose their contractile phenotype and may begin to secrete a number of proteins that affect plaque development. SMCs may also ingest lipids and become foam cells. Lipid-laden foam cells may die, and the presence of proinflammatory cytokines may promote apoptosis of various adjacent cells, all of which contribute to the inflammatory nidus by creating pools of modified lipids and cellular debris (C). DCs and macrophages both avidly sample their environments and present peptide fragments loaded onto MHC molecules, which when accompanied by appropriate co-stimulatory molecules activate T cells (D). Activated DCs in plaques are thought to migrate to lymph tissues, where further interactions with immune cells occur, including promoting an adaptive response and clonal expansion of T cells and B cells. These immune cells may also interact with DCs and macrophages within the plaque. The relative proportions of various subclasses of T cells found in lesions appears to be controlled by the specific types and amounts of cytokines produced in the plaque, and has very important consequences for plaque development and stability. These processes collectively increase the bulk of the lesion, creating stenoses, and an imbalance of proteolytic degradation processes that is a part of normal ECM homeostatic turnover may eventually cause structural weakening of the plaque that in turn can directly instigate plaque rupture and subsequent arterial thrombosis, the direct cause of clinical events such as myocardial infarction, stroke, and sudden cardiac death.





artery may migrate into the lesion, where they may undergo phenotypic modulation, gradually losing their typical contractile abilities and assuming a more and more synthetic phenotype that expresses and secretes proteins that are normally not expressed by SMC. SMCs may also ingest lipids and become foam cells, or, under some circumstances, they may counteract inflammation, and begin elaboration of extracellular matrix. If the overlying endothelium becomes damaged, platelets may adhere, generate growth factors and cytokines and influence local thrombotic/fibrinolytic homeostatic balance. Macrophages in lesions are prone to undergo apoptosis, releasing inflammatory lipids and cellular debris. This tends to exacerbate inflammation, and a number of cell types attracted to the plaque site express cytokines and proteases that weaken the structure of the plaque and promote further inflammation. Eventually, the plaque may become structurally weakened by the action of proteases, and when this occurs the plaque is said to be vulnerable to rupture, tearing, or erosion. Subendothelial tissues are highly thrombogenic, so deterioration of plaque structure will usually cause arterial thrombosis. If thrombosis progresses sufficiently, the arterial lumen can rapidly occlude, and usually myocardial infarction, stroke or sudden death is immediate unless collateral blood vessels have developed to a point where they can compensate for the compromised blood supply.

Toll-like receptors and atherosclerosis: *In vitro* evidence

TLR expression in atherosclerotic lesions

Several reports have documented the expression of TLR4, TLR1, TLR2 and to a lesser extent TLR5 in both human plaques and murine models of atherosclerosis [22, 23], where they appear to be mainly expressed by macrophages and endothelial cells. Moreover, TLR4 expression in macrophages is upregulated by oxidized LDL, a proinflammatory and pro-atherogenic lipoprotein, but not by native LDL [22, 24]. Furthermore, it has been shown that in addition to endothelial cells and macrophages, murine vascular smooth muscle cells also express TLR2 and TLR4/MD-2 while human arterial smooth muscle cells express only TLR4/MD-2 [25, 26]. TLR expression might also be influenced by and dependent on local shear stress. *In vitro* data have shown that in particular TLR2 is downregulated under laminar flow [27]. Stimulation with the appropriate TLR ligand induces signaling that promotes a proinflammatory phenotype in vascular SMCs, which suggests that these cells may potentially play an active role in vascular inflammation via the release of chemokines and proinflammatory cytokines. In recent years DCs have become more and more appreciated as a key player in the development of atherosclerosis [28, 29]. Vascular DCs, which are present in small numbers in the subendothelial layer of non-diseased arteries, become activated in early stages of athero-

sclerosis by signaling pathways most likely involving TLRs and migrate into atherosclerotic plaques. In the atherosclerotic plaques the DCs cluster with T cells suggesting presentation of antigens such as HSP70 and MM-LDL [30, 31]. DCs locate particularly in rupture-prone regions within the atherosclerotic plaques and are in highest abundance in vulnerable plaques, which leads to the conclusion that DCs might contribute to plaque destabilization [30]. Dislipidemia has been shown to alter DCs function in ApoE-deficient mice [32]. While in the early stages of atherosclerosis the number of Langerhans cells in the skin is reduced, later on the migration of DCs to the lymph nodes is impaired in ApoE-deficient mice, which explains high susceptibility to infection in these mice [33].

Endogenous TLR ligands

Evidence from diverse sources and experimental models has provided a wealth of data suggesting that TLRs could affect atherosclerosis in multiple ways. Minimally modified LDL (MM-LDL), a strong inducer of proinflammatory cytokines and chemokines with pro-atherogenic potential [5, 34], is recognized by both TLR4 and CD14 on macrophages, and interaction of this lipoprotein with TLR4 leads to actin polymerization and macrophage spreading [35, 36]. Data on the potential role of other TLRs in atherosclerotic lesions is still largely unknown.

Furthermore, evidence for an involvement of an immune response towards heat shock proteins (HSPs) in the development of arteriosclerosis is accumulating (for a recent review, see [37]). Heat shock proteins are among the most highly conserved protein families and are ubiquitously expressed in almost all mammalian tissues. In particular prokaryotic and human HSP60 share a high amino acid sequence homology (>70%). Immunologic cross-reaction between bacterial (e.g., Chlamydial) and human HSP60, which has been detected on the surface of stressed endothelial cells, might be involved in atherogenesis. Both, bacterial and human HSP60 signal through TLR4 and/or TLR2 and lead to the activation of NF- κ B-dependent proinflammatory gene targets [38–41]. Chlamydial HSP60 was shown to lead to human SMCs proliferation in a TLR4-dependent manner [41]. Nasal vaccination with mycobacterial HSP65 has been demonstrated to reduce inflammation and decrease atherosclerosis in aortic arches in LDL receptor-deficient mice [42]. Furthermore, fragments of fibronectin have been associated with tissue injury and tissue remodeling in response to inflammation, and the extra domain A of fibronectin (EDA) can be recognized by TLR4 [43]. Depletion of EDA results in decreased atherosclerosis in apoE^{-/-} mice [44], suggesting that the role of TLR4 during atherogenesis is multifaceted and might include both endogenous (MM-LDL, HSP60, EDA) and exogenous (ligands derived from various pathogens) ligands.

Ox-PAPC (oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine), a bioactive component of mildly oxidized LDL [45], utilizes TLR4 as a sig-

naling receptor, but also has a direct effect on TLR signaling. Ox-PAPC inhibits the activation of IL-8 and MCP-1 by TLR4 and TLR2 ligands via their corresponding receptor in endothelial cells or macrophages by disrupting lipid rafts/caveolae [24]. The integrity of lipid rafts/caveolae is thought to be essential to LPS-induced cellular activation, since raft-disrupting drugs inhibit LPS-induced cytokine secretion [46]. Inhibition of TLRs by Ox-PAPC might contribute to downregulation of the acute-phase response to bacterial lipid-containing products and propagate a more chronic inflammation typical of the progression of atherosclerosis.

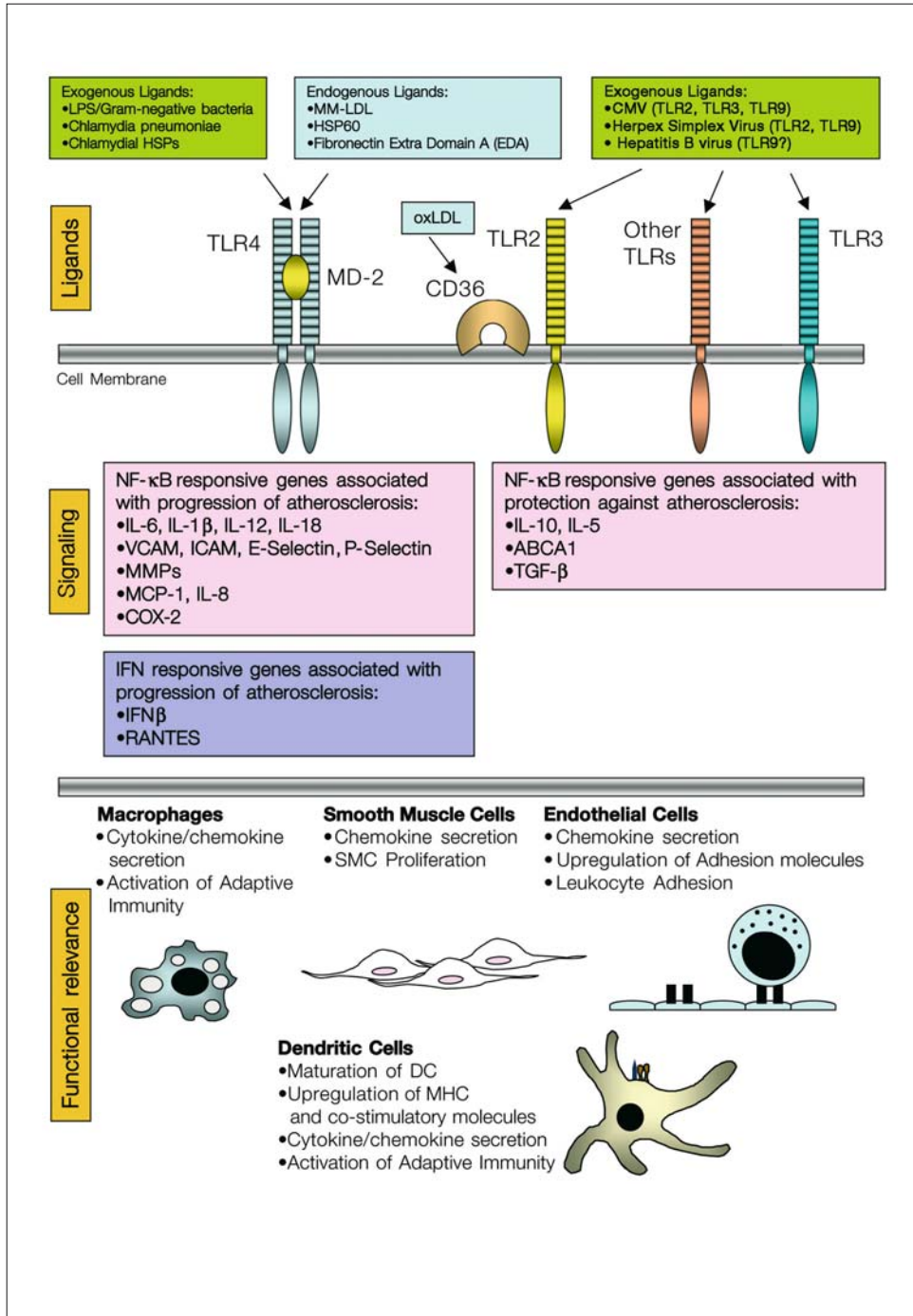
Exogenous TLR ligands implicated in the progression of atherosclerosis

A number of infectious agents have been associated with atherosclerotic cardiovascular disorders, including *Chlamydia pneumoniae* [47], *Helicobacter pylori* [48], cytomegalovirus (CMV) [49], Epstein-Barr virus [50], human immunodeficiency virus [51], herpes simplex viruses (HSV)1 [52], HSV2 [53], and hepatitis B [54] and C [55]. More recent models emphasize the relationship of atherosclerosis to total “infectious burden” rather than specific pathogens [56]. The above mentioned infectious agents or derived PAMPs have been shown to signal through one or more TLRs (Fig. 2). Furthermore, the recently identified cytoplasmatic PRR NOD1 (nucleotide-binding oligomerization domain) has been proposed to be involved in *Chlamydia* and *Helicobacter* induced signal transduction [57, 58]. It is intriguing to

Figure 2

TLR signaling pathway and its relevance to atherogenesis

Endogenous and exogenous molecules associated with lesion formation are thought to signal through TLR4 but the potential role of other TLRs has not yet been investigated. Interaction between ligand and receptor leads to the activation of NF- κ B- or IRF3-responsive genes associated with progression of or protection against atherosclerosis. Endogenous or exogenous ligands can stimulate TLR-bearing cells, such as macrophages, dendritic cells (DCs), endothelial cells (EC), and smooth muscle cells (SMC). Minimally modified LDL (MM-LDL) can stimulate EC and SMC secretion of proinflammatory cytokines and chemokines, and also upregulates expression of adhesion molecules. Secreted chemokines attract monocytes to the endothelium, where these EC adhesion molecules facilitate leukocyte retention and subsequent migration into the subendothelial space, where they differentiate into macrophages under the influence of proinflammatory cytokines and growth factors. Uptake of modified LDL by macrophages via scavenger receptors initially protects ECs and SMCs from stimulation by modified LDL. However, persistent hypercholesterolemia leads to excessive cellular uptake of modified LDL, intracellular accumulation of cholesteryl esters, formation of foam cell, and eventually apoptosis and release of proinflammatory oxidized lipid derivatives into the plaque, further exacerbating the inflammatory nidus.



speculate if NODs are also involved in or associated with the development of atherosclerosis.

Crosstalk between TLRs and lipid metabolism by liver X receptors

Interestingly, TLRs can directly interfere with cholesterol metabolism in macrophages [59]. The accumulation of cholesterol-loaded macrophages in the arterial wall is a very important event in the development of early atherosclerotic lesions [5]. Macrophages normally activate a compensatory pathway for cholesterol efflux mediated by the ABCA1 transporter in response to lipid loading but this mechanism is overwhelmed in systemic hypercholesterolemia [60]. Castrillo and co-workers recently demonstrated that activation of TLR3 and TLR4 by their corresponding ligands blocks the induction of liver X receptors (LXR) target genes, such as the cholesterol transporters ABCA1 and ABCG1 and the lipoprotein apoE *in vitro* and *in vivo* leading to an inhibition of cholesterol efflux from macrophages [59]. However, LXR agonists also inhibit LPS- and cytokine-induced expression of proinflammatory genes [61]. New studies deepen the connection between LXR and inflammation by uncovering a protective role of LXR in immunity to *Listeria monocytogenes*, a Gram-positive intracellular bacterium [62]. Mice deficient in both isoforms of LXR (LXR $\alpha/\beta^{-/-}$) are more susceptible to *Listeria monocytogenes* infection, develop higher bacterial burdens which leads to higher mortality. Interestingly, immune response to *Listeria monocytogenes* seems to be independent of TLR2 and 4 and might depend on the intracellular pattern recognition receptor NOD2 [62, 63]. Nevertheless, crosstalk between signaling pathways controlling innate immunity and macrophage cholesterol metabolism may help explain the ability of bacterial and viral pathogens to accelerate atherosclerosis.

Toll-like receptors and atherosclerosis: *In vivo* evidence

Recently, Bjorkbacka et al. [64] and our own group [65] independently reported the first direct evidence demonstrating that MyD88-dependent TLR signaling plays an important role in development of atherosclerotic plaque. Genetic MyD88-deficiency in atherosclerosis-prone hypercholesterolemic mice (apoE $^{-/-}$) led to a significant decrease in plaque size, lipid content, expression of proinflammatory genes and systemic expression of proinflammatory cytokines and chemokines (IL-12, MCP-1). In addition, we demonstrated that genetic deficiency of TLR4 is also associated with a significant reduction of aortic plaque size, lipid content, and macrophage infiltration in apoE-null mice [65]. These effects did not involve CD14, since apoE/CD14 double knockout mice exhibited similar atherosclerosis

compared to controls [64]. This is consistent with *in vitro* data showing that ox-PAPC binds to TLR4 to activate endothelial cells without the use of CD14 [24]. The fact that TLR4 deficiency in apoE mice leads to less atherosclerosis [65] but CD14 deficiency has no effect [64] implies that TLR4 signaling contributes to atherosclerosis, and either LPS is not the ligand, which is supported by lack of reduction of atherosclerosis in apoE^{-/-} mice kept under germ-free conditions [66], or some other ligand is interacting with TLR4 in a CD14-independent manner to influence plaque development [67].

Unraveling these complexities will be an important goal for future studies, and clues will no doubt emerge from rapidly evolving insights into the molecular mechanisms of TLR signaling. MyD88 also participates in the IL-1R pathway as a downstream adaptor, so TLR signaling might not have been entirely responsible for observed effects in apoE/MyD88 double knockout mice; a role for IL-1 signaling can not be excluded, particularly since IL-1 signaling accelerates atherosclerosis in apoE-deficient mice [68]. However, a reduction in the extent of atherosclerosis in the apoE/MyD88 double knockout mice does not seem to be entirely due to diminished IL-1 signaling, but is also caused by a lack of TLR4 [65]. These findings also raise the possibility that other TLRs that utilize MyD88 might also be involved in the development of atherosclerosis. Indeed, TLR2 has been shown to impact development of atherosclerosis in apoE-deficient mice [69].

TLR4 could also have divergent downstream effects. Of potential relevance, NF- κ B is a downstream target of TLR4/MyD88 signaling [70], and considerable evidence suggests a pro-atherogenic function for NF- κ B [71]. However, NF- κ B might have a variety of effects on atherosclerosis, since macrophage-restricted inhibition (via genetic IKK-2 deletion) of the NF- κ B pathway accelerates atherosclerosis [72], yet bone marrow transplantation using p50-null donor bone marrow and pro-atherogenic LDL receptor knockout mice recipients decreased atherosclerosis [73], even though plaques were more inflamed. Thus NF- κ B may have complex functions both to promote and to limit inflammation.

There are suggestions that TLR signaling might affect plaque vulnerability as well. Epidemiologic and clinical data indicate that chronic inflammation and infection increases the risk of devastating cardiovascular events such as stroke and myocardial infarction [74], but how this might occur is unclear, particularly with regard to infection; prospective clinical trials of antibiotics have failed to decrease risk of cardiovascular events [75]. Coronary events and stroke are mostly precipitated by rupture or erosion of structurally weakened plaque [76]. In more advanced stages of atherosclerosis, extensive outward arterial remodeling tends to preserve luminal diameter and arterial blood flow but is often associated with a more vulnerable plaque phenotype that is thought to be due to structural weakening of the overlying cap by proteases such as matrix metalloproteinases (MMPs) [77]. Evidence from animal studies indicates that TLRs may also be involved in this outward arterial remodeling [78]. Remodeling occurred either in the presence or absence of

exogenous LPS, and was reduced in TLR4-deficient mice, suggesting that endogenous ligands such as EDA or HSP60 that are upregulated in surrounding tissue [78] could be interacting with TLR4 to influence plaque development and remodeling processes.

Toll-like receptors and atherosclerosis: Genetic evidence

Several polymorphisms in the genes encoding TLRs have been investigated in the context of infectious diseases [79], autoimmune disorders [80], allergies [81], periodontal disease [82], and renal disorders [83], but have also focused on a potential role of TLR polymorphisms on vascular disease and its clinical sequelae. Patients either heterozygous or homozygous for two different single nucleotide polymorphisms (Asp299Gly and Thr399Ile) that map to the extracellular domain of TLR4 are hyporesponsive to a challenge with LPS [84]. Atherosclerosis is characterized by chronic local inflammation [5, 85], and thus a blunted response to LPS might place the host at a disadvantage in eradicating invading microorganisms yet could diminish cardiovascular risk because of decreased systemic inflammation. Therefore, patients harboring Asp299Gly and Thr399Ile polymorphisms might be imbued with greater cardiovascular protection. A population-based epidemiologic study did indeed show that subjects carrying the Asp299Gly were less susceptible to carotid artery atherosclerosis [86]. These results have been supported by reports that this polymorphism is associated with protection from carotid and femoral artery atherosclerosis and acute coronary events [84, 86, 87], as well as greater benefit from statin therapy [88]. But studies in patients with familial hypercholesterolemia failed to show any protection against development of carotid atherosclerosis [89], and expression of inflammatory markers was also not associated with the Asp299Gly polymorphism. The Southampton Atherosclerosis Study also failed to show an association between the TLR4 Asp299Gly polymorphism and either severity of or susceptibility to coronary artery diseases [90], and the Stockholm Heart Epidemiology Program (SHEEP) found that men with both the Asp299Gly and Thr399Ile polymorphisms actually had an increased risk of myocardial infarction [91]. However, since this analysis included only subjects with a first nonfatal myocardial infarction and no recurrence within 3 months, selection bias could have affected the results in unanticipated ways. Moreover, most of these studies involved relatively few subjects, and larger, prospective clinical studies will be required to reconcile these discordant results. A recent study in patients with restenosis after angioplasty or angioplasty plus stent implantation showed that the Arg753Gln SNP in the gene encoding TLR2 was more prevalent in patients with restenosis compared to those without restenosis [92]. In contrast, the TLR4 SNP Asp299Gly was similarly distributed among the patients. Although the process leading to restenosis

is not atherosclerosis, this study also suggests a more general involvement of TLR signaling in vascular diseases.

Conclusions: Future investigations and therapeutic potential

Innate immunity represents an attractive source of therapeutic targets because it is linked directly to development of atherosclerosis, and also because of its strategic position in controlling inflammation, autoimmunity, and antibody responses. Ligands for specific TLRs are already being developed and evaluated in clinical trials as vaccines or adjuvants [93, 94]. Innate immune cells, particularly DCs, are being evaluated for possible roles in delivering antigen and inducing antitumor immunity [95] and in controlling autoimmunity [96]. Therapies aimed at tipping the Th1/Th2 balance toward a Th2 response may be a fruitful area of investigation; among these are molecules that target specific aspects of TLR signaling [93]. NK cells and NK T cells may be useful in treating cancer [97], and since both have been recently linked to atherosclerosis [98, 99], the possibility arises that cellular therapy utilizing these lineages might also prove useful in atherosclerosis. Although still in early development, therapeutic utilization of T regulatory cells in diseases such as type 1 diabetes is promising [100] and could be explored for treating atherosclerosis as well. Since TLR signaling has been implicated in neointimal proliferation after arterial injury [78], myocardial reperfusion injury [101], heart failure [102], and unfavorable ventricular remodeling after myocardial infarction [103], the potential utility of TLR inhibitors in drug-eluting stents and in acute coronary syndromes is being explored.

Thus, intensive investigation of a number of diseases is increasingly directed toward therapeutic manipulations of various aspects of innate immunity, and similar approaches in atherosclerosis warrant development. Manipulating innate immunity in ways that limit or even prevent atherosclerotic plaque development and destabilization carries the risk of compromising host defense. A major challenge to future translational investigations will be to achieve the goal of limiting pro-atherogenic innate immune influences while simultaneously maintaining adequate and appropriate innate immune defense mechanisms. Continued rapid progress in our understanding of how TLR signaling works and particularly how it is involved in vascular disease can be expected to enable us to one day achieve these goals in a way that could dramatically limit the two major threats to global wellbeing – cardiovascular disease and infection.

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Toll-like receptors and inflammatory bowel disease

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Toll-like receptor (TLR) signaling in the gut

The surface epithelium serves a critical function in the gastrointestinal (GI) tract as the front line of the mucosal innate immune system against luminal pathogens. The recognition of intestinal pathogens is one of the most important functions of intestinal epithelial cells and is at least partially dependent on TLR signaling. TLRs are members of a conserved interleukin-1 (IL-1) superfamily of transmembrane receptors that recognize pathogen-associated molecular patterns (PAMPs) and are a subset of pathogen-recognition receptors (PRRs). The expression and function of several TLRs have been examined in the gut. So far, almost all of the TLRs 1–9 have been reported to be expressed in intestinal epithelial cells as well as in other types of cells in the intestine [1]. TLR11, which is a mouse specific TLR, recognizes uropathogenic *E. coli* [2] and is expressed in the intestine. A description of the specific TLR-ligand interactions and the TLR signaling pathways have been discussed elsewhere in this book.

Despite the extremely high concentrations of bacteria and their products in the intestine, intestinal epithelial cells do not activate proinflammatory pathways in the normal state yet are able to control against microbial invasion. One would assume that if intestinal epithelial cells respond to normal commensal bacteria, it might result in excessive immune activation leading to dysregulated mucosal inflammation as is seen in inflammatory bowel disease (IBD). How then does the mucosal immune system regulate a homeostatic balance between tolerance and immunity to the numerous bacteria and dietary constituents of the gut lumen? Although the principal role of TLR signaling in the intestine is the same as that in other tissues, i.e., defense against pathogens, it may need to play a unique role in the specific situation of the gut. Due to the close proximity and high density of PAMPs in the intestinal lumen, we postulate that a variety of mechanisms have evolved to protect against dysregulated inflammation in the presence of commensal bacteria (Tab. 1). In order to avoid an excessive immune response and dysregulated inflammation, intestinal epithelial cells have to control PAMP recognition. In this context, TLR signaling in normal intestinal epithelial cells appears to be downregulated [3, 4]. This downreg-

Table 1 - Characteristics of TLR expression in a variety of cells in the gut

Site of expression	TLR signaling
Epithelial cells	Majority of TLRs expressed in human intestinal epithelial cell lines; level low Almost all of the TLRs except for TLR10 expressed in colonic epithelial cells <i>in vivo</i> or primary cells (low levels) Function of TLRs <i>in vivo</i> under investigation
Lamina propria macrophages	TLR 2, 4, and 5 expressed but not TLR1 and 3 Other TLRs have not been examined TLRs and CD14 are normally downregulated
Dendritic cells	Inferred expression of TLR3 and TLR4 Signaling <i>in vivo</i> unknown
Myofibroblast	All of the TLRs except TLR10 and 11 are expressed TLR2 and TLR4 are functional
Endothelial cells	Functional expression of TLR4 and TLR5
Polymorphonuclear cells	Express all TLRs except TLR3 Only TLR2 and TLR4 have been confirmed functionally

ulation of signaling may be strictly controlled through decreased receptor expression on the epithelial cell surface and increased expression of inhibitors of TLR signaling. We and others have shown that intestinal epithelial cells are hyporesponsive to LPS with very low expression of TLR4 and MD-2; but transgenic expression of TLR4 and MD-2 permits LPS-dependent signaling [5–7]. In addition to the intestinal epithelial cells, lamina propria macrophages in the uninfamed intestine also have low expression of TLR2 and TLR4 and do not express CD14 and are LPS unresponsive [8, 9]. Moreover, a recent study demonstrated that human intestinal macrophages do not express other innate response receptors including CD89, CD64, CD32, CD16, CD11b/CD18, and CD11c/CD18, and have defective production of proinflammatory cytokines [10]. These macrophages are able to phagocytose normally, however. Surprisingly, this “quietness” of lamina propria macrophages results from exposure to intestinal stromal cell-derived products. Exposure of blood monocytes to intestinal stromal cell products causes not only morphological changes but also a dose- and time- dependent reduction in the expression of innate response receptors [10]. This phenotypic change of blood monocytes also includes a downregulation of chemokine receptors. Since anti-TGF- β could block the effect of stromal cell products [10], TGF- β may play a critical role in the intestinal innate immune response.

On the other side of the equation, we also have shown that Tollip, one of the inhibitors of TLR signaling, is highly expressed in intestinal epithelial cells suggesting that this is another factor in LPS hyporesponsiveness [11]. In addition, Tollip

expression increases in LPS-treated or lipoteichoic acid (LTA)-treated intestinal epithelial cells which then become hyporesponsive to PAMPs [4].

In contrast to the low expression of TLR4 or TLR2 by intestinal epithelial cells, colonic epithelial cells express TLR5 [12]. Expression of TLR5 is on the basolateral surface of polarized intestinal epithelial cells [12, 13]. This expression pattern of TLR5 may protect against dysregulated inflammation since the ligand flagellin would be found on the apical surface in the lumen. Others, however, have described apical expression of TLR5 on intestinal epithelial cells by immunohistochemistry but it is difficult to assess whether this is functional [14].

Besides the intestinal epithelial cells and lamina propria macrophages, other cell types in the gut express TLRs [1, 15, 16]. Dendritic cells are present in the gut [17]. TLR signaling largely regulates dendritic cell maturation [18], which may play a critical role in the generation of regulatory T cells in the gut providing further support to the notion that TLR signaling is likely involved in the regulation of tolerance to commensal bacteria [19, 20]. Although much is known about dendritic cells in general, little is known about gut-specific dendritic cells. All together, regulated expression and function of TLRs by each cell type may contribute to gut homeostasis and prevent dysregulated mucosal inflammation.

Innate immune responses to commensal bacteria in inflammatory bowel disease

Crohn's disease and ulcerative colitis are the two major forms of IBD characterized by acute and chronic inflammation in the absence of a known pathogen. These inflammatory disorders are distinguished by the depth and location of inflammation with ulcerative colitis being limited to the mucosa of the colon and Crohn's disease involving both the small intestine and the colon in a transmural fashion. The pathogenesis of Crohn's disease and ulcerative colitis is multifactorial, resulting from the interplay of genetic predisposition, environmental and immunological factors [21]. Initiation and perpetuation of the intestinal inflammation in this chronic disorder has been thought to result from dysregulated immune response to commensal bacteria in the genetically-susceptible host. For instance, the efficacy of fecal diversion and the recurrence when the fecal stream is restored [22, 23], the existence of subpopulations who can be improved by antibiotics or probiotic treatment [24], and the loss of tolerance observed with commensal bacteria [25, 26] all imply that enteric bacteria have a critical role in the intestinal inflammation in patients with IBD. These clinical observations have been supported by the evidence that commensal bacteria are indispensable for the development of inflammation in most murine models of inflammatory bowel disease [27].

Recent clinical and laboratory investigations appeared to show the answer for why and how IBD patients have dysregulated immune responses to commensal bac-

teria. Firstly, the disease-susceptibility gene identified in Crohn's disease named NOD2/CARD15 encodes a protein with a leucine-rich repeat domain that has pattern recognition receptor (PRR) function. Thus the genetic predisposition in Crohn's disease is at least partly due to an abnormality of host recognition system for commensal bacteria. Secondly, the intestinal mucosa in patients with IBD may be defective in clearance of luminal bacteria. IBD patients have been found to have a dramatic increase in the number of bacteria adherent to the intestinal mucosa, even in mucosa that is not inflamed [28]. Increased intestinal permeability has been found in patients with Crohn's disease as well as in symptom-free first-degree relatives [29]. These observations suggest that there exists defective barrier function even before the onset of a dysregulated immune response in patients with IBD.

Much work has been done to identify specific bacteria or bacterial components which can serve as a dominant antigen for the aberrant host response seen in IBD. A unique bacterial DNA sequence, termed I2, has been identified in lamina propria mononuclear cells in patients with Crohn's disease [30]. This bacterial sequence derived from *Pseudomonas fluorescens* can act as a super-antigen in CD4⁺ T-cell activation [31]. In addition, aberrant T cell responses to the host flora and expression of antimicrobial antibodies have been found in patients with IBD [32–34]. Serologic markers consisting of antibodies against microbial substances such as yeast (anti-*Saccharomyces cerevisiae*), *E. coli* (anti-OmpC) and anti-flagellin [26, 35] are expressed by patients with IBD, especially Crohn's disease. These unique serological markers associated with abnormal responses to commensal bacteria have begun to contribute to the clinical management of IBD patients.

The next issue to address is whether inappropriate recognition of PAMPs by TLRs plays a role in IBD pathogenesis. Part of the way in which TLRs may be involved in control of local bacterial populations in the gut is through the expression of defensins. Defensins are antimicrobial peptides that may be expressed by Paneth cells (cryptdins) or intestinal epithelial cells. Paneth cells located at the base of small intestinal crypts also express a wide range of TLRs [36]. Stimulation through TLR9 causes degranulation of Paneth cells [37]. We have recently demonstrated that TLR4- and TLR2-dependent pathways can stimulate β -defensin-2 expression by human intestinal epithelial cells [38]. In Nod2^{-/-} mice, cryptdin expression by Paneth cells is decreased suggesting a role for PRRs in Paneth cell function [39]. Therefore, secretion of various antimicrobial peptides by Paneth cells and intestinal epithelial cells can be regulated by TLR-mediated recognition of PAMPs. Defects in the ability to sense microbes through TLRs or nucleotide oligomerization domains (Nods) may result in decreased ability to clear bacteria from the apical surface of the epithelium.

The expression and functional characteristics of TLRs in the IBD mucosa are still under investigation (Fig. 1). Human intestinal epithelial cells normally express TLR3 and TLR5, while TLR2 and TLR4 are only barely detectable [3–5, 40]. However, immunohistochemical examination has revealed that TLR4 is strongly up reg-

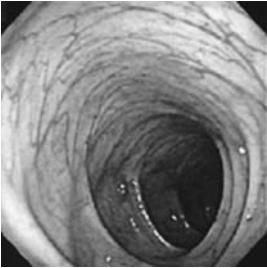
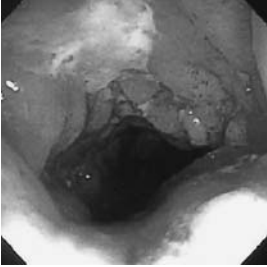
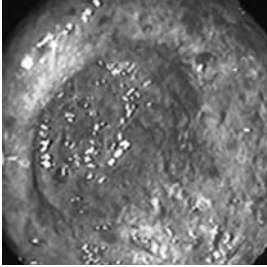
Endoscopic views	Clinical presentations	Characteristics in TLR expression
	Normal intestine with flat, smooth surface, showing fine vascular pattern.	Limited repertoire of TLR expression. TLR 2 and TLR 4 have low expression. TLR signaling is normally down-regulated. Polarized (basolateral) TLR expression.
	May affect entire digestive tract especially in the terminal ileum.	Discrete and transmural inflammation; linear ulcer formation. TLR4 is upregulated in IEC. Expression of TLR2 and TLR4 is increased in lamina propria macrophages.
	Rectum involved and may extend to proximal colon; does not affect small intestine. Continuously inflamed mucosa; no involvement beneath submucosa.	TLR4 is upregulated in IEC. TLR3 is downregulated in IEC. Expression of TLR2 and TLR4 is increased in lamina propria macrophages.

Figure 1

Differences in clinical presentation and characteristics of mucosal TLR expression in normal and IBD intestine

Mucosal bacterial recognition plays a critical role in the pathogenesis of both ulcerative colitis and Crohn's disease. Differences in clinical presentations between these two major forms of IBD may result from alterations in TLR signaling. Characteristic abnormalities of TLR signaling associated with clinical subtypes of IBD are illustrated.

ulated in both Crohn's disease and ulcerative colitis, while the expression of TLR2 and TLR5 remains unchanged [40]. Distribution of TLR4 in the intestinal epithelial cells is different between Crohn's disease and ulcerative colitis. The overexpression in Crohn's disease is in the apical aspect of the cells, whereas in ulcerative colitis it is in the basolateral aspect [40]. Most of these data are based on immunohistochemical characterization but the antibodies against TLRs have not been optimal. The expression of TLR3 in the intestinal epithelial cells is down-regulated in active Crohn's disease, but not in ulcerative colitis [40]. In addition, expression of TLR4 and TLR2 is increased in lamina propria macrophages in IBD [8]. A recent study, on the other hand, has demonstrated that colitic animals and patients with Crohn's disease express serum antibody reactivity against flagellin derived from commensal bacteria [35]. The response to flagellin was against a specific peptide sequence derived from a limited array of bacterial species. These data suggest that TLR5-dependent recognition of flagellin may play a role in the dysregulated immune response to commensal bacteria in IBD. Therefore, IBD may be associated with distinctive changes in TLR expression and function in intestinal epithelial cells as well as other types of cells in the gut. However, the numerous possible combinations and crosstalk of TLRs and other signal transduction pathways make the pathogenesis of IBD difficult to elucidate.

The role of TLRs in cytoprotection and damage control in the gut

Normal intestinal function may be regulated by bacteria through TLR signaling. TLR signals may be important for maintenance of normal epithelial homeostasis in the intestine. Recent studies in germ-free mice colonized with *Bacteroides thetaiotaomicron*, a prominent component of the normal intestinal microflora, have revealed global intestinal transcriptional responses to colonization [41–43]. The microarray data demonstrated that colonization of bacteria augment the expression of genes involved in modulating various intestinal functions including junctional proteins, enzymes involved in digestion, absorption and metabolism. In addition, germ-free mice show a greatly reduced rate of intestinal epithelial proliferation [44]. These studies, however, did not address whether TLRs mediate this phenomenon or which cell type is responding to the presence of bacteria or PAMPs. For example, lamina propria mononuclear cells may be exposed to PAMPs and secrete cytokines or other peptides that act in trans on the epithelium to mediate the proliferative effect.

Cario et al. have recently shown that TLR2 signaling via protein kinase C is associated with enhanced transepithelial resistance in intestinal epithelial cells correlated with apical tightening and sealing of the tight junctional protein ZO-1 [45]. We have recently shown that healing of injured intestinal epithelium and clearance of intra-mucosal bacteria require the presence of intact TLR signaling [46]. Decreased epithelial proliferation is also found in myeloid differentiation marker (MyD88)

knockout (MyD88^{-/-}) and TLR4 knockout (TLR4^{-/-}) mice [46, 47]. At the same time, LPS has been shown to induce cytoprotective heat shock protein expression by intestinal epithelial cells and protects against radiation-induced injury [48, 49]. In addition, TLR signaling can induce HGF, TGF-beta1, VEGF and other cytoprotective molecules [50]. Rakoff-Nahoum et al. have shown that administration of dextran-sodium sulfate (DSS) to TLR4^{-/-}, TLR2^{-/-}, or MyD88^{-/-} mice results in higher mortality than wild-type mice [47]. Furthermore, administration of oral broad-spectrum antibiotics to wild-type mice brings them closer to the MyD88^{-/-} phenotype suggesting that signals provided by the luminal bacteria via TLRs protect against DSS damage. Although, others have reported the opposite results regarding the effect of antibiotics on DSS-induced colitis [51], it is difficult to know the exact effect of antibiotics on the model depending on the background of the mice used.

Learning from animal studies

Animal studies have provided a wealth of information with respect to normal and abnormal host-microbial relationships. A number of models of IBD have been described over the last decade in which chronic intestinal inflammation occurs spontaneously in mice with a specific genetic background or genetically manipulated (i.e., transgenic, knockout) mice. The importance of indigenous luminal bacteria in the development of intestinal inflammation has been revealed in those mice [27, 52, 53]. Interestingly, a sub-strain of C3H/HeJ mice develop spontaneous colitis which is dependent on luminal bacteria [54]. At least in this model, TLR4 is not necessary for the development of colitis. Intestinal epithelial cells from IL-2-knockout mice in which colitis is initiated by exposure to commensal bacteria have shown changes in TLR responsiveness during the development of colitis, characterized by augmented TLR2 responsiveness and concomitant reduction in TLR4 responsiveness [55]. We and others have shown that MyD88^{-/-} and TLR4^{-/-} mice have increased signs of colitis compared with wild-type littermates in response to DSS [46, 47]. A comparison of the histology demonstrated that TLR4^{-/-} and MyD88^{-/-} mice have significantly fewer neutrophils in the lamina propria and submucosa compared with control mice. The cause for decreased neutrophils in the intestine is due to diminished chemokine expression by lamina propria macrophages. The consequences of the decreased neutrophil recruitment include Gram-negative bacterial translocation to mesenteric lymph nodes in knockouts but not wild-type mice. These data demonstrate that TLR signaling in response to the presence of bacteria in the lamina propria following epithelial injury is critical for elimination of bacteria before they disseminate. The increase in bacterial dissemination is made worse by the decrease in intestinal epithelial proliferation in these mice.

On the other hand, other data implicating TLRs in the generation of a regulatory response to the commensal flora relate to the observation that oral or systemic

administration of bacterial DNA ISS-ODN or CpG ODN, the ligand for TLR9, ameliorates inflammation in several animal models of colitis [56] but not in TLR9^{-/-} mice [57]. The balance however between the requirement of TLR signaling to initiate and sustain acute and chronic inflammation *versus* its role in healing of the epithelium is probably contextual and depends on other factors as well as the nature of the injury.

Genetic contribution of TLR signaling in the pathogenesis of IBD

IBD is thought to result in the genetically-susceptible host following a triggering event. Genetic factors play a more dominant role in Crohn's disease than ulcerative colitis based on accumulated data from identical twin studies and familial clustering [58]. Polymorphisms in the NOD2/CARD15 gene (R702W, G908R and 1007fs) have been identified in patients with Crohn's disease [59, 60]. Nucleotide oligomerization domain (Nod) proteins are PRRs with homology to plant disease resistance proteins [61, 62], which confer responsiveness to peptidoglycan through Rip2/RICK kinase, a mediator of NF- κ B activation [63–65]. Approximately a third of patients with Crohn's disease carry one of three allelic variants of NOD2/CARD15 compared with 10–15% of the normal population or ulcerative colitis patients [66–69]. Homozygosity increases the relative risk of developing Crohn's disease by as much as 40-fold as compared to simple heterozygosity [59, 70, 71]. Clinical phenotypic associations with NOD2/CARD15 mutations include a slightly younger age of onset, ileal involvement, and fibrostenotic disease [72–75]. Children with mutations in NOD2/CARD15 have an accelerated course towards their first surgery [76]. Nod2 protein is highly expressed by monocytes and Paneth cells [77] and is also seen in the inflamed colon in Crohn's disease [78]. Polymorphisms in this gene result in a decreased ability to bind to the bacterial ligand, muramyl dipeptide (MDP), and altered activation of the NF- κ B system leading to a reduced capacity to activate proinflammatory signals in the presence of intracellular bacteria [63, 79].

Initially it was difficult to reconcile why those mutations would increase susceptibility to a disease characterized by exuberant inflammation in response to commensal bacteria. Cytokine production in response to MDP is abolished in Nod2 knockout (Nod2^{-/-}) mice and they do not develop any intestinal pathological changes spontaneously [80, 81]. More recently, Nod2 appears to play an important role in innate immunity against pathogens. Nod2^{-/-} mice have low levels of cryptdin expression by Paneth cells [39]. When these mice are orally infected with *Listeria monocytogenes*, there is dissemination of the *Listeria* that is not seen with wild-type mice. More germane to the issue of IBD, mice carrying a mutation that is analogous to the Crohn's disease susceptibility allele, 3020insC, exhibit elevated NF- κ B activation in response to MDP and more efficient processing and secretion of the cytokine interleukin-1 β [51]. In addition, mice carrying the Crohn's disease-associ-

ated Nod2 mutation are more susceptible to DSS-induced colitis with greater expression of proinflammatory cytokines [51]. Although these findings in the animal model are interesting, the induction of acute inflammation with DSS is quite dissimilar to the ileal inflammation seen in patients with Crohn's disease. On the other hand, patients homozygous for the 3020insC frameshift-mutation in the Nod2 gene demonstrate defective release of IL-10 from peripheral blood mononuclear cells after stimulation with TLR2 ligands [82]. Thus, in the setting of Crohn's disease-associated mutations of Nod2, TLR2 signaling is associated with diminished secretion of anti-inflammatory cytokines. Another model for why mutations in Nod2 may predispose to Crohn's disease is through aberrant clearance of intracellular pathogens, since overexpression of Nod2 in intestinal epithelial cells protects against *Salmonella typhimurium in vitro* [83]. Studies examining NOD2 mutations in Japanese, Korean and African-American individuals with Crohn's disease have not shown an association [84–86]. Therefore other genes await identification as IBD-susceptibility genes.

TLR genes may be candidate genes for IBD, while recent studies have demonstrated mixed results with respect to the association between the TLR4 polymorphisms and IBD. Two common co-segregating missense mutations in the extracellular domain of TLR4, Asp299Gly and Thr399Ile, have been found to result in diminished response to inhaled LPS and protection against atherosclerosis [87–89]. The allele and carrier frequencies for the Thr399Ile mutation in the TLR4 gene were positively associated with ulcerative colitis in a German population [90]. In addition, association of the TLR4 Asp299Gly polymorphism was found in both Crohn's disease and ulcerative colitis in a Belgian population [91]. However, TLR4 (A299G) and CD14 (T-159C) variants did not differ between Crohn's disease and controls in Scottish and Irish patients [92]. A recent study in Japanese patients with ulcerative colitis yet again failed to detect any increase in TLR4 polymorphisms in IBD patients [93].

Additional genetic evidence points to the recognition of PAMPs in the pathogenesis of IBD. Polymorphisms in the IL-1 receptor antagonist gene may affect severity and extent of disease in ulcerative colitis patients, particularly in patients positive for perinuclear antineutrophil cytoplasmic antibody (pANCA) [94]. In addition, a common functional promoter region polymorphism (T-159C) of the CD14 gene has been identified and has a weak association with both Crohn's disease and ulcerative colitis in German and Japanese patients [95, 96]. These studies imply a role of innate immune response genes in the pathogenesis of IBD.

Conclusions and future directions

The fundamental purpose of the innate immune system is to protect a host against pathogens. In this context, the innate immune system in the gastrointestinal tract is

unique because of the numerous foreign organisms such as bacteria living within it. Intestinal epithelial cells are the interface between host and microbes. Only in the last few years with the discovery of TLRs, are we beginning to understand the delicate balance between an inflammatory response and tolerance to microbial flora. At least one mechanism involves careful regulation of TLR expression and function in the intestine. The risk, however, is having too little response and thereby pathogenic invasion. Through a better understanding of the innate immune response to commensal bacteria, we can develop targeted therapy for patients with IBD.

Agonist or antagonists for individual TLRs may be used to generate tolerance or inhibit signaling in IBD. Future therapy may also include manipulation of the microbial environment combined with targeting of inflammatory pathways to reset the immunologic balance.

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Toll-like receptor signaling in the liver

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Introduction

The liver is an indispensable organ that performs essential metabolic functions including the synthesis, storage and redistribution of carbohydrates, amino acids and fat and also has a major role in the detoxification and excretion of many ingested toxins. The role of the liver in innate and adaptive immunity has received little attention for a long time. Evidence is accumulating that the liver has very specific immunologic properties and that it contains a large number of resident and non-resident cells that participate in the regulation of inflammatory and immune responses [1]. Due to its anatomical links to the gut through the portal vein, which carries nutrient-rich blood from the intestine to the liver, the liver is the major target of gut-derived bacteria and bacterial products and acts as a first line of defense. The liver functions as a major filter organ for bacterial products. For example, up to 80% of intravenously injected endotoxin is detected in the liver within 20–30 min [2, 3]. Kupffer cells, the resident macrophages of the liver, are able to efficiently take up endotoxin and phagocytose bacteria carried through portal vein blood and are considered to play a major role in the clearance of systemic bacterial infection [4–6]. Despite the constant exposure to low levels of gut-derived bacteria and bacterial products, there are no signs of ongoing inflammation in the healthy liver. This lack of response is to some extent explained by the high degree of tolerance that is found in the liver. Liver tolerance is typified by graft survival across major histocompatibility antigen disparities, induction of systemic tolerance to food antigens and persistence of some viral infections for decades [7]. Continuous exposure to low levels of lipopolysaccharides (LPS) may induce LPS tolerance [8] through several mechanisms including the LPS-induced downregulation of TLR4, the major receptor for LPS [9]. Indeed, TLR4 expression in the liver differs from other organs in that TLR4 levels are low in the liver and cannot be further induced by proinflammatory mediators such as IL-1 β and TNF- α [10]. Furthermore, hepatic dendritic cells and Kupffer cells show a much lower expression of TLR4 and CD14, respectively, than elsewhere in the body which may also contribute to the hepatic tolerance towards LPS [11, 12]. It has also been shown that the healthy liver contains low mRNA levels of

Table 1 - Toll-like receptor expression in hepatic cell populations

Cell population	Main functions	TLR expression	TLR signals
Hepatocyte	Albumin synthesis, metabolic (protein, fat, carbohydrate), detoxification	TLR2,3,4	TLR2: Modest increase in NF- κ B activation and SAA production TLR3: Upregulation of IFN-responsive genes TLR4: Modest increase in NF- κ B activation and SAA production
Hepatic stellate cell	Retinoid storage, fibrogenesis	TLR4	TLR4: NF- κ B and JNK activation, chemokine secretion, effects on fibrogenesis unknown
Biliary epithelial cell	Lining of bile ducts	TLR2,3,4,5	TLR4: NF- κ B activation and TNF- α secretion
Sinusoidal endothelial cell	Lining of sinusoids, filtration of sinusoidal blood	Not known	Not known
Kupffer cell	Phagocytosis, antigen presentation, cytokine secretion	TLR2,(3)*,4,(9)*	TLR2,3*,4*: NF- κ B activation and increased TNF- α , IL-1 β , IL-6, IL-12 and IL-18 secretion TLR9*: Increased TNF- α , IL-6 and IL-12 secretion
Dendritic cell	Professional antigen presentation cytokine secretion	TLR4,(9)	TLR4: Increased TNF- α , IL-1 β , IL-6, IL-12 and IL-18 secretion TLR9: Increased TNF- α , IFN- α , IL-6, IL-12

* unpublished data. Brackets indicate TLR expression as suggested by functional assays.

TLR1,2,4,6,7,8,9,10, MD-2 and MyD88 in comparison to other organs [13]. Additionally, it is likely that a decrease in IRAK and Gi-protein expression as well as an increase in SOCS-1, IRAK-M and I κ B levels also contribute to this tolerance. Under conditions like alcoholism, liver cirrhosis, ischemia-reperfusion and partial hepatectomy, the levels of portal blood endotoxin are significantly increased [14, 15]. Endotoxin is a key player in the pathophysiology of these diseases and initiates signaling cascades that trigger proinflammatory, pro-apoptotic and proliferative responses in the liver. Liver injury induced by carbon tetrachloride, choline-deficient diet or alcohol is mitigated by nonabsorbable oral antibiotics, colectomy or germ-free conditions suggesting a prominent role for LPS in the pathophysiology of these

diseases [16–18]. Conversely, it has been shown that the injection of LPS augments liver injury by alcohol or choline-deficient diet [19–21]. The hepatic response to LPS is mediated by several hepatic cell populations that express TLRs and are part of a cellular network involved in the hepatic wound healing and regenerative response. In this chapter, we will describe the role of TLRs in hepatocytes, biliary epithelial cells, hepatic stellate cells, Kupffer cells and hepatic dendritic cells and highlight the importance of TLR receptors in the pathophysiology of several disease states including alcoholic liver disease, hepatic regeneration, hepatic ischemia reperfusion injury, hepatic infection and hepatic fibrosis.

TLR expression in the liver

Hepatic cell populations and hepatic architecture

On their apical side, hepatocytes form bile canaliculi which merge into bile ducts (see Fig. 1). Bile ducts are formed by biliary epithelial cells and transport bile from the hepatic bile caniculi to the intestine. On their basolateral side, hepatocytes are surrounded by the subendothelial space of Disse. Sinusoidal endothelial cells form a fenestrated endothelium which separates the liver sinusoids, the microvascular end unit of the liver, from the space of Disse. Hepatic stellate cells, the mesenchymal cell population of the liver, reside within the space of Disse. Kupffer cells and dendritic cells reside in the sinusoidal space under normal conditions. The liver has a dual blood supply. The hepatic artery supplies a relatively small amount of oxidized blood, whereas the portal vein carries a large amount of nutrient-rich blood from the intestine and the spleen.

Kupffer cells

Kupffer cells are resident macrophages of the liver which are located in the hepatic sinusoids. Kupffer cells perform multiple functions, including phagocytosis, antigen processing and presentation, and generate various proinflammatory products, including cytokines, prostanoids, nitric oxide, and reactive oxygen intermediates. Due to their anatomical localization, Kupffer cells are among the first cells in the liver to be hit by gut-derived toxins including LPS and orchestrate the inflammatory response within the liver under many pathological conditions. Kupffer cells express TLR4 and are responsive to LPS [22]. Due to the continuous exposure to low amounts of LPS, Kupffer cells seem to have evolved mechanisms to evade some of the proinflammatory actions of LPS. In comparison to peripheral blood monocytes, Kupffer cells express low levels of CD14 [12]. Upon LPS stimulation however, Kupffer cells upregulate CD14 expression and thus may become more responsive

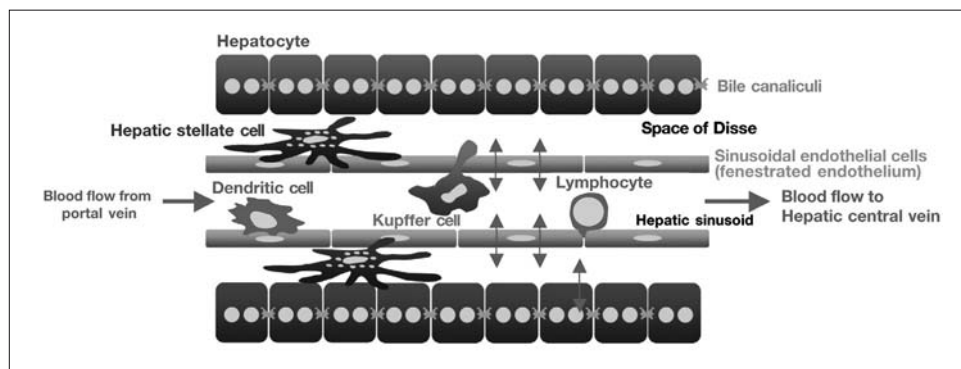


Figure 1

The microenvironment of liver

The liver has two blood supplies, one from hepatic artery, the other from portal vein. These two blood supplies mix into the hepatic sinusoids surrounded by sinusoidal endothelial cells. Nutrient rich blood traverses through the fenestrated endothelium to the subendothelial space of Disse, where it gets into contact with hepatocytes. Kupffer cells, liver lymphocytes and dendritic cells usually reside in sinusoidal space. Hepatic stellate cells exist in the space of Disse. Following liver injury, Kupffer cells, lymphocytes and dendritic cells migrate into the liver parenchyma.

to LPS. Accordingly, high amounts of bacterial products such as LPS are among one of the most potent activator of Kupffer cells. After LPS stimulation, Kupffer cells produce TNF- α , IL-1 β , IL-6, IL-12, IL-18 and several chemokines [23, 24]. Kupffer-cell derived IL-12 and IL-18 in turn activate hepatic NK cells to produce IFN- γ , a key cytokine involved in microbial eradication and hepatic wound healing [25]. Kupffer cells also seem to express functional TLR2 since TLR2 deficient Kupffer cells show a greatly diminished response towards *Listeria monocytogenes* [26]. Activated Kupffer cells play an important role in activating hepatic stellate cells (see below) through the secretion of profibrogenic mediators such as TGF- β 1, MMPs, PDGF and ROS [27].

Hepatocytes

In the adult liver, hepatocytes mainly fulfil metabolic functions such as the production of albumin, regulation of glucose and lipid metabolism, detoxification and production of bile acid, but appear to have little involvement in immunoregulatory processes. Although hepatocytes express TLR2 and TLR4 receptors and are responsive to LPS, this response is fairly weak with only two-fold elevated levels of serum amyloid A (SAA) after LPS [28]. Similarly, stimulation with the TLR2 ligand bacter-

ial lipoprotein in hepatocytes induces NF- κ B activation and a weak induction of SAA [28]. The expression of TLR2 in hepatocytes is upregulated by LPS, TNF- α , bacterial lipoprotein and IL-1 β in an NF- κ B-dependent manner indicating that hepatocytes become more responsive to TLR2 ligands under inflammatory conditions [28]. On the other hand, TLR4 expression in hepatocytes is not upregulated by proinflammatory mediators [10]. Hepatocytes are also believed to play a role in the uptake of endotoxin and its removal from the systemic circulation through secretion into the bile [29]. Based on the available data, it seems plausible that hepatocytes mainly serve to remove LPS from the systemic circulation and for this reason express low levels of TLR4 and only weakly respond to LPS. It is likely that the uptake of LPS in hepatocytes is not mediated by CD14 or TLRs, but by scavenger receptors including scavenger receptor type BI which plays a role in LPS uptake and is expressed at high levels in hepatocytes [30, 31]. A recent report shows that the transformed hepatocyte cell line PH5CH8 expresses TLR3 and upregulates IFN- β promoter activity and interferon-responsive genes in response to poly-I:C [32] but it is not known whether primary hepatocytes express TLR3 to activate anti-viral signaling pathways.

Hepatic stellate cells

In the normal liver, hepatic stellate cells store a large amount of retinoids in lipid droplets. After liver injury hepatic stellate cells undergo an activation process which results in the loss of retinoids, a phenotypic change and high expression of large numbers of extracellular matrix proteins, cytokines and chemokines [33]. Activated stellate cells are the main producers of extracellular matrix proteins in the fibrotic liver [33]. Hepatic fibrogenesis is regulated by a cellular network in which Kupffer cells and hepatic stellate cells are considered key players. TLR-dependent and TLR-independent activation of Kupffer cells is believed to drive hepatic stellate cells activation through several profibrogenic mediators [27]. However, activated hepatic stellate cells also express TLR4/CD14 and respond to LPS with the activation of IKK/NF- κ B and JNK as well as the secretion of proinflammatory cytokines [34]. Thus, LPS may induce fibrogenic responses in the liver through two TLR4-dependent pathways: One pathway which first activates Kupffer cells followed by cytokine synthesis and subsequent hepatic stellate cell activation and a second pathway in which activated hepatic stellate cells are directly stimulated by LPS to perpetuate hepatic stellate cell activation.

Biliary epithelial cells

Biliary epithelial cells form the biliary tree which connects the liver with the intestinal lumen to deliver bile to the intestine. Through its connection with the intestine

the biliary system is susceptible to infection from gut-derived bacteria. Therefore, biliary epithelial cells express molecules with roles in innate immunity including CD14, MD-2 and TLR2, 3, 4 and 5 [35]. Lipopolysaccharide treatment induces the production of TNF- α , and nuclear translocation of NF- κ B and increased NF- κ B-DNA binding in cultured cells. This induction of TNF- α is partially inhibited by anti-TLR4 antibody [35].

Hepatic dendritic cells

Hepatic dendritic cells are the professional antigen-presenting cells of the liver. During inflammation, dendritic cells are recruited into the liver sinusoids from where they can migrate to periportal and pericentral areas. Hepatic CD11c⁺/CD11b⁺ dendritic cells express TLRs on their cell surface and respond to LPS, peptidoglycan, poly(I:C) and CpG-DNA to produce inflammatory cytokines, such as IL-12 and TNF- α and to express co-stimulatory molecules, such as CD40, CD80 and CD86 on their cell surface (Seki and Tsutsui, unpublished data). Liver CD11c⁺, CD8 α ⁻ (myeloid) and CD11c⁺, CD8 α ⁺ ("lymphoid-related") DC express lower TLR4 mRNA compared with their splenic counterparts [11]. Lower TLR4 expression correlates with the reduced capacity of LPS-stimulated, but not anti-CD40-stimulated liver DC to induce naive allogeneic (C3H/HeJ) T cell proliferation. In contrast to LPS-stimulated splenic DC, these LPS-activated hepatic DC induce alloantigen-specific T cell hyporesponsiveness *in vitro* and are inferior allogeneic T cell stimulators compared with splenic DC [11]. These data suggest that the low expression of TLR4 by liver DC may contribute to the reduced or altered activation of hepatic adaptive immune responses.

TLR signaling in hepatic disease

Alcohol-induced liver injury

Orally ingested alcohol disrupts the intestinal epithelial barrier causing an enhanced permeability [36] and subsequent elevations of endotoxin levels in the portal vein [37, 38]. LPS-mediated activation of Kupffer cells plays a crucial role in ethanol-induced liver injury (as outlined in Fig. 2). Liver injury is strongly reduced when the Gram-negative microflora is eliminated from the gut by lactobacillus or antibiotics, or when Kupffer cells are destroyed with gadolinium chloride [16, 39, 40]. The activation of Kupffer cells in alcoholic liver disease largely depends on TLR4 since TLR4-mutated C3H/HeJ mice display strongly reduced levels of proinflammatory mediators in the liver and blunted liver injury despite elevated endotoxin levels [41]. NADPH-oxidase is a crucial downstream mediator of TLR4 in Kupffer cells during

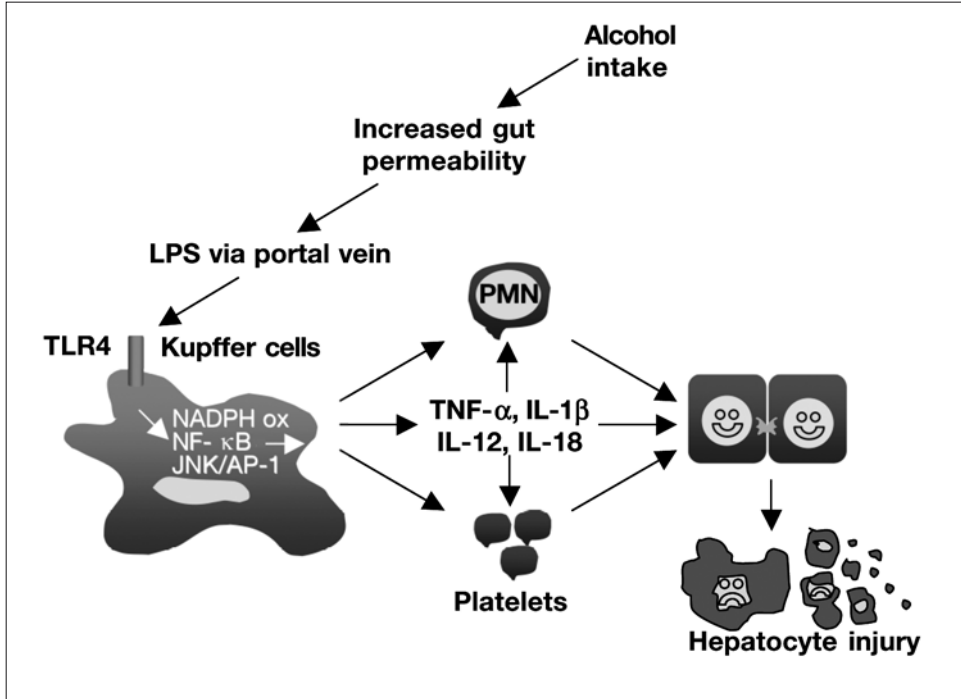


Figure 2

TLR signaling in alcoholic liver injury

Orally ingested alcohol destroy the intestinal barrier and increases the levels of LPS in the portal vein. LPS binds to TLR4 which is express on Kupffer cells. TLR4 activated NF-κB, JNK/AP-1 and NADPH oxidase. These factors are involved in the release of TNF-α, IL-1β, IL-12 and IL-18 which then promote hepatocyte injury through the recruitment of neutrophils and platelets and through direct effects on hepatocytes.

alcohol-induced liver injury [42]. Mice deficient in p47phox, the main cytosolic component of NADPH oxidase, show an absence of free radical production, activation of NF-κB, TNF-α mRNA induction and liver pathology after ethanol treatment [42].

Liver fibrosis

Hepatic fibrosis is a reaction to chronic hepatic injury induced by a variety of stimuli including viral hepatitis, alcohol, autoimmune and metabolic disease [33]. Fibrogenesis is usually preceded by hepatocyte injury and an inflammatory envi-

ronment. Although there is an abundance of data demonstrating that LPS is elevated in patients with cirrhosis [43–45] and a key player in liver injury and inflammation, there are only a few studies addressing the role of LPS signaling in liver fibrosis. Studies from the middle of the last century have suggested a role for bacteria in the development of fibrosis induced by CCl₄ or choline-deficient diet [17, 18]. Furthermore, it has been shown that TLR4 is expressed on two key mediators of hepatic fibrogenesis, Kupffer cells and hepatic stellate cells. Kupffer cells initiate fibrogenesis by secreting proinflammatory and profibrogenic cytokines which act on other cells types including the hepatic stellate cell (as outlined in Fig. 3). The role of LPS and TLR4 in the early stage of alcoholic liver disease has been clearly demonstrated in the TLR4-mutated C3H/HeJ strain, but fibrogenesis has not yet been studied in this mouse model [41]. Activated hepatic stellate cells are the main source of collagen in the fibrotic liver and are highly responsive to LPS through a TLR4-dependent pathway [33, 34]. In hepatic stellate cells, LPS induces IL-8 and MCP-1 production and activates transcription factor NF- κ B and c-Jun through TLR4 indicating that LPS exerts direct effects on hepatic stellate cells during fibrogenesis [34]. The central question whether LPS and TLRs are involved in the activation process of hepatic stellate cells has not been studied yet and is required to understand the role of TLR signaling in hepatic fibrogenesis. Another interesting question is whether hepatic stellate cells express TLR2 and may thus be stimulated during Hepatitis C virus (HCV) infection by the TLR2 ligands HCV core and NS3 [46, 47].

HCV infection

Hepatitis C virus (HCV) is a single-stranded hepatotropic RNA virus which causes a chronic infection of the liver that may lead to the development of cirrhosis and hepatocellular carcinoma. Hepatitis C virus has developed several strategies to evade the immune system resulting in failure to eradicate the virus in most infected individuals [48]. A recent report provides evidence that HCV may evade the attack from the innate immune system by inducing the degradation of TRIF, one of the TLR adaptor proteins [49]. NS3 impedes both IRF-3 and NF- κ B activation by reducing functional TRIF abundance and by generating cleavage products with dominant-negative activity [49]. Thus, HCV has not only developed strategies to avoid attack from the adaptive immune system, but also from the innate immune system. HCV core and NS3 proteins may also activate TLR2 in monocytes and macrophages to induce TNF- α , IL-6 and IL-8 production through NF- κ B, JNK/p38/AP-1 and ERK pathways [46]. These changes may contribute to the proinflammatory environment seen in later stages of chronic HCV infection which is associated with hepatocyte damage, fibrosis and hepatocellular carcinoma development. As mentioned previously, it has recently been demon-

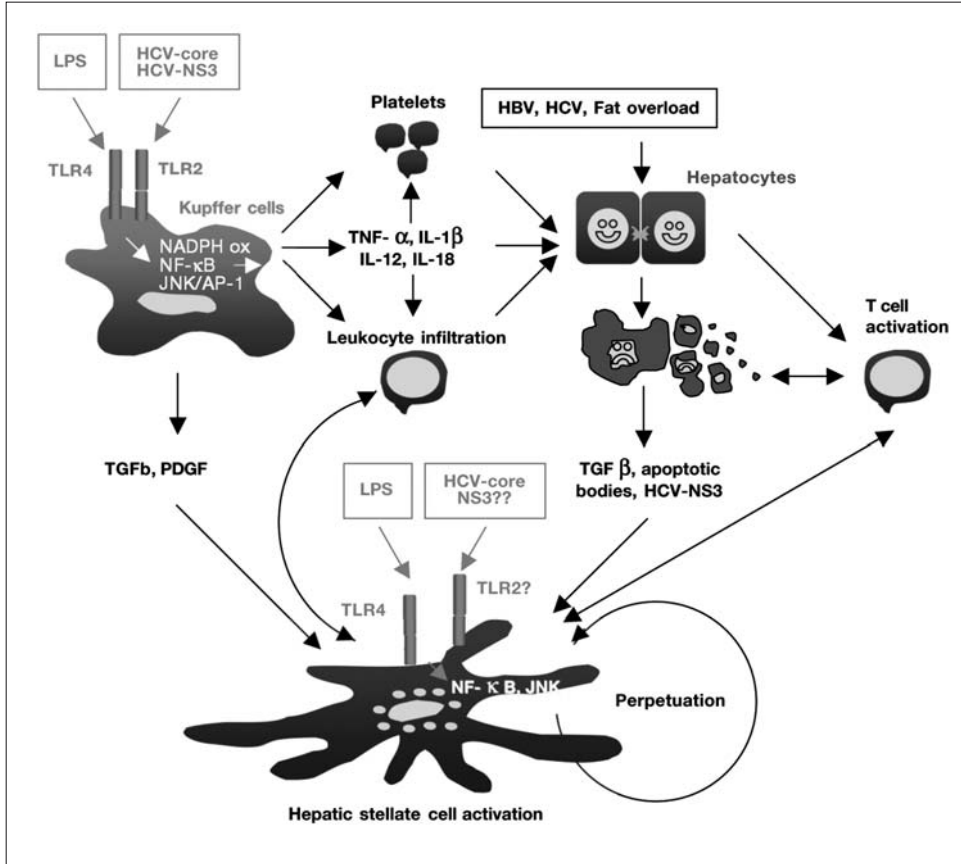


Figure 3

TLR signaling in hepatic fibrogenesis

During liver injury due to alcohol, HCV or fat overload, LPS levels are increased and believed to contribute to fibrogenesis through 2 pathways: 1. LPS and HCV-core/NS3 stimulate TLR4 and TLR2 on Kupffer cells to (a) enhance hepatocyte damage (b) increase leukocyte infiltration (c) secrete profibrogenic cytokines such as TGF-β and PDGF. These factors are believed to then act in concert to induce activation of hepatic stellate cells and fibrogenesis. 2. LPS and TLR ligands, e.g., HCV-NS3 and HCV-core, may bind TLR4 and possibly TLR2 to activate NF-κB and JNK and perpetuate hepatic stellate cell activation.

strated that the transformed hepatocyte cell line PH5CH8 expresses TLR3 and upregulates IFN-β promoter activity and interferon-responsive genes after poly-I:C exposure which may represent a potential defense mechanism against HCV infection [32].

Ischemia/reperfusion liver injury

Ischemia/reperfusion injury of the liver occurs in procedures such as partial hepatectomy and liver transplantation. Kupffer cells play a prominent role in ischemia/reperfusion injury of the liver (as outlined in Fig. 4). After activation, Kupffer cells produce proinflammatory cytokines that damage hepatic endothelial cells, hepatocytes and neutrophils leading to the recruitment of T lymphocytes and hepatic inflammation. After liver transplantation, not only LPS, but also endogenous TLR ligands such as hsp60 and hsp70 are strongly elevated in serum and liver [50, 51]. In TLR4-deficient mice and mice deficient in IRF-3, ischemia/reperfusion-induced liver inflammation and hepatocellular damage is almost completely prevented, whereas ischemia/reperfusion liver injury was unchanged in MyD88-deficient mice [52]. These findings indicate that ischemic-reperfusion induces liver injury through TLR4-mediated IRF-3-dependent, but MyD88-independent signaling pathway [26].

Liver regeneration after partial hepatectomy

As the main detoxifying organ in the body, the liver has a high likelihood of toxic injury and has amazing regenerative properties that will restore the liver to full size and ensure survival [53]. The regenerative capacity of the liver was first described in Greek mythology and was confirmed in 1931 by the studies of Higgins and Anderson, who demonstrated that the liver regenerates to full size after a two-thirds partial hepatectomy (PH) within 7–10 days [54]. During liver regeneration, a complex network of cytokines (TNF- α and IL-6), growth factors (HGF, EGF), kinases (Erk, JNK) and transcription factors (AP-1, NF- κ B, Stat3) drives hepatocytes out of G₀ phase to enter 1–2 rounds of replication. After PH, LPS is elevated in portal vein blood and may contribute to the initiation of liver regeneration [55]. LPS triggers the secretion of TNF- α and IL-6 in Kupffer cells which then initiate liver regeneration (see Fig. 4). However, the currently available data on the role of TLRs and TLR ligands is not sufficient to draw a clear picture. Whereas some studies have reported that liver regeneration after CCl₄ is suppressed in TLR4-mutant mice [55, 56], we do not find a role for TLR 2, 4 and 9 in liver regeneration after PH [57]. On the other hand, MyD88-deficient mice display decreased TNF- α and IL-6 levels, impaired NF- κ B nuclear translocation in Kupffer cells and decreased levels of immediately early genes [57], suggesting that liver regeneration is likely to be driven by other TLRs which may possibly bind endogenous TLR ligands to signal through MyD88. In addition, strong activation of TLRs may also impair liver regeneration. Injection of the TLR4 ligand LPS or the TLR3 ligands, murine CMV and poly(I:C) suppress liver regeneration after hepatectomy [58]. Taken together, TLR/MyD88 signaling is required for beginning of

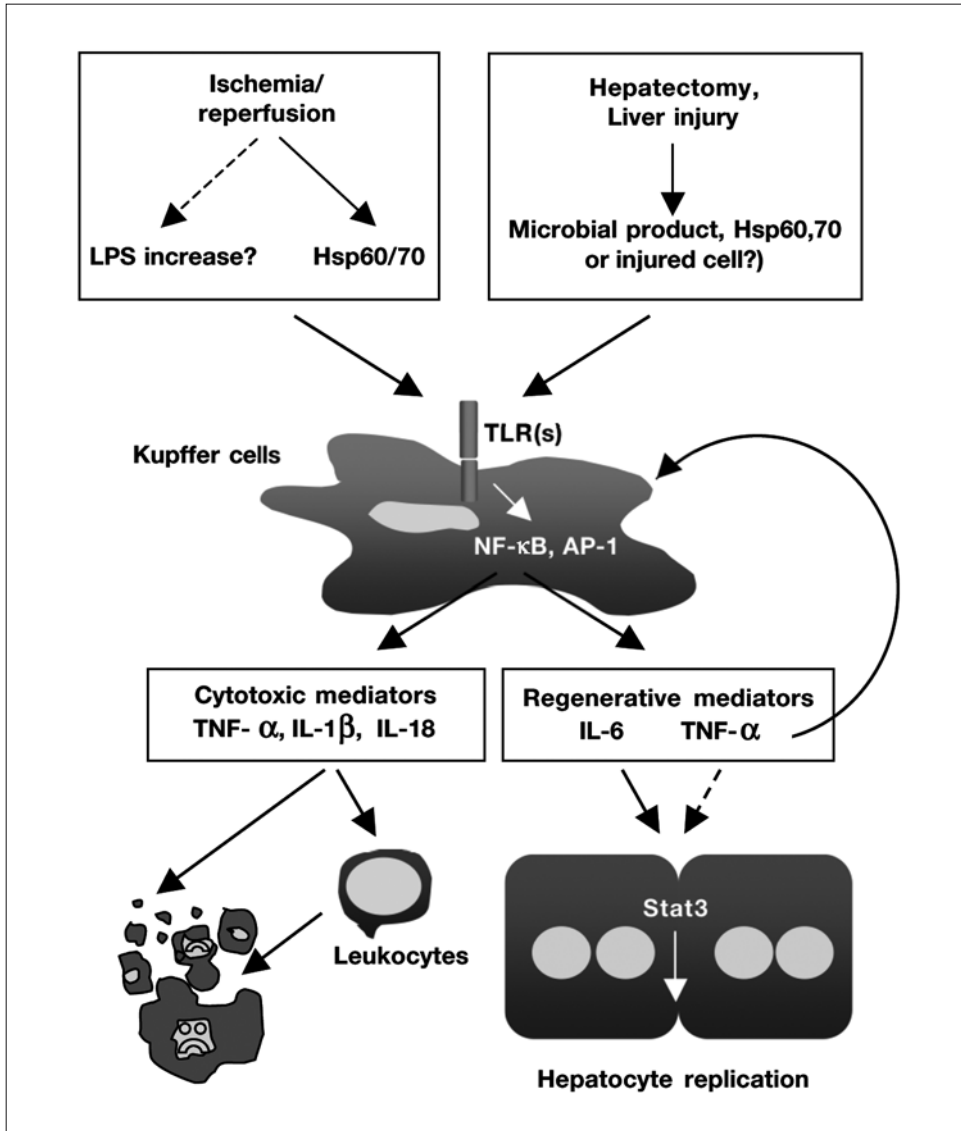


Figure 4

Dual role of toll-like receptor signaling in liver injury

After ischemia or partial hepatectomy, TLR ligands increase to activate Kupffer cells. In the case of liver regeneration, a modest amount of TLR stimulation induces NF-κB and AP-1 activation in the Kupffer cells which leads to IL-6 and TNF-α secretion and a regenerative hepatocyte response. Strong TLR stimulation, e.g., after ischemia-reperfusion injury, leads to the activation of cytotoxic mediators which directly and indirectly cause hepatocyte injury.

liver regeneration, but strong activation of TLR/MyD88 may also induce opposite signals and suppress liver regeneration.

Endotoxin-induced liver injury

Endotoxin-induced fulminant hepatitis is a common clinical complication during sepsis and accounts for a high percentage of sepsis-associated mortality. In comparison to humans, rodents are highly resistant to LPS. However, after treatment with heat-killed *P. acnes*, mice become highly susceptible to LPS challenge. *P. acnes* leads to a recruitment of macrophages and dendritic cells into the liver and a differentiation of hepatic T-helper lymphocytes into type 1 T-helper cells. The sensitization by *P. acnes* in the liver depends on IFN- γ , IL-12 and IL-18 [59–61]. TLRs are involved in both the *P. acnes*-priming phase and the LPS challenge phase. Indeed, hepatic granulomas, consisting of macrophages, dendritic cells and lymphocytes, were not found in MyD88-deficient mice after *P. acnes* administration and liver injury was blunted (Seki, unpublished data). *P. acnes* sensitizes towards TLR4-, but not TLR2 ligands, and upregulates the hepatic levels of TLR4 and its co-receptor MD-2 [62]. Whereas the *P. acnes*-induced upregulation of MD-2 seems to be at the level of mRNA, the increase of in TLR4 appears to be regulated at the posttranscriptional level [62]. This data suggests that the TLR4/MD-2/MyD88 signaling pathway is essential in endotoxin-induced liver injury.

TLRs and microbial infection in the liver

The liver is a target of a wide range of microbes including *Listeria monocytogenes* (*L. monocytogenes*), *Salmonella* and *Plasmodium* species. *L. monocytogenes* is a Gram-positive facultative intracellular bacterium which infects hepatocytes and Kupffer cells, leading to bacterial replication and the destruction of the host cell. *L. monocytogenes*-infected Kupffer cells produce proinflammatory cytokines, such as TNF- α and IL-12 via TLR2/MyD88-dependent signaling. In MyD88-deficient mice, proinflammatory cytokine production and *L. monocytogenes* clearance from host are almost abolished causing high mortality. In TLR2-deficient mice, the levels of proinflammatory cytokines are decreased, but *L. monocytogenes* clearance is almost identical to that observed in WT mice. The discrepancy in *L. monocytogenes* eradication between TLR2-deficient and Myd88-deficient mice suggests that TLR2 is involved in cytokine production in response to *L. monocytogenes*, but that the activation of multiple TLRs is required to achieve *L. monocytogenes* eradication [26].

Concerning *Salmonella* infection TLR4-mutated C3H/HeJ mice display an enhanced susceptibility to *S. typhimurium* [63, 64]. Functional TLR4 is required to upregulate TLR2 mRNA, downregulate TLR4 mRNA and initiate granuloma for-

mation in the liver [65]. Furthermore, C3H/HeJ mice show an impairment in their NO-dependent antimicrobial activity and display high levels of *S. typhimurium* in Kupffer cells [64]. *S. choleraesuis* infection induces liver injury through upregulation of Fas-ligand on NKT cells which is dependent on TLR2, but not TLR4 [66]. However, in this model of liver injury, eradication of *S. choleraesuis* does not depend on TLR2 and TLR4, suggesting that *Salmonella* infection may activate other TLRs in addition to TLR2 and TLR4.

Plasmodium berghei, a lethal strain of mouse malaria, induces stage-specific pathological changes in the host including asymptomatic changes during the liver stage and symptomatic changes during the erythrocyte stage, characterized by the presence of apoptotic and necrotic hepatocytes and dense infiltration of lymphocytes. Liver injury in this model is mediated by infiltrating lymphocytes in response to increased IL-12 production. In mice deficient in MyD88, IL-12 secretion and liver injury are completely blunted suggesting that the TLR-MyD88 pathway is an essential mediator of hepatic manifestations of malaria infection [67].

Conclusion

TLR-mediated innate immune responses are indispensable for the host defense against microbial infection. Through its anatomical links with the intestine the liver is the main target of gut-derived bacteria and bacterial products. The liver not only serves as first line of defense against bacteria and endotoxin, but also plays a major role in eliminating endotoxin from the body. Hepatic cell populations possess mechanisms that limit the response to bacterial products, such as the low expression of CD14 and TLR4, to prevent inflammation due to the constant exposure to gut-derived endotoxin. When this balance is disrupted and endotoxin levels exceed threshold levels as seen during microbial infection, ethanol-induced loss of intestinal barrier function or loss of functional liver mass, TLRs on several hepatic cell populations mediate proinflammatory and pro-proliferative responses which are at the center of the pathophysiology of these diseases. TLRs also seem to be involved in the pathology of chronic HCV infection by downregulating the innate immune response in early phases and inducing inflammation in later stages of the disease. There is still a fundamental lack of knowledge about the functional expression of TLR and TLR adapter molecules in the liver under normal and pathological conditions. Future studies need to further investigate (1) the role of TLRs in hepatic fibrogenesis and (2) characterize the “good” signals induced by TLR, i.e., as enhanced immune response, protection from apoptosis and proliferation, *versus* the “bad” signals such as hepatotoxicity and chronic inflammation. Further understanding of the TLR signaling pathways in the liver will help to shape new concepts with TLRs and their downstream mediators as pharmacological targets in liver disease.

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Toll-like receptors as key sensors of viral infection

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Introduction

Since their initial discovery, an explosion of research carried out in the Toll-like receptor field has certainly established TLRs as the major sensory molecules of the innate immune system. The pivotal role these receptors play in detecting invading pathogens and initiating a host immune response to such foreign material is underlined by the fact that they are so widely conserved in mammals and insects. While the link between TLRs and the sensing of bacterial and fungal infection by host innate immune responses has been apparent since their initial discovery, an analogous association between TLRs and viruses has only come to light more recently. Nevertheless, a function for TLRs in sensing primary viral infection is now clear. In the same way that individual TLRs have been implicated in detecting distinct bacterial and fungal structural moieties, or pathogen associated molecular patterns (PAMPs) as they have become known, a similar picture is now emerging for the recognition of viral components by TLRs. A number of viral structural entities have been identified as targets for TLR recognition. Examples include viral single-stranded RNA which is sensed by both TLR7 and TLR8, double stranded RNA (TLR3), viral DNA (TLR9), as well as a number of specific viral structural proteins which have been shown to activate signal transduction through TLR2 and TLR4. The signalling pathways induced by different TLRs are determined by their adaptor molecule usage and as such myeloid differentiation factor 88 (MyD88) and TIR domain-containing adaptor inducing IFN- β (TRIF) represent two key adaptors which transduce signals on activation of TLRs in response to viral infection (see below).

The cascade of events which occurs following engagement of TLRs by viral components may be considered two-fold. Firstly, the ensuing signalling events culminate in the activation of an inflammatory response characterised by the production of a wide range of proinflammatory cytokines and chemokines such as TNF, IL-6, IL-8 and RANTES. Crucially, the activation of TLRs by viruses results in the production and release of type I interferons (IFN- α/β). These antiviral cytokines function specifically to inhibit viral replication and *de novo* synthesis of viral proteins, for exam-

ple, through the activation of PKR and 2'-5' oligoadenylate synthase. Thus, TLRs instigate an initial rapid response to viral infection in an attempt to limit viral spread within the host. A second critical function of TLR activation by viruses is the priming of the adaptive arm of the host immune response. This is not entirely separate from the IFN-inducing function of TLRs as the potent activation of cytotoxic T lymphocytes (CTLs) seen on triggering TLR signalling has been shown to be dependent on IFN- α/β at least for TLR3, 4, 7 and 9 [1]. CTL are potentially the most important protective component of host immunity to an array of viruses. Thus, the significance of TLRs in preventing virus-induced disease may lie in this cross-talk between the innate immune response and antigen-specific adaptive immunity.

Whether the outcome of the complex interplay between TLRs and viruses should favour the virus or the host is not always self-evident. TLR-mediated inflammation can, potentially, induce an antiviral state, thus preventing infection of target cells and subsequent viral spread within the host. This could then, of course, help to limit the horizontal transmission of the virus. On the other hand, it would seem that, in some cases, the severe inflammation occurring as a result of TLR activation by viral infection can contribute to the pathogenesis and severity of virus-induced disease. Teasing out these complex interactions between TLRs and viruses will potentially open up a myriad of therapeutic strategies for treating virus-associated and other diseases. These could range from the use of adjuvants to prime TLR-induced inflammatory responses, to the targeted inhibition of TLR-mediated inflammation in the case of some viral infections and auto-immune diseases.

Which TLRs play a specific role in 'sensing' viral PAMPs?

As indicated above, TLRs have a distinct and vital part to play in host immunity to viral infection and there is now a wealth of evidence supporting this. Individual TLRs have been specifically implicated in the cellular activation seen upon viral infection. Furthermore, TLRs have been shown to transduce signals which lead to the production of IFN- α/β and the subsequent establishment of an antiviral state. Finally, and potentially most indicative of an essential role for TLRs in mediating antiviral immunity, some viruses have evolved specific immuno-evasive strategies targeting these innate immune receptors. Lessons learned from viral immunomodulatory mechanisms targeting other branches of host antiviral immunity would suggest that the acquisition of specific machinery to inhibit TLR signalling accentuates the importance of TLR-mediated innate immunity in response to viral infection.

TLRs may be considered to 'sense' viral PAMPs in that they respond to viral infection by inducing signalling cascades which result in the activation of cellular transcription factors and gene expression and ultimately a powerful inflammatory response. Whether or not the interaction between a particular virus and a TLR facilitates host innate immunity or serves to favour the virus probably differs from virus

Table 1 - Sensing of viral PAMPs by TLRs. See text for details and references.

TLR	Species	Viral PAMP	Virus
TLR2	Human	Virion protein?	CMV
	Human and mouse	HA	Measles
	Mouse	Unknown	HSV-1
	Human	Core, NS3 proteins	HCV
TLR3	Human, Mouse	dsRNA	Most viruses
TLR4	Mouse	F protein	RSV
	Mouse	Env protein	MMTV
	Mouse	Env protein	Moloney murine leukemia virus
	Human	Virion component?	Coxsackievirus B4
TLR7	Mouse	ssRNA	VSV, Influenza, HIV-1
TLR8	Human	ssRNA	VSV, influenza, HIV-1
TLR9	Mouse	DNA	HSV-1, HSV-2, mCMV

to virus. Thus, it may be that some viruses actively trigger TLRs to create a cellular environment which promotes viral replication and enhances infectious virion production.

Very little is known about how viral PAMPs interact with TLRs. Nevertheless, Table 1 illustrates the range of TLRs that have been shown to sense viral components and activate downstream signalling pathways in response to viral infection. The following paragraphs describe the known virus/TLR interactions identified to date. No doubt this list will expand considerably over the coming years.

TLR4

The first indication of a viral PAMP being recognised by a TLR was the case of the fusion (F) protein of respiratory syncytial virus (RSV) and TLR4 [2]. F protein stimulated secretion of IL-6 from wild-type monocytes, but not cells isolated from CD14 knockout mice, C3H/HeJ mice (which have a non-functional TLR4) or C57BL10/ScCr mice (in which the gene encoding TLR4 is deleted). Furthermore, RSV replicated to a higher titre and persisted longer in the C57BL10/ScCr mice compared to control mice suggesting that TLR4 is important for clearing RSV infection. A subsequent study showed that C57BL10/ScCr mice challenged with RSV exhibited impaired natural killer cell and CD14⁺ cell pulmonary trafficking, deficient natural killer cell function, impaired IL-12 expression and impaired virus clearance compared to normal mice [3]. One concern in these studies is that the

C57BL10/ScCr mice are also reported to have defects in the gene encoding IL-12R β 2 [4], and hence some of the defects could be attributed to this. However, Haerberle et al. have shown that NF- κ B activation in response to RSV in the lung is impaired in C3H/HeJ mice [5], providing more direct evidence for a role for TLR4. Also, RSV infection resulted in increased expression of functional TLR4 in airway epithelial cells [6]. Interestingly, despite structural similarities between the F proteins of RSV and Sendai virus (SeV), TLR4 is not involved in host defence against respiratory tract infection by SeV [7].

RSV infection has, in fact, been associated with enhanced cell surface expression of TLR4 on peripheral blood lymphocytes and monocytes [8]. Acute RSV infection resulted in the upregulation of surface TLR4 expression on monocytes isolated from a sub-population of infants with RSV bronchiolitis. This returned to control levels 6–8 weeks post-infection. TNF- α production in these infants was normal compared to control levels. In contrast, an inverse correlation was seen between surface TLR4 expression and minimal oxygen saturation suggesting a possible link between TLR4 expression and disease severity. The impact of this modulation of TLR4 expression during RSV infection on the efficacy of the consequent immune response to RSV remains to be determined. There is some evidence to suggest that TLR4 is not essential for the induction of an effective immune response to murine RSV infection [9]. However, the recently established link between a number of common TLR4 mutations and the severity of RSV disease strongly suggests that TLR4 expression does indeed influence the outcome of the immune response to RSV infection in humans [10]. In this study, there was no association between severe RSV disease and the CD14 polymorphism investigated.

Coxsackievirus, a member of the *Picornavirus* genus of enteroviruses, is also sensed by TLR4 [11]. Coxsackievirus B4 (CBV4) has long been known to be associated with insulin-dependent diabetes mellitus (IDDM). Infection with this virus has been shown to induce secretion of proinflammatory cytokines such as IL-1 β and TNF- α and it is thought that this inflammation may be responsible for the destruction of insulin-producing cells and, hence, the pathogenesis of IDDM seen following CBV4 infection. This cytokine production has been specifically attributed to TLR4 expression. As TLR4-reactive antibodies specifically blocked the induction of IL-6 production by both untreated and UV-inactivated CBV4, a direct interaction between TLR4 and a component of the CBV4 virion is likely to mediate this response. Critically, anti-TLR4 antibodies did not inhibit overall binding of virus to the surface of target cells and had no effect on virus infectivity as determined by plaque assays. Thus, TLR4 appears to play a central role in sensing CBV4 and mounting an inflammatory response to this virus but is not implicated in any stage of viral entry or the infection cycle of the virus. The role of TLR4 in responding to enterovirus infection is emphasised by the finding that enteroviral replication is associated with TLR4 expression in the myocardium of patients suffering from dilated cardiomyopathy [12].

TLR2

Cellular activation seen on infection with human cytomegalovirus (HCMV) has been found to be dependent on TLR2 expression. In cells lacking TLR2 or CD14 (a co-receptor for TLR2 and TLR4), UV-inactivated HCMV virions could no longer activate NF- κ B or induce IL-6 and IL-8 [13]. It has also been proposed that mouse mammary tumour virus (MMTV) can utilize TLR2 to activate cells [14]. Use of TLR2- and MyD88-deficient mice has further demonstrated a role for TLR2 signalling in mounting a proinflammatory response to lymphocytic choriomeningitis virus (LCMV) [15]. Reduced expression of IL-6 and monocyte chemotactic protein-1 was observed in both TLR2-null and MyD88-null mice following infection with LCMV. Conversely, while induction of type I IFN in response to LCMV was dependent on TLR2 expression, it was found to be MyD88-independent. Despite this, MyD88-deficient mice failed to induce an efficient CD8⁺ CTL response to the virus and defective MyD88 expression was associated with reduced viral clearance. Surprisingly, TLR2-null mice developed a CD8⁺ CTL response comparable to the wild-type response and cleared viral infection normally. Together, these data suggest that while TLR2 plays a critical role in the proinflammatory response to LCMV, it is the CD8⁺ CTL response that is crucial for effective clearance of the virus and that MyD88 plays an essential role in priming this CTL response (see section below).

The core and non-structural 3 (NS3) proteins of Hepatitis C virus (HCV) can both induce inflammatory responses in monocytes and TLR2 has now been identified as the host receptor involved in mediating this inflammatory response [16]. An absolute requirement for MyD88 expression was similarly demonstrated adding further weight to the argument for a role for TLR2-induced signalling pathways in detecting HCV infection. Furthermore, activation of IRAK and the MAP kinases p38, ERK and JNK, as well as the key transcription factors, AP-1 and NF- κ B, by both the core protein and NS3 protein was seen. A direct interaction between TLR2 and these HCV proteins has not yet been verified. Nevertheless, it seems likely that TLR2 plays a key role in sensing this viral pathogen through binding of one or both of these viral PAMPs. Crucially, internalisation of core and NS3 proteins did not require intact TLR2 expression, indicating that HCV does not use TLR2 to infect its target host cell.

TLR2 has also been implicated in the host immune response to primary herpes simplex virus (HSV)-1 infection. Cells stably expressing TLR2 were shown to activate NF- κ B upon infection with HSV-1 while TLR3, TLR4 and TLR9-expressing cells showed no response when exposed to this virus [17]. Similarly, cells isolated from mice lacking TLR2 demonstrated much reduced IL-6 cytokine release upon HSV-1 challenge. Notably, in the absence of TLR6 expression, IL-6 levels released were comparable to wild-type cells inferring that TLR6 does not play a role in the host response to HSV-1. This is of particular interest in the context of TLR2 signalling as this receptor is believed to function as a heterodimer with either TLR1 or

TLR6. Any role for TLR1 in sensing HSV-1 has yet to be established. Absence of TLR2 was associated with reduced brain inflammation and enhanced survival suggesting that TLR2 contributes to the severe encephalitis induced in mice challenged with HSV-1. Knocking out TLR2 expression did not reduce virus titres in the brains of infected mice indicating that TLR2 is not used by HSV-1 to gain entry to the brain.

TLR3

Most viruses will produce double-stranded (ds) RNA at some stage during their life cycle and so dsRNA may be considered as a major marker of viral infection. The IFN-inducible dsRNA-dependent protein kinase (PKR) has long been recognised as an intracellular receptor for dsRNA, inducing an antiviral state upon detection of both native and synthetic dsRNAs, including poly(I:C), a widely-used analogue of viral dsRNA. Nevertheless, the fact that cells isolated from mice deficient in PKR expression could still respond to poly(I:C) suggested that other cellular sensors of dsRNA existed. Alexopoulou et al. showed that TLR3 could activate NF- κ B and stimulate the production of type I IFNs in response to poly(I:C) [18]. Knocking out TLR3 expression resulted in a much attenuated inflammatory response to poly(I:C) treatment and significantly protected mice from poly(I:C)-induced shock. Crucially, cellular activation induced by native viral genomic dsRNA purified from a mammalian retrovirus was impaired in TLR3-deficient cells. Despite being the first TLR to be implicated in sensing a viral nucleic acid PAMP, demonstrating a role for TLR3 in the induction of an antiviral response to natural viral infection has proved difficult. Nevertheless, recent reports point to a significant role for TLR3 as an innate immune receptor inducing a potent inflammatory response to viral infection.

Using *Tlr3*^{-/-} mice, Tabeta et al. have now shown that TLR3 contributes to murine CMV-induced type I IFN production and to overall protection against the virus [19]. TLR3 has very recently been implicated in the response to both influenza A virus [20] and RSV [21]. Infection of bronchial epithelial cells with influenza A led to the simultaneous upregulation of TLR3 protein expression, a modulation that was similar to that seen with poly(I:C) stimulation [20]. Infection also led to a substantial increase in IL-8 and RANTES secretion. IL-8 induction was dependent on p38, ERK and PI3K while RANTES production was only dependent on p38 and PI3K demonstrating the induction of divergent signalling pathways by influenza A through TLR3 to stimulate these cytokines. A role for TRIF in NF- κ B activation by influenza A was established while MyD88 was found to be redundant, confirming that TLR3 is likely involved in mediating an immune response to this virus.

Combined use of cell lines stably expressing TLR3 and siRNA blockade of TLR3 protein expression have defined a role for TLR3 in the production of chemokines in response to RSV infection [21]. Interestingly, in this study introducing TLR3 into cells or downregulating TLR3 expression did not effect RSV replication in any way.

However, whether the same holds true *in vivo* remains to be determined. RSV appears to trigger both TLR3 and TLR4-dependent responses so it would be of major interest to assess the influence of both these inflammatory pathways on RSV infection in parallel *in vivo* studies. Targeting either or both of these TLR signalling pathways may be a useful therapeutic approach in the treatment of RSV-associated pulmonary disease.

The most compelling evidence for a distinct role for TLR3 in mounting an effective response to viral infection is provided by the significant protective effect induced by TLR3 in response to HSV-2 infection [22]. Ashkar and colleagues found that prior stimulation of TLR3 by treatment with poly(I:C) resulted in 100% survival of mice challenged intravaginally with a lethal dose of HSV-2. Conversely, administering LPS prior to virus challenge had only a very slight positive effect on host survival while CpG treatment protected the mice from virus infection similarly to poly(I:C). Absolutely no release of virus was observed in mice pretreated with TLR3 and TLR9 agonists indicating that both TLR3- and TLR9-induced responses completely blocked HSV-2 infection. The fact that TLR3 could confer a much more potent protective effect against HSV-2 than TLR4 is in keeping with the observation that TLR3 could induce antiviral genes to a much stronger extent than TLR4 and that this antiviral response was much more prolonged in response to TLR3 compared to TLR4 [23].

TLR9

TLR9 was originally shown to be activated by bacterial DNA sequences containing unmethylated CpG dinucleotides [24]. These motifs are also found in abundance in some viral genomes, such as HSV. Both HSV-1 and HSV-2 have been shown to activate plasmacytoid dendritic cells (pDCs) to produce type I IFNs through TLR9 [25, 26]. In addition, purified HSV-2 DNA was capable of inducing IFN- α from pDCs. In TLR9^{-/-} mice injected with HSV-2 no IFN- α was detected [25], although mice lacking either TLR9 or MyD88 were capable of controlling HSV-1 replication after local infection, suggesting that TLR9- and MyD88-independent pathways in cells other than pDCs can effectively compensate for defective responses to HSV-1 [26]. The importance of a MyD88-independent route to type I IFN induction in protection against virus infection is becoming apparent [27] and may explain this paradox (see section below). Recognition of HSV-2 by pDCs did not require virus replication and was through an endocytic pathway that was inhibited by chloroquine or bafilomycin A. This is consistent with the fact that TLR9 is located in and signals from intracellular endosomal compartments [28, 29].

Although the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) cannot replicate in mammalian cells, there are numerous lines of evidence suggesting that it can confer protection against infection with other viruses [30, 31].

TLR9 is now believed to mount an inflammatory response to this virus as cytokine production in immune cells isolated from both TLR9- and MyD88-deficient mice was much reduced compared to wild-type cells challenged with AcNPV [32]. The importance of this TLR9-induced innate immune response lies in the potential of utilising AcNPV as an adjuvant to prime immune responses directed against other infectious agents.

TLR7 and TLR8

TLR7 and 8 are the most recent family members to be implicated in responding to viral PAMPs. Originally, two imidazoquinoline compounds termed imiquimod and resiquimod (or R-848), that were known to have potent antiviral properties, were shown to activate murine macrophages in a MyD88- and TLR7-dependent manner [33]. It was subsequently shown that human TLR7 or TLR8, but not murine TLR8, could also confer cells with responsiveness to R-848 [34]. Several guanosine analogues were then shown to mediate cellular activation via TLR7, in a process requiring endosomal maturation [35]. These initial studies on TLR7 and TLR8, together with the observation that G- and U- rich ssRNA oligonucleotides derived from HIV-1 stimulate DCs and macrophages to secrete IFN- α and proinflammatory cytokines, led Heil et al. to show that recognition of GU-rich ssRNA was mediated by human TLR8 and murine TLR7 [36]. In addition, Diebold et al. showed that the production of large amounts of IFN- α by pDCs in response to wild-type influenza virus required endosomal recognition of influenza genomic RNA and signalling via murine TLR7 and MyD88 [37]. Further, this study showed that ssRNA molecules of non-viral origin (such as polyU) also induced TLR7-dependent production of inflammatory cytokines. In corroboration with these studies, Lund et al. demonstrated that as well as recognising influenza, TLR7 was required for pDC and B cell responses to another ssRNA virus, vesicular stomatitis virus (VSV) [38].

Together, TLR7, 8 and 9 form a functional subgroup within the TLR family that recognises viral PAMPs in endosomal or lysosomal compartments [36]. Thus, these TLRs are likely to be important for the detection of viruses which gain entry into the cell via endocytosis. For example, an ssRNA virus would reach the endosome through receptor-mediated uptake of a viral particle. There is evidence accumulating that, like TLR3 [39], these TLRs can respond to ‘self’ nucleic acid, which has been found to be immunostimulatory and may act as a ‘danger signal’, depending on its compartmentalisation [36, 37]. Hence, it may be more correct to think of TLR7, 8 and 9 as detecting the abnormal localisation of nucleic acid rather than structures or motifs absent from the host [37]. Overall it appears that cell surface TLR2 and TLR4 may recognise viral glycoproteins present on virions, while intracellular TLR3, TLR7, TLR8 and TLR9 may detect naked viral nucleic acid. Hence,

there is ample evidence that TLRs have an important role in viral PAMP-induced gene induction which leads to activation of host innate immune responses.

Other cellular sensors of viral dsRNA

As mentioned above dsRNA is a major marker of viral infection. Consequently, the detection of dsRNA is a critical function of innate immunity. The function of TLR3 may, however, be somewhat limited by its subcellular localisation. It may be simplest to consider TLR3 (like TLR7, 8 and 9) as having a role in sensing viruses that achieve entry into the host cell by endocytosis and so may come in contact with TLR3 in endosomal compartments. Naturally, this is not true for all viruses. A large number of viruses replicate in the cytoplasm of host cells and so generate vast quantities of dsRNA in the cytoplasm during their replicative cycle. Intuitively, other intracellular sensors of viral dsRNA are being identified and it is becoming clear that their importance in initiating an antiviral response is paramount.

One such receptor is retinoic acid inducible gene I (RIG-I), a DExD/H box RNA helicase molecule containing tandem caspase recruitment domains (CARD). Full-length RIG-I protein and a truncated form, comprising the CARD motifs alone (denoted Δ RIG-I), hugely enhanced transcription from an IRF regulatory sequence and the native IFN- β promoter in response to poly(I:C) transfection and infection with Newcastle disease virus (NDV) [40]. This study elegantly demonstrated the antiviral action of RIG-I using siRNA to specifically knock-down RIG-I expression. While NDV infection was associated with a clear induction of IRF-3 dimerisation in cells stably transfected with a control siRNA sequence, targeted siRNA knock-down of RIG-I expression specifically inhibited this activation. Furthermore, IFN- α , IFN- β , IP10 and RANTES gene induction in response to NDV infection was completely blocked in cells where RIG-I protein expression was impaired. In this study, experiments showing that full-length RIG-I could substantially impede the formation of any visible cytopathic effect on infection with both vesicular stomatitis virus (VSV) and encephalomyocarditis virus (EMCV) emphatically underlie the importance of RIG-I in controlling virus infection. Thus, RIG-I would appear to play a central role in establishing an antiviral state and preventing the spread of invading viruses.

The RNA-binding activity of RIG-I has been mapped to its helicase domain, which has intrinsic ATPase activity. Critically, the RIG-I helicase domain bound the HCV 5' and 3' nontranslated regions [41]. Thus, the interaction of RIG-I with these highly structured regions of the HCV genome represents an affinity of RIG-I for a natural dsRNA-like viral PAMP. Unstructured single-stranded RNA from HCV was not recognised by RIG-I, confirming the specificity of RIG-I for dsRNA. Melanoma differentiation associated gene-5 (Mda-5), a closely related CARD domain containing DExD/H box RNA helicase, similarly enhanced IRF-3 activation in response to

both poly(I:C) and NDV infection [40]. Interestingly, V proteins encoded by a range of paramyxoviruses, including SeV, human parainfluenza virus 5 and mumps virus, could antagonise the induction of type I IFNs by mda-5 [42].

As mentioned above PKR is a long-standing intracellular receptor for dsRNA. Direct binding of dsRNA induces PKR dimerisation and the resulting activation of PKR via auto-phosphorylation allows this kinase to catalyse the phosphorylation of its primary target, eukaryotic initiation factor (eIF) 2 α [43]. PKR is central to the induction of an antiviral state by type I IFNs, blocking the translation of viral mRNAs and hence, preventing *de novo* synthesis of viral proteins. PKR can also signal to activate NF- κ B and MAP kinases [44, 45] but does not appear to regulate IRF-3 activation [46]. Furthermore, signalling through TLR3, TLR4 and TLR9 appears to induce autophosphorylation of PKR [47, 48]. Thus, the antiviral functions of PKR are many fold.

In summary, although TLR3 does appear to function as an antiviral innate immune receptor and can mount a strong inflammatory response on detection of viral dsRNA, it may be that other cytoplasmic pattern recognition receptors, such as RIG-I, are more suitably located to act as major sensors of dsRNA released into the cytoplasm during viral infection. The cell type-specific subcellular localisation of TLR3 might reflect a divergence in TLR3 function in different tissue types. Human fibroblasts and epithelial cells display both a cell surface and an intracellular pattern of TLR3 expression while monocyte-derived immature dendritic cells (iDCs) and CD11c⁺ blood DCs express TLR3 solely in intracellular vesicles [49]. Thus, it may be that, where TLR3 is exposed at the cell surface, it can serve to detect the presence of dsRNA of both cellular and viral origin released upon lysis of virally infected cells. This is supported by the fact that anti-TLR3 antibodies could block activation of fibroblasts in response to poly(I:C) treatment [50], indicating that this is a cell surface event. The observation that TLR3-mediated IFN induction by DCs was inhibited by chloroquine or bafilomycin strongly implies that this signalling cascade is initiated in endosomal compartments [51]. While endosome-localised TLR3 may serve to alert host innate immunity to invading endocytic viruses, a growing body of evidence suggests a major role for TLR3 expressed within the endosomal compartments of DCs in activating the adaptive cellular immune response to viral infection.

Cross-priming of adaptive immunity to viral infection by TLRs

TLRs present an initial barrier to invading pathogens, alerting the host to their presence and mounting an initial rapid inflammatory response in a bid to curtail any further spread of the infectious agent. Of course, a major role of innate immunity is to prime the adaptive immune response to activate specific cell-mediated immunity to foreign antigens. Dendritic cells (DCs) are the frontline of the innate immune sys-

tem, providing a link between the innate and adaptive responses. DCs acquire viral antigens indirectly via the phagocytosis of virally infected cells. They can then present this foreign antigenic material in an MHC class I-restricted fashion to CD8⁺ CTL, thus priming naïve CTL to mount a specific cytotoxic response. Nevertheless, the cross-talk between DCs and this cellular arm of the adaptive immune response does not always result in CTL activation. The stimulatory signals required to allow DCs to prime CD8⁺ CTLs to mount a cytotoxic immune response, as opposed to simply inducing cross-tolerance (inactivation of CTLs) have remained ambiguous. However, it would now appear that signals induced via the engagement of TLR3 by dsRNA in DCs strongly enhance cross-priming of naïve CD8⁺ CTL while the absence of this TLR3-mediated signal transduction results in a much weaker CTL response [52]. This constitutes a completely novel role for TLR3 in host antiviral immunity.

Incubation of CD8 α ⁺ spleen DCs with cells transfected with poly(I:C) and subsequently exposed to UV light to initiate cell death resulted in DC activation and enhanced IFN- α , IFN- β , TNF- α and IL-6 transcription. Despite both empty cells and cells loaded with poly(I:C) being phagocytosed to a similar degree, exposure to cells alone in the absence of poly(I:C) had little or no effect on the levels of expression of the above genes. Culturing DCs with either Semliki Forest virus (SFV)- or EMCV-infected cells led to an even greater increase in IL-6 production than that seen for poly(I:C)-transfected cells. Poly(I:C)-facilitated DC activation occurred independently of PKR and MyD88 but an absolute requirement for TLR3 was identified for both poly(I:C)- and virus-induced effects. Exposure to virally infected cells or cells carrying poly(I:C) substantially augmented CTL responses to ovalbumin antigen *in vivo*. This CTL cross-priming was also completely dependent on intact TLR3 expression.

A somewhat conflicting study by Chen et al. reported that MyD88 was in fact necessary for optimal cross-priming of CTL responses by DCs [53]. Both wild-type and MyD88-deficient DCs directly infected with a SFV replicon encoding ovalbumin efficiently processed and presented the OVA antigen and stimulated IFN- γ release by T cells to a similar extent when pure populations of infected DCs were used as the stimulus, hence eliminating any contribution of cross-presentation by uninfected DCs. Where mixed populations of uninfected and infected DCs were used to activate CD8⁺ CTLs, DCs lacking MyD88 did not appear to stimulate as effectively as wild-type cells. Likewise, MyD88-null DCs co-cultured with SFV-infected fibroblasts demonstrated impaired IL-12 production compared to wild-type cells. This impaired IL-12 production in MyD88 knockout cells corresponded to severely reduced IFN- γ and IL-2 release from target CTLs. Schulz et al. only looked at cross-priming by MyD88-deficient DCs in the context of poly(I:C) [52]. While one might expect signal transduction induced by poly(I:C) to mirror that of native viral dsRNA this is not always the case. Indeed, TLR3-deficient mice could still mount low level CTL responses when primed with cells infected with ovalbumin

antigen-carrying SFV. This suggests that other TLR3-independent signals can similarly facilitate cross-priming of CD8⁺ CTL by DCs. Poly(I:C) has been implicated in the induction of CD8⁺ CTL responses to specific HIV antigens [54]. Whether or not this enhanced cross-priming facilitated by poly(I:C) is TLR3 dependent has not been demonstrated.

Viral subversion of innate immunity through TLRs

It is becoming increasingly apparent that viruses can harness TLR-induced signalling pathways and taper them to their own merit, thus using host responses to facilitate efficient viral replication (see Fig. 1). This adds further complexity to the relationship between TLRs and viruses and the importance of the balance of the ensuing response which can be tipped in favour of the host or the virus depending on the interaction in question.

TLR4 has been shown to be important for murine retrovirus activation of B cells: MMTV was unable to induce B cell activation markers or NF- κ B from C3H/HeJ mice [55]. Additionally, the envelope (Env) proteins from both MMTV and Moloney murine leukaemia virus could be co-immunoprecipitated with TLR4. MMTV binding to TLR4 also induced maturation of bone marrow-derived dendritic cells [56]. MMTV-induced TLR4 signalling helped to sustain infection and, furthermore, wild-type virus was eliminated by the cytotoxic immune response in C3H/HeJ mice [57]. Thus, MMTV activation of TLR4 may serve to potentiate virus replication and, as such, may represent a subversion strategy.

The haemagglutinin (HA) protein of wild-type, but not vaccine strains of measles virus activates murine and human cells via TLR2, leading to induction of proinflammatory cytokines such as IL-6 in human monocytic cells, and upregulation of surface expression of CD150, the receptor for measles virus [58]. Hence, activation of TLR2-dependent signalling by wild-type measles virus is likely to contribute to both immune activation and viral spread and pathogenicity. Thus, the loss of TLR2-activating capability may be considered as an attenuation marker.

It has long been recognised that contraction of a mycobacterial infection potentially leads to accelerated progression of AIDS in HIV-positive individuals. In recent years, it has come to light that there may be a role for TLRs in this synergistic exacerbation of HIV-1 infection. Báfica and colleagues demonstrated that while exposure to individual mycobacterial cell wall components and the live mycobacteria, *M. tuberculosis* and *M. avium*, led to vast increases in HIV-1 p24 antigen secretion from wild-type spleen cells from HIV-1 transgenic (Tg) mice, this effect was not observed with spleen cells isolated from TLR2-deficient HIV-1 Tg mice [59, 60]. Expression of the HIV-1-encoded *env* and *gag* mRNAs was substantially elevated in TLR2^{-/-} Tg mice infected with *M. avium in vivo* when compared to levels seen in TLR2-normal Tg mice. Conversely, saline-treated or *Toxoplasma gondii*-treated

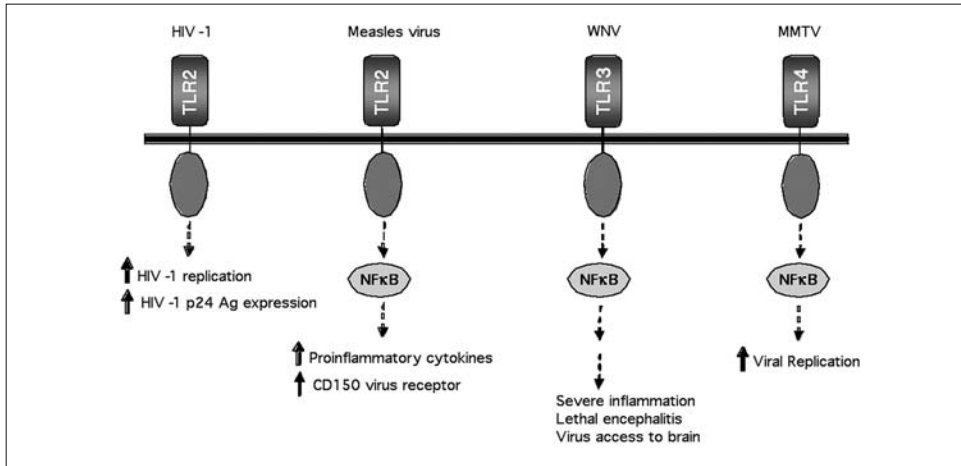


Figure 1

Viral subversion of the TLR response

A number of viruses have been found to utilise the activation of TLR signalling pathways to their own advantage. Thus, the interaction of some viruses with host TLRs favours the virus and results in enhanced viral replication and a more severe virus infection. HIV, human immunodeficiency virus; MMTV, mouse mammary tumour virus; WNV, West Nile virus

mice demonstrated no increase in HIV-1 gene expression in TLR2-deficient Tg mice compared to wild-type mice. TLR2 stimulation similarly led to increased HIV-long terminal repeat *trans*-activation and virus replication [61]. Co-transfection of human TLR2 cDNA was a prerequisite for the induction of a HIV-LTR-luc construct by soluble *Mycobacterium tuberculosis* factor (STF) and the *Staphylococcus epidermis*-encoded phenol-soluble modulin (PSM). Interestingly, in this study dual-stimulation of TLR2 and TLR4, with ligands STF and LPS respectively, resulted in synergistic *trans*-activation of HIV-1 LTR.

The observation that TLR2 expression is increased on the surface of monocytes isolated from HIV-positive individuals and that the HIV-1 envelope protein gp120 is sufficient to mediate upregulation of surface TLR2 expression on monocytes *in vitro* [62] certainly suggests an interplay between TLR2 and HIV during the course of virus infection. Moreover, the fact that TLR2 stimulation in HIV-infected patients resulted in enhanced viral replication and TNF- α production distinctly points to a role for TLR2 in chronic immune activation and regulation of viral replication during HIV infection [62]. Another angle to the story of the interplay between TLR2 and HIV-1 infection is that the protease inhibitors used to block HIV-1 aspartyl protease, similarly inhibited TLR2-mediated NF- κ B activation [63]. Thus, it seems that these antiretroviral drugs not only block HIV protease activity but also stem

pathogen-induced TLR activation. Consequently, they may provide a two-tiered mechanism of improving prognosis of HIV infection.

Recent studies defining a role for TLR3 in mounting an antiviral response have suggested that TLR3 may act as friend or foe to the host depending on the viral infection in question. An elegant study by Wang et al. revealed that TLR3 was not only involved in mounting an inflammatory response to West Nile virus (WNV) but also indirectly facilitated passage of this ssRNA flavivirus across the blood brain barrier [64]. TLR3-deficient mice demonstrated enhanced survival compared to wild-type mice when exposed to a lethal dose of WNV and this correlated to a reduced viral load and dampened inflammatory responses in the brains of these animals. While this study suggests that TLR3 can detect WNV infection and go on to induce a proinflammatory response, this appears to be detrimental to the host.

Antiviral signalling pathways activated by TLRs

The induction of type I IFNs (IFN- α , IFN- β , IFN- ω and IFN- τ) and the ensuing activation of IFN-stimulated gene (ISG) expression and activity is a major defense against viral infection. This upregulation of ISGs such as PKR, 2'-5' oligoadenylate synthase, IFNs themselves and IRFs leads to an array of antiviral, antiproliferative and immunoregulatory responses [65]. Although viral activation of IRFs is known to be important in IFN induction, and many viruses have been shown to trigger NF- κ B activation, it was unclear how exactly this virus-mediated transcription factor activation occurred. Therefore, the discovery and elucidation of TLR-mediated signalling pathways to both NF- κ B and IRFs, leading to type I IFN production, provides a crucial missing link in the understanding of exactly how viral infection leads to the IFN response. That TLR signalling can induce an antiviral state was clearly shown by Doyle et al. (2002) in that pretreatment of cells with poly(I:C) or lipid A (the moiety of LPS recognised by TLR4) inhibited the replication of a murine herpesvirus in macrophages [66].

Viral activation of NF- κ B through TLRs

Numerous viruses, including HIV, HTLV, hepatitis B virus, HCV, EBV and influenza virus have been shown to activate NF- κ B upon infection [67]. It is often unclear whether this represents viral appropriation of a cellular signalling pathway or a host antiviral response. Viruses have evolved many strategies to specifically block NF- κ B activation which suggests that triggering NF- κ B is often a host response to viral infection [68]. All TLRs studied to date have the ability to activate NF- κ B and the list of viruses known to activate NF- κ B in a TLR-specific manner on infection is likely to continue to expand.

The downstream signalling components induced by different TLRs along the pathway to NF- κ B activation is governed by the specific receptor-proximal Toll/IL-1 receptor resistance (TIR) domain-containing adaptor molecules utilised by each receptor. All TLRs, with the notable exception of TLR3, recruit the adaptor protein myeloid differentiation factor 88 (MyD88) via homophilic TIR/TIR domain interactions. A second TIR adaptor, MyD88 adaptor-like (Mal) or TIR domain containing adaptor protein (TIRAP), is used as a linker molecule by TLR2 and TLR4 to activate NF- κ B via MyD88. MyD88 is believed to form a complex with IL-1 receptor-associated kinase (IRAK) family members which then potentially go on to activate TRAF6. IRAK1 was believed to be central to this induction of TRAF6 by MyD88 but its role in the pathway from MyD88 to NF- κ B is now being questioned. It may be that IRAK1 is more important in the activation of IRFs through MyD88 [69, 70]. TRAF6 can activate TGF- β -activating kinase (TAK1) which goes on to induce the I κ B kinases IKK α and IKK β [71]. These kinases catalyse the phosphorylation of I κ B which targets it for polyubiquitination and subsequent proteasomal degradation allowing the translocation of NF- κ B into the nucleus where it can initiate the transcription of target genes [72].

TLR3 provides the only example of a TLR that does not signal to NF- κ B through MyD88 as activation of this transcription factor on treatment with poly(I:C) was normal in the absence of MyD88 [18]. Rather, another TIR adaptor, termed TIR domain-containing adaptor inducing IFN- β (TRIF), has been implicated in all downstream signalling cascades induced by TLR3 [73, 74]. TRIF binds directly to TLR3 and activates NF- κ B through its C-terminal domain [75] by recruiting the kinase receptor interacting protein 1 (RIP1) via a RIP homotypic interaction motif [76]. TRIF also contributes to TLR4-induced NF- κ B activation and hence, TLR4 can mediate both MyD88-dependent and MyD88-independent induction of NF- κ B. In the case of LPS signalling via TLR4, NF- κ B activation was delayed but not completely blocked in cells from MyD88-null mice [77]. This MyD88-independent late activation of NF- κ B occurs through the recruitment of TRIF [74]. TRIF is likely to associate indirectly with TLR4 via another linker TIR adaptor, TRIF-related adaptor molecule (TRAM), which is solely involved in TLR4 signalling [78, 79].

Viral activation of IRF-3 through TLRs

IRF-3 is one of the most important transcription factors involved in the induction of type I IFNs in the host response to viral infection. The fact that both TLR3 and TLR4 could stimulate the upregulation of IFN and IFN-dependent genes in MyD88-null mice pointed to a MyD88-independent mechanism of IFN activation by these receptors [18, 77]. Thus, much attention has focused on TRIF-mediated IFN induction and more specifically how TRIF leads to IRF-3 activation [73, 74]. Overexpression of TRIF, but not MyD88 or Mal, was shown to activate IRF-3 and induce

the IFN- β promoter and furthermore, IRF-3 could be co-immunoprecipitated with TRIF [80]. Consistent with this, a requirement for TRIF in LPS-induced IRF-3 activation has been demonstrated [81]. There are, however, key differences between TLR3- and TLR4-mediated IRF-3 activation, suggesting that TRIF function may differ in these two pathways. The TIR adaptor TRAM was shown to play a role in TLR4 but not in TLR3-induced IRF-3 activation [78, 81]. Moreover, poly(I:C) treatment led to phosphorylation of the critical C-terminal serine residue 396 (Ser-396) in IRF-3 while LPS was unable to induce phosphorylation at this site [82]. Thus, the exact mechanism of IRF-3 activation by TRIF on engagement of TLR3 and TLR4 remains to be elucidated. Furthermore, the discovery of a critical role for RIG-I in the activation of IRF-3 and type I IFNs in response to some viruses, including, SeV, NDV and HCV [40, 41] questions the relative role of TRIF-induced IRF-3 in the antiviral response. Nevertheless, given the wide number of viruses shown to stimulate TLR3 and TLR4-dependent signalling pathways and the existence of viral inhibitory mechanisms that specifically target TLR3 signalling, it remains likely that the regulation of IRF-3 by TRIF is a central part of the host innate immune response to some viruses.

IKK ϵ and TBK1 have been identified as two key kinases involved in the virus-mediated phosphorylation of IRF-3 [83, 84]. Both kinases are downstream of TRIF, further linking TLR pathways to viral induction of the IFN response. The essential role of TRIF in the virus-mediated activation of type I IFNs was confirmed using mice containing a mutation in the *Trif* locus (termed *Lps2*). When normal mice were infected with murine CMV, high levels of type I IFNs were detected in the serum, while for *Lps2* mice, IFN production in response to the virus was completely impaired [73]. In addition, macrophages from *Lps2* mice supported replication of vaccinia virus (VV) to a significantly higher titre than wild-type cells. Hence, TRIF was conferring a broad antiviral response, presumably through TLR-mediated signalling pathways.

Viral activation of IRF-7 through TLRs

Plasmacytoid DCs (pDCs) are the primary source of type I IFNs in the blood, and as such are specifically referred to as IFN-producing cells. These cells do not express TLR3 or TLR4 but express high levels of TLR7 and TLR9 and while wild-type pDCs demonstrated potent IFN- α production on infection with influenza virus and VSV, this cytokine release was grossly impaired in pDCs isolated from TLR7-deficient mice [37]. Similarly, pDCs from TLR9-null mice were unable to stimulate IFN- α release when infected with HSV-1 and HSV-2 [26]. How these TLRs could facilitate the induction of IFN in a MyD88-dependent manner posed a major question until very recently. Unlike IRF-3, IRF-7 expression is highly restricted. pDCs, however, express high levels and, in keeping with this, a pivotal role for IRF-7 in TLR7

and TLR9-mediated type I IFN production is emerging. IRF-7 was shown to bind to MyD88 and co-transfection with MyD88 led to a huge increase in IFN- α transcription [85]. IFN- α promoter activity was vastly reduced when IRF-7 and MyD88 were co-expressed in TRAF6-deficient MEFs compared to wild-type cells, implicating TRAF6 in this signalling complex. IRAK1 was found to interact with IRF-7 and catalyse its phosphorylation and the TLR7 and TLR9-mediated IFN- α response was almost completely ablated in IRAK1-deficient mice while IFN- α induction in response to poly(I:C) was normal [70].

IRF-7 appears to be essential for both MyD88-dependent and the more classical MyD88-independent pathways to IFN production and so a global role for IRF-7 as a major regulator of type I IFN induction has been unveiled [27]. The IFN response to ssRNA (VSV, EMCV) and dsDNA (HSV-1) viruses was almost completely abolished in IRF-7-deficient MEFs while MyD88 was not required. VSV and HSV-1-induced IFN in pDCs displayed a dual dependency on IRF-7 and MyD88. Importantly, the *in vivo* IFN- α response to HSV-1 and EMCV infection was completely abrogated in IRF-7-deficient mice. Interestingly, knocking out MyD88 expression only diminished IFN- α production slightly, suggesting that, although MyD88 plays a role in the IFN response to these viruses, the MyD88-independent pathway can compensate in its absence. IRF-7 on the other hand is absolutely essential for all type I IFN production in response to these viruses. A study by Honda et al. has shed some light on the question of how pDCs can induce type I IFN production through the MyD88/IRF-7-dependent pathway so efficiently. In pDCs, the CpG ligand for TLR9 was retained in endosomal compartments for longer periods than in other cells and so the rapid shuttling of CpG to lysosomes in conventional DCs may explain the lack of a robust IFN response in these cells [86].

Thus, it would appear that while both IRF-3 and IRF-7 are essential for the activation of type I IFNs in response to viral infection, IRF-7 plays a more global role in this function. IRF-3 is potentially more critical for TLR3 and TLR4-mediated IFN- β induction through TRIF. The specific importance of IRF-7 in the induction of an antiviral state is emphasised by the fact that Kaposi's sarcoma-associated herpesvirus (KSHV) targets this transcription factor for proteasomal degradation [87]. Notably, although IRF-5 is induced in response to TLR7 and TLR9 ligands along with IRF-7, it is redundant for the activation of antiviral IFNs. In contrast, IRF5 would appear to be a central regulator of proinflammatory cytokine expression on stimulation of a range of TLRs [88].

Targeted viral evasion of TLR-mediated immunity

Not only can viruses subvert TLR-induced responses to facilitate their replication but a number of viruses have now been shown to encode proteins which specifically target components of TLR signalling pathways to block TLR function. These

viral inhibitory strategies are illustrated in Figure 2 and provide us with a major clue as to the significance of TLRs in protecting the host from viral infection.

Vaccinia virus (VV) is a member of the *Poxviridae* family of large dsDNA viruses and like other poxviruses has acquired a vast array of immunomodulatory proteins which specifically target many different branches of host immunity [89]. Suitably, TLR induction of inflammatory responses is similarly targeted and to this end VV encodes two distinct proteins, namely A46R and A52R, which interfere with TLR signalling in distinct ways. Initially, A46R and A52R were individually transfected into mammalian cells and tested for their ability to block activation of an NF- κ B-regulated reporter gene. A46R was found to inhibit IL-1-induced NF- κ B activation which proceeds via MyD88, while A52R could antagonise NF- κ B activation stimulated by both IL-1 and TLR4 [90]. More recent work has shown that both A46R [91] and A52R [92] were capable of inhibiting signalling by other TLRs. A52R was particularly potent against TLR3-mediated NF- κ B activation and, moreover, it was found to specifically sequester the TLR signalling components IRAK2 and TRAF6. A virus deletion mutant lacking the A52R gene was attenuated compared to wild-type and revertant controls in a murine intranasal model of infection [92]. Hence, inhibition of TLR function by A52R is likely to be important for the suppression of host immunity by VV.

A detailed study has revealed that A46R is an extremely broad-acting immunoregulatory molecule which targets many of the TLRs proximal to the receptor end of the signalling cascade [91]. A46R was shown to associate directly with all the TIR adaptors which have known functions in TLR signalling, namely MyD88, Mal, TRAM and TRIF. A46R did not interact with TRAF2 or SARM, the fifth known cytosolic TIR domain-containing protein whose function has yet to be deduced, indicating the specificity of this viral protein for the TIR adaptors indicated above. It is of interest that A46R is expressed early in VV's replicative cycle as any TLR-induced IFN response is likely to be initiated rapidly following virus infection. Thus, to effectively inhibit such a response *in vivo* A46R expression would be required very early on. Crucially, a VV deletion mutant lacking the A46R gene showed an attenuated phenotype when administered to mice intranasally confirming that A46R does indeed contribute to viral virulence *in vivo*. Unlike A52R, A46R did not inhibit TLR3-dependent NF- κ B activation despite targeting TRIF [91]. The reason for this is unclear but it does highlight distinct differences between A46R and A52R. Similarly, A52R did not inhibit TRIF-dependent IRF-3 activation while A46R did. Thus, VV encodes two distinct antagonists of TLR function which target different components of TLR signalling, presumably, to ensure complete inhibition of TLR-mediated innate immunity.

Interestingly, the HIV-1 protein Vpu, a known NF- κ B inhibitor, specifically blocked Toll-mediated innate immune responses when expressed in *Drosophila* [93]. Given the conservation of the Toll and TLR signalling pathways in flies and mammals, Vpu may also target TLR-dependent host defences. In fact, now that it is

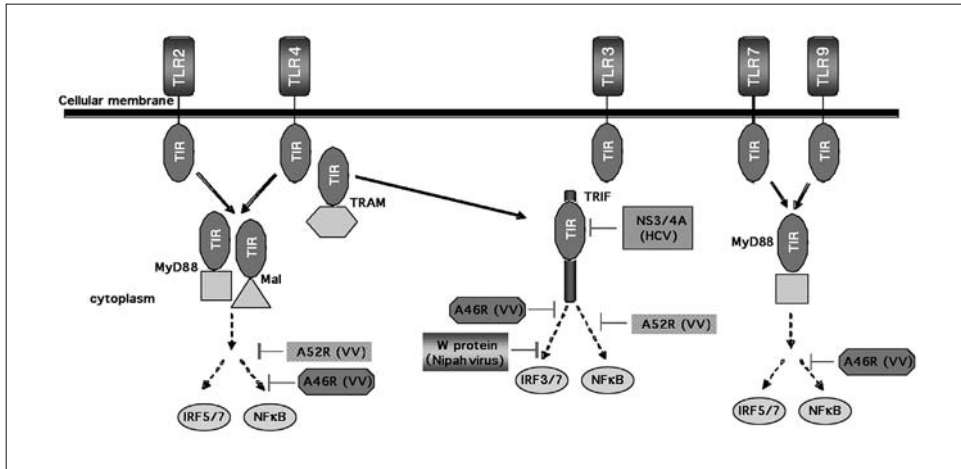


Figure 2

Evasion of TLR-mediated innate immunity by viruses

Some virally encoded proteins specifically target components of TLR signalling pathways to inhibit TLR-induced innate immune responses. VV encodes two such proteins (A46R and A52R) which block distinct arms of TLR-mediated signalling cascades. Thus, VV expresses two functionally independent proteins which inhibit TLRs by different mechanisms, emphasising the importance of TLR signalling in the host innate immune response to viral infection. HCV, Hepatitis C virus; VV, Vaccinia virus

known that TLRs have a role in viral NF- κ B induction, many of the already identified viral inhibitors of NF- κ B [94] may, in fact, be found to mediate their effects via targeted inhibition of TLR signalling. Likewise, the study of the many viral strategies that counteract the IFN system [65] will now have to take TLRs into account. For example, the poxvirus-encoded E3L protein, a known PKR and dsRNA antagonist, has been shown to inhibit IRF-3 activation in a PKR-independent manner [95]. So conceivably, E3L could also be targeting TLR-induced IRF-3 directly. The observation that the Nipah paramyxovirus-encoded W protein, a viral protein previously known to antagonise the IFN function, could specifically inhibit TRIF-induced IRF-3 phosphorylation illustrates this point [96].

HCV provides us with a further example of a viral immune evasion strategy evolved to specifically target TLR-mediated innate immunity. The HCV-encoded NS3/4A serine protease is known to inhibit viral activation of IRF-3, a function that is solely dependent on its protease activity [97]. Potential NS3/4A cleavage sites were identified within TLR3, TRIF, IKK ϵ and TBK1 but of all these only TRIF was specifically degraded [98]. Individually, the two polypeptides generated by proteolysis of TRIF were no longer able to activate transcription of the IFN- β promoter.

Furthermore, although the peptides were quite stable in extracellular *in vitro* cleavage assays, no by-products of TRIF cleavage were observed in cell culture based assays, indicating that they are rapidly degraded following cleavage by NS3/4A. Overexpression of NS3/4A dramatically reduced both poly(I:C) and SeV-induced IFN- β promoter activity as well as poly(I:C)-stimulated activation of the NF- κ B-dependent PRDII promoter. Similarly, upregulation of two IRF-3-dependent genes, ISG56 and ISG15, in response to poly(I:C) stimulation was delayed in the presence of NS3/4A. Thus, by targeting TRIF, a receptor-proximal component of TLR3 signalling, HCV can broadly inhibit both NF- κ B and IRF-3-mediated signals.

Like so many other virally encoded immunomodulatory proteins, NS3/4A employs a multi-faceted approach to inhibit host innate immunity. While TLR3 delivers a transmembrane signal alerting the cell to the presence of viral dsRNA, RIG-I is now deemed a major cytoplasmic radar for this viral PAMP. Intriguingly, HCV NS3/4A targets both of these branches of host innate immunity. NS3/4A completely abolished the nuclear translocation of IRF-3 induced by overexpression of the RIG-I CARD motifs along with RIG-I-mediated IRF-3 dimerisation and phosphorylation at serine-386 [99]. Given that RIG-I can detect HCV genomes directly [41], it makes sense that this virus would develop an evasion strategy to inhibit this arm of the host innate immune response. In support of this, Foy et al. demonstrated an absolute requirement for RIG-I in the induction of an interferon response to HCV dsRNA and that RIG-I could in fact repress replication of an HCV replicon [99].

Attempts to pinpoint which particular component of the RIG-I signalling cascade is specifically cleaved by NS3/4A have thus far been unsuccessful [99]. The TBK1 and IKK ϵ protein kinases are both believed to be involved in signal transduction from RIG-I to IRF-3. However, as indicated above, neither of these molecules act as proteolytic substrates for this virally encoded protease [98, 99]. It would seem potentially very appealing for the virus to target RIG-I directly as a strategy to prevent activation of an antiviral response. Nevertheless, overexpression of NS3/4A had no demonstrable effect on cellular protein levels of RIG-I [99]. It is therefore conceivable that the NS3/4A serine protease degrades an as yet unidentified downstream mediator of RIG-I signalling. Use of this viral antagonist of TLR function could provide a useful tool to further tease out the molecular intricacies of RIG-I signalling.

Future perspectives

A growing body of evidence suggests that TLRs do indeed have a significant part to play in antiviral host defence. Despite a huge surge in research into the multifaceted interactions between TLRs and viruses in recent years, it would appear that we are only scratching the surface of this complex area. Further research is required to elu-

cidate the precise role of TLRs in host immune responses to viral infection *in vivo* and moreover, to define the outcomes of the many complicated relationships between TLRs and viruses.

The exact role of TLRs in viral disease progression needs to be elucidated. The innate immune response to mCMV has been shown to involve both TLR3 and TLR9 [19]. Both the TLR3/TRIF and the TLR9/MyD88 pathways led to the production of type I IFN. However, mice lacking TLR9 died quickly after infection, while the absence of TLR3 did not significantly reduce survival. In another study, the absence of TLR3 did not alter viral pathogenesis or impair the generation of adaptive antiviral responses in infections with either VSV, LCMV, mCMV or reovirus [100]. TLR3-induced inflammatory responses to WNV infection contributed to viral compromising of the blood brain barrier and in doing so greatly exacerbated inflammation and encephalitis in the brain [64]. Thus, TLR3-deficient mice showed enhanced survival compared to wild-type animals administered with a lethal dose of the virus. Similarly, the observation that mice defective in TLR2 expression were protected from a lethal dose of HSV-1 while a normal TLR2 phenotype led to lethal viral encephalitis [17] implies that virus-induced TLR-mediated inflammation can indeed be detrimental to the host. On the other hand, TLR4 expression *in vivo* promotes effective and efficient clearance of RSV, minimising disease progression [2]. The observation that mutations in human TLR4, previously linked to hyporesponsiveness to LPS and increased infection with Gram-negative bacteria [101, 102], are associated with severe RSV bronchiolitis in infants [10] suggests that TLR4 may be equally important in controlling RSV disease in humans. Thus, a systematic analysis of the overall outcome of each TLR/virus interaction will be necessary in order to clarify the importance of TLRs in protection against viral infection.

Crucially, the direct binding of any individual viral PAMP, of protein or nucleic acid origin, to a TLR remains to be examined in detail. A lot of information has emerged as to how two bacterial PAMPs, LPS and flagellin, directly interact with the TLR4 and TLR5 receptor complexes, respectively, but no such structural studies have been carried out for any of the viral PAMPs identified to date. A related issue is whether or not the responses elicited by a given TLR are the same for different activators (e.g., LPS and RSV F protein in the case of TLR4). The existence of co-receptors, or indeed upstream pattern recognition receptors that would mediate specific PAMP recognition, and then activate TLRs, is still a possibility.

The lessons to be learned from the intricacies of TLR/virus interactions are potentially invaluable from the point of view of the development of therapeutic strategies, not only for the treatment of virus-associated diseases but also for the treatment of a vast array of inflammatory disorders. Virally encoded immunomodulatory molecules specifically targeting TLR-mediated immunity, such as the VV-encoded A46R and A52R proteins or HCV-encoded NS3/4A could potentially be exploited to suppress inappropriate TLR signalling in a number of clinical contexts.

No doubt the number of such viral TLR antagonists available for therapeutic manipulation will grow in the coming years.

Conversely, artificial TLR activation could also be of therapeutic value. Local mucosal delivery of CpG oligodeoxynucleotides to the genital tracts protected mice from a lethal HSV-2 challenge, through inducing dramatic changes in the genital mucosa, the recruitment of innate immune cells and the inhibition of HSV-2 replication [103]. Thus, the induction of TLR9-mediated mucosal innate immunity could provide protection against HSV infection. Use of the innocuous baculovirus, AcNPV, as a viral vector for gene delivery to mammalian cells has the added advantage of an adjuvant effect induced by the virus to prime TLR9-mediated immunity [32]. Finally, viral-TLR interactions have recently been suggested to be beneficial in cancer immunotherapy. In an established mouse model, vaccinia and adenoviral vectors could break CD8⁺ tolerance in the presence of regulatory T cells, while other cell-based vaccines could not, due to persistent virally induced TLR activation [104]. In conclusion, the potential rewards to be reaped from thoroughly understanding the complicated interactions between TLRs and viruses are boundless.

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IRAK-4: A key kinase involved in toll-like receptor signaling and resistance to bacterial infection

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Introduction

Sensing of microbial pathogens is essential for mounting a strong antibacterial immune defense and for survival of the host. Because of molecular heterogeneity and rapid evolution of pathogens, a recognition strategy by host cells has evolved that is based on detection of conserved molecular patterns that are unique to microbes and highly conserved among entire classes of pathogens. During infection, cells of the innate immune system recognize conserved molecular structures of pathogens either in the context of the bacterial membrane or when released in circulation [1, 2]. Recognition of certain bacterial structures, e.g., lipopolysaccharide (LPS) and lipoarabinomannan (LAM), is initiated by high affinity co-receptors, e.g., CD14 [3–7] that bind various pathogen products and ultimately present them to Toll-like receptors (TLRs) that trigger signal transduction. TLRs are evolutionarily conserved, non-clonally distributed signaling receptors expressed predominantly on monocytes, macrophages, and neutrophils [1, 2, 8, 9]. All 10 human TLRs express an extracellular leucine-rich region, a transmembrane portion, and a cytoplasmic tail with a conserved “Toll-IL-1 resistance” (TIR) domain that is essential for mediating signal transduction of both TLRs and the IL-1 receptor (IL-1R) [8–10]. A lack of LPS sensitivity in TLR4-mutant or knockout (KO) mice [10–14], coupled with a gain of LPS responsiveness upon TLR4 expression in LPS-unresponsive cell lines [15–17], point to TLR4 as the primary signal transducing receptor for LPS. In addition to its ability to transduce intracellular signals that enable responsiveness to most lipopolysaccharide (LPS) species, TLR4 also responds to structurally unrelated microbial structures, e.g., the fusion (F) protein of respiratory syncytial virus [18], Chlamydial heat shock protein (HSP) 60 [19], and pneumolysin [20], as well as paclitaxel (TAXOLTM) [21], a plant-derived diterpenoid whose specificity for TLR4 is restricted to murine cells. In addition, TLR4 has been reported to respond to endogenous agonists (reviewed in [22]) including heat shock proteins 60 and 70, fibrinogen, fibronectin, surfactant protein A, and murine β -defensin 2. This suggests the possibility that such endogenous agonists may play critical roles in alerting the

host to “danger” since many are associated with cellular damage. Thus, TLR4 represents, perhaps, the most versatile of the all TLRs. TLR2 enables responses to components of Gram-positive bacteria (e.g., lipoteichoic acid (LTA) and lipopeptides) [23, 24], mycobacterium (e.g., LAM) [24, 25], mycoplasma lipopeptides [24–26], and HSP70 [27, 28]. TLR1 and TLR6 do not elicit signaling on their own, but are required for TLR2-mediated responses [29, 30]. TLR3 and TLR5 agonists include viral double-stranded RNA and many species of flagellin, respectively [31, 32], while TLR9 responds to unmethylated CpG motifs found in bacterial DNA [33]. Antiviral imidazoquinoline compounds, imiquimod and resiquimod, activate cells via human TLR7 and TLR8 [34, 35], respectively, and murine TLR7 is capable of recognizing another synthetic compound, loxoribine [36]. Because of structural similarities of both imidazoquinoline and loxoribine to guanosine nucleoside, TLR7 and TLR9 were predicted to recognize a nucleic acid-like structure of viruses. This has recently proven to be the case, as TLR7 and TLR8 recognize quanosine- or uridine-rich single-stranded RNA from human immunodeficiency virus, vesicular stomatitis virus, and influenza virus [37]. Thus, TLRs 1, 2, 4, 5, and 6 are more involved in recognition of bacterial products and, possibly, some host proteins, whereas TLRs 3, 7, 8, and 9 detect viral or bacterial nucleic acids preferentially (Fig. 1). Interestingly, whereas TLR4 and TLR5 recognize their ligands at the cell surface, TLR2 is expressed on the cell membrane and is recruited to the macrophage phagosomes after exposure to zymosan. In contrast, TLR3, TLR7, and TLR9 agonist sensing requires endosomal maturation and appears to occur in endosomes (reviewed in [38]; Fig. 1). Thus, TLRs discriminate diverse microbial and viral structures, as well as endogenous eukaryotic proteins released after cell damage as a consequence of wounding and/or infection. This elicits the host defense against infection and enables responses to “danger signals” associated with cellular damage [21].

TLR signaling pathways

Binding of microbial products to co-receptors, e.g., CD14, and their subsequent presentation to TLRs, initiates downstream signaling cascades that are triggered by TLR oligomerization (e.g., TLR4) [39] or by heterodimerization of TLR2 with TLR1 or TLR6 [30, 40, 41]. TLR4 signaling also requires an accessory protein, MD-2 [17, 21, 42], a soluble molecule that has been shown to bind LPS and to associate with the extracellular domain of TLR4 [18, 42–46]. Cross-linking studies revealed an interaction of LPS with CD14, MD-2, and TLR4, demonstrating that these proteins are in close proximity within an integrated “LPS receptor complex” [43]. Agonist-induced TLR oligomerization is believed to bring TIR domains into close proximity, altering their conformation and/or creating novel scaffolds within the cytoplasmic domain. This initiates recruitment of adapter proteins and IL-1R-associated kinases (IRAKs) to TLRs, forming new signaling platforms to which

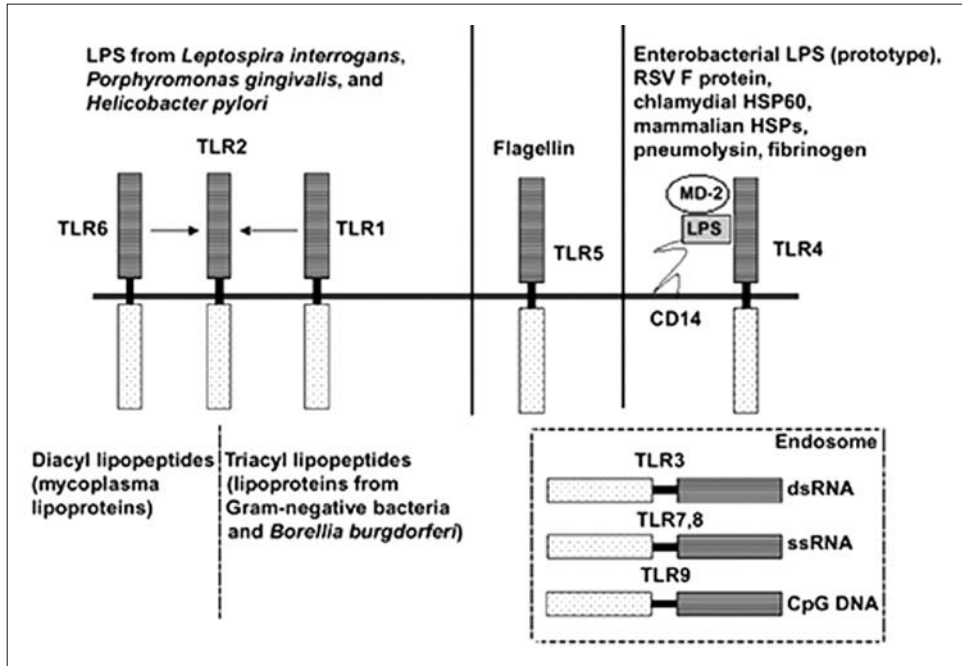


Figure 1

TLRs and their agonists

TLR2, in cooperation with TLR1 or TLR6, recognizes triacyl and diacyl lipopeptides, TLR3 responds to viral dsRNA, whereas TLR4 senses LPS and many other structurally unrelated agonists. MD-2, an accessory protein, binds to the extracellular domain of TLR4 and is necessary for TLR4-mediated LPS recognition and signaling. TLR5 and TLR9 are essential for recognition of flagellin and CpG DNA, respectively, while TLR7 and TLR8 are important for sensing viral-derived ssRNA. TLR recognition of their agonists takes place in different cellular compartments. While sensing of LPS by TLR4 and recognition of flagellin by TLR5 is mediated at the cell surface, TLR3, TLR7, and TLR9 respond to their agonists primarily in intracellular compartments such as endosomes.

additional kinases and downstream adapter molecules are recruited [2, 8, 9, 45]. These processes lead to activation of various mitogen-activated protein kinases (MAPK) and transcription factors that mediate expression of genes for cytokines involved in antibacterial immune defense [8, 9]. The use of mice with targeted mutations in genes that encode adapter proteins has revealed the existence of two major signaling pathways. The myeloid differentiation factor 88 (MyD88)-dependent pathway is critical for B cell proliferation and cytokine secretion, early NF- κ B translocation, and MAPK activation. In contrast, the MyD88-independent pathway

drives dendritic cell maturation, phosphorylation of interferon (IFN)-regulatory factor (IRF)-3, IFN- β and STAT-1-dependent gene expression, and delayed NF- κ B and MAPK activation [47–51]. Mice with targeted mutations in genes that encode proteins necessary for either the MyD88-dependent or MyD88-independent pathways are more resistant to LPS, but exhibit increased susceptibility to both Gram-positive and Gram-negative infections *in vivo*. This indicates that both pathways contribute significantly to antimicrobial defense and endotoxicity.

Currently, four adapter molecules are known to participate in TLR signaling, as illustrated in Figure 2 for adapter protein utilization in the case of TLR4. MyD88 is required for responses to most TLR agonists [52–54], although TLR3 signaling is MyD88-independent [10]. Stimulation of cells with bacterial products triggers recruitment of MyD88 to TLRs [8], association of MyD88 with IRAK-4 and IRAK-1, and subsequent phosphorylation reactions by IRAK-4 and IRAK-1 [52–55]. Tollip, an inhibitory protein that is bound to IRAK-1 in unstimulated cells [56, 57], undergoes phosphorylation by IRAK-1 and dissociates from it in response to TLR agonists. Once hyperphosphorylated, IRAK-1 dissociates from the TLR complex and interacts with TNFR-associated factor 6 (TRAF-6; [58–61]), leading to activation of MAPK and transcription factors (e.g., NF- κ B and AP-1) [60–66]. A second adapter, TIR domain-containing adapter protein (TIRAP; also called Mal), is involved in signaling pathways emanating from TLR2 and TLR4, but not from TLR3, 5, 7, and 9 [67–70]. Macrophages from Mal/TIRAP knockout (KO) mice retain the capacity to activate interferon regulatory factor-3 (IRF-3) in response to LPS [69–71], indicating that the “MyD88-independent” pathway is intact. Two other adapter proteins mediate MyD88-independent signaling triggered by TLR3 or TLR4. TIR domain-containing adapter inducing IFN- β (TRIF) was first implicated in the MyD88-independent signaling pathway elicited by TLR3 [72–75]. TRIF KO mice showed impaired IFN- β induction and IRF-3 activation in response to TLR3 and TLR4 agonists [74, 75]. Another adapter, TRIF-related adapter molecule (TRAM), interacts with TLR4, but not with TLR3, and is involved in TLR4-mediated MyD88-independent signaling [76–79]. Vogel et al. [80] proposed that association of individual TLRs with different combinations of adapter molecules results in distinct repertoires of genes that are expressed in response to agonist-induced stimulation. For example, in the case of TLR4, Mal/TIRAP and TRAM appear to be constitutively associated with the receptor, and, in response to LPS, recruit TRIF (to TRAM) and MyD88 (to TIRAP and TLR4). This forms a signaling “platform”, enabling recruitment of IRAK-4 and IRAK-1 to MyD88, while TBK-1 and IKK ϵ are recruited to TRIF (Fig. 2). As a result of these interactions, these kinases become activated and signal activation of various transcription factors, ultimately leading to cytokine gene expression and secretion (reviewed in [80]). Thus, TLR-mediated engagement of either the MyD88/TIRAP or the TRIF/TRAM components of the pathway initiates multiple downstream signaling pathways whose hallmarks include extreme complexity, the possibility for cross-talk, and a high potential for mutations

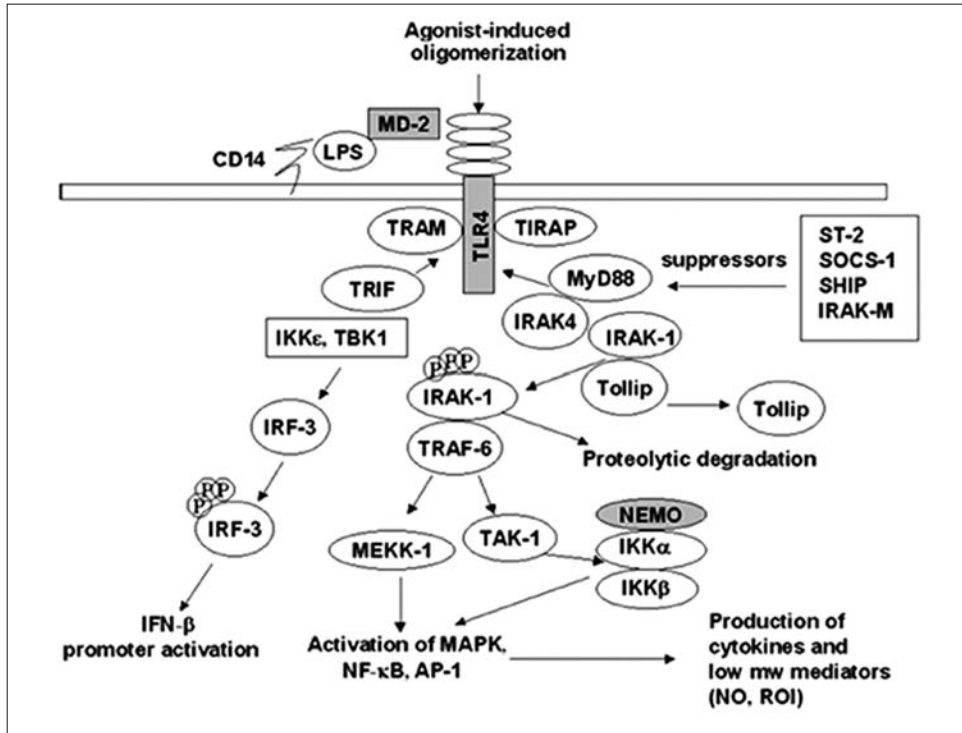


Figure 2

Schematic representation of MyD88-dependent and independent TLR4 signaling pathways. LPS binds to accessory proteins CD14 and MD-2 leading to TLR4 engagement and oligomerization. This results in conformational changes within the TIR cytoplasmic domain, recruitment of adapter proteins and kinases and activation of MyD88-dependent and independent signaling pathways. The MyD88 signaling pathway is initiated by engagement of Mal/TIRAP and association of MyD88 with the cytoplasmic region of TLR4 via their TIR domain interactions followed by recruitment of IRAK-4 and IRAK-1-Tollip complex. This triggers phosphorylation of IRAK-1, dissociation of Tollip, interaction of IRAK-1 with TRAF-6, and stimulation of MAP kinases and transcription factors (e.g., NF-κB and AP-1) via the activation of TAK-1 and MEKK-1 kinases and phosphorylation of MKK 3, 4, 6 and the IKK complex (IKKα, IKKβ, and NEMO/IKKγ). TLR4 stimulates MyD88-independent pathway via engagement of TRAM/TRIF adapter proteins that signal activation of IRF-3 via stimulation of two non-typical IKKs, IKKε and TBK-1 and, ultimately, induction of IFN-β and IFN-β-dependent genes.

(reviewed in [81]) given the number of individual gene products that must act in a coordinated manner to elicit responses to TLR agonists. Microarray analysis revealed a >3-fold change (i.e., upregulation or downregulation of gene expression)

in ~800 genes in mouse macrophages within 3 h of LPS stimulation (~10% of the genes examined) ([82] and unpublished observations). Extending this observation, more recent microarray data suggests that LPS induces significantly more genes through the MyD88-independent than the MyD88-dependent pathway [83]. Another important feature of TLR4 regulation that has only recently come to light is that certain proteins, e.g., ST2, SIGIRR, Tollip, IRAK-M, MyD88s, SOCS, SHIP and others exert suppressive effects on TLR4 signaling (reviewed in [10, 81]; Fig. 2). Thus, the regulation of TLR4 signaling represents a carefully orchestrated balance of positive and negative regulatory signals.

The IRAK family and IL-1R/TLR signaling

To date, four distinct IRAK species have been identified in humans that belong to the Ser/Thr kinase family and are involved in initiation and regulation of signal transduction emanating from the IL-1R and several members of the TLR family. These include two active kinases, IRAK-4 and IRAK-1, as well as two inactive kinases, IRAK-2 and IRAK-M, that all share a similar structural organization comprised of the N-terminal death domain (DD), and a central kinase domain (KD) [54, 55, 59, 84, 85]. The human *IRAK-1*, *IRAK-2*, *IRAK-M*, and *IRAK-4* genes map to chromosomes Xq28, 3p25.3-3p24.1, 12q14.1-12q15, and 12q11.22, respectively (reviewed in [84]). Human IRAK-1, IRAK-2, and IRAK-4 mRNA exhibit wide tissue distribution, although IRAK-4 was found to be expressed at lower levels [55], whereas IRAK-M mRNA expression is predominantly expressed in primary peripheral blood leukocytes and monocytic cell lines [86]. In contrast to IRAK-1, IRAK-2, and IRAK-M, IRAK-4 does not express a unique C-terminal domain which is required for interaction of IRAK-1 with TRAF-6 [55, 84]. IRAK-4 is a key early enzyme in the TLR4 and IL-1 signaling pathways and appears to represent the only IRAK that requires its kinase activity for signaling. Indeed, in contrast to other IRAKs, a kinase-inactive IRAK-4 KK 213AA variant (obtained by mutating two lysine residues in the ATP binding pocket to alanine) fails to activate an NF- κ B reporter and mediates a dominant-negative effect in IL-1 signaling [55]. In addition, overexpression of IRAK-4 KK213AA or mutant IRAK-4 variants identified in a patient we studied (see Fig. 3) inhibit IL-1-mediated IRAK-1 phosphorylation and kinase activity [55, 87]. In contrast to the lack of activity of kinase-deficient IRAK-4 proteins, kinase-deficient IRAK-1 variants are still able to mediate MAP kinase activation and NF- κ B induction upon overexpression [89, 90]. Similarly, although IRAK-2 and IRAK-M are catalytically inactive because of a substitution of a critical aspartate residue to asparagine or serine that renders the kinase domain non-functional [84], they both enable signaling [86]. *In vitro*, IRAK-4 directly phosphorylates Thr387 and Ser376 in the activation loop of IRAK-1 that is critical for IRAK-1 kinase activity [55]. An indispensable role for IRAK-4 in TLR/IL-1R sig-

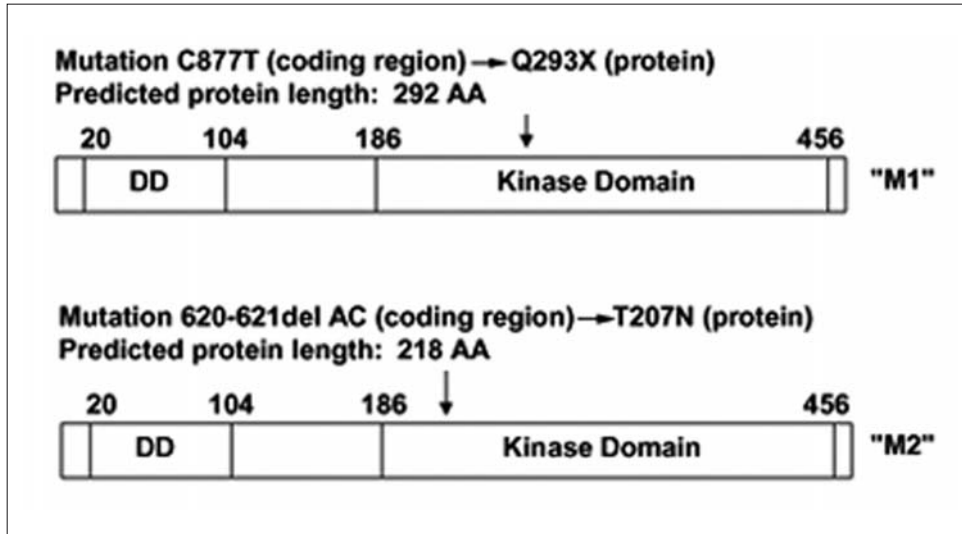


Figure 3

IRAK-4 mutations in a patient with a compound heterozygous genotype

Two IRAK-4 mutations identified in the coding mRNA regions in our patient (i.e., C877T substitution, M1, and 620-621del AC, M2) are illustrated [87]. In addition, their predicted effects on amino acid sequence at the protein level, Q293X (M1) and T207N (M2) are shown. Arrows indicate approximate location of truncation in IRAK-4 protein.

naling is strongly supported by the profound hyporesponsive phenotype seen in IRAK-4 KO mice and in patients with mutations that result in IRAK-4 kinase domain truncations (discussed below). Despite a recent report which has demonstrated that the “kinase-dead” IRAK-4 KK213AA variant restored IL-1 signaling in IRAK-4-deficient human fibroblasts upon stable transfection, a truncated IRAK-4 species (1–191 amino acids) failed to elicit NF-κB reporter activation [91]. The latter finding is consistent with our results that have shown significantly suppressed LPS- and IL-1-mediated NF-κB activation, MAPK phosphorylation, and cytokine production in the patient who expresses truncated IRAK-4 variants [87]. Additional studies will be required to determine the extent to which the catalytically active kinase domain is required for proper functioning of IRAK-4.

IRAK-4 is thought to form a complex with MyD88 and IRAK-1 and initiates phosphorylation of IRAK-1, leading to a conformational change within IRAK-1 that results in its autophosphorylation [55, 84]. As a consequence, Tollip, an inhibitory protein that is bound to IRAK-1 in unstimulated cells through a death-domain-death domain (DD-DD) interaction [56, 57], undergoes phosphorylation by IRAK-1 and dissociates from it. Hyperphosphorylated IRAK-1 then dissociates

from the TLR complex and interacts with TNFR-associated factor 6 (TRAF-6) via binding to the TRAF domain [58–61, 84]. This, in turn, induces activation of TRAF-6 by clustering its effector N-terminal domain, interaction with a pre-existing complex of TGF- β -activating kinase (TAK)-1 and two TAK-binding proteins, TAB 1 and 2, leading to activation of TAK-1 [65, 92]. Activated TAK-1 subsequently phosphorylates IKK α/β and MAPK kinase (MKK) 3, 4, and 6, resulting in activation of MAPK and transcription factors (e.g., NF- κ B and AP-1) [60–66]. Activated, hyperphosphorylated IRAK-1 is subsequently ubiquitinated and degraded which is thought to ensure balanced regulation of its function and prevents chronic cellular activation.

Another level of regulation of TLR signaling is achieved by other full-length or alternatively spliced IRAK species. For instance, IRAK-M is induced upon TLR stimulation, and overexpression of IRAK-M was found to prevent dissociation of IRAK-1 and IRAK-4 from MyD88 and formation of IRAK-TRAF6 complexes [86]. Functional characterization of IRAK-M demonstrated increased cytokine production upon TLR/IL-1 stimulation and bacterial challenge of IRAK-M-deficient cells, and IRAK-M KO mice showed increased inflammatory responses to bacterial infection [86]. In addition, induction of endotoxin tolerance, a mechanism that has been postulated to protect the host against endotoxin shock, was significantly reduced in IRAK-M KO cells [86]. Thus, in contrast to IRAK-4 and IRAK-1, IRAK-M seems to represent a negative regulator of TLR signaling. Interestingly, Hardy and O'Neill [93] demonstrated that the murine, but not human, *IRAK-2* gene encodes four alternatively spliced isoforms, two of which, *Irak2c* and *Irak2d*, also exert inhibitory effects on LPS-mediated activation of a NF- κ B reporter. LPS was found to upregulate *Irak2c* expression, suggesting that, at least in the mouse, alternatively spliced IRAK-2 can mediate negative feedback regulation of TLR signaling [93].

The necessity of IRAK-4 in TLR signaling has been unambiguously demonstrated in IRAK-4 KO mice as evidenced by their extreme susceptibility to infection, resistance to LPS-induced shock, and failure to respond to stimulation through IL-1RI, TLR4, TLR2, and TLR3 receptors [94]. IRAK-4-deficient cells fail to activate p38 and JNK MAPK, to induce the transcription factor NF- κ B, and to produce cytokines (TNF- α , IL-6, and IFN- γ) and nitric oxide in response to IL-1 or LPS stimulation [94]. Since virus infection triggers IFN- γ production in an IL-18-dependent manner [95], IRAK-4 has been suggested to be required also for IL-18R signaling [94]. Compared to the severe effect of IRAK-4 deficiency on IL-1R and TLR signaling, only partial inhibition of these responses were found in IRAK-1 KO mice [96, 97], a phenomenon that might be explained by functional redundancy among IRAK-1 and other IRAKs [84]. For instance, the overexpression of either IRAK-1 or IRAK-2, but not IRAK-4, was shown to activate NF- κ B in IRAK-1 KO cells [54, 86, 89], suggesting that IRAK-4 functions upstream of IRAK-1 and that IRAK-1 and IRAK-2 are functionally redundant. Since overexpression of either MyD88 or TIRAP/Mal failed to activate an NF- κ B reporter in IRAK-4-deficient mouse embry-

onic fibroblasts, whereas overexpression of TRAF-6 stimulated NF- κ B activation, IRAK-4 was suggested to lie downstream of MyD88/Mal, but upstream of TRAF-6 [94]. An alternatively spliced, truncated variant of MyD88, MyD88s, that lacks the intermediate domain between the N-terminal DD and the C-terminal TIR domain, is still capable of interacting with IRAK-1, similar to full-length MyD88. However, in contrast to the interaction of IRAK1 with full-length MyD88, the IRAK-1-MyD88s interaction does not trigger IRAK-1 phosphorylation [98], suggesting that the interdomain region of MyD88 is required for IRAK-1 phosphorylation, most likely, through the recruitment of IRAK-4. This conclusion is further supported by Burns et al. [99] who reported that MyD88s fails to recruit IRAK-4, suggesting that IRAK-4 binds the intermediate region of MyD88 positioned between the DD and TIR domains. Hence, it appears that IRAK-4 and IRAK-1 binding to distinct domains of MyD88 ensures their sufficient proximity that could facilitate IRAK-4-mediated IRAK-1 phosphorylation [85]. Thus, IRAK-4 is a critical upstream kinase required for efficient signaling from IL-1RI and many TLRs, e.g., TLR2, TLR3, and TLR4, whose mutations are predicted to greatly affect the ability of the host to mount efficient antibacterial immune responses.

Known mutations in human IRAK-4

Within two years of the first report of IRAK-4 deficiency, mutations in the human *IRAK-4* gene were identified in 13 patients within nine unrelated families suffering from repeated, life-threatening bacterial infections early in life who also exhibit hyporesponsiveness to LPS, IL-1, and IL-18 [87, 100, 101]. These individuals are highly susceptible to Gram-positive infections, although one patient had a documented *N. meningitidis* infection [101], show no developmental abnormalities, and manifest this pattern of life-threatening bacterial infections in childhood. Interestingly, if the IRAK-4-deficient child survives the early life-threatening bacterial infections, this phenotype dissipates with age, suggesting that repeated immunization and exposure to pathogens elicits a level of adaptive immunity that is sufficient to elicit, at least partially, a protective immune response.

Picard et al. [100] first described autosomal recessive mutations in exons 7 and 8 of the *IRAK-4* gene in three unrelated patients suffering from infections caused by pyogenic bacteria and hyporesponsiveness to IL-1, IL-18, LPS, as well as to TLR2, TLR3, TLR5, and TLR9 agonists. These defects were found to be specific for the TLR pathway as stimulation of patients' cells with either TNF- α or PMA and ionomycin resulted in normal levels of cytokine production [100]. One patient had a homozygous deletion of thymidine in exon 7 (821delT in mRNA), whereas two other patients had a point mutation in exon 8 (C877T substitution in mRNA), both of which resulted in premature stop codons and the lack of expression of functional full-length IRAK-4 mRNA and protein [100]. The health state of two of these

patients gradually improved with age, whereas the third patient has had recurring bacterial and fungal infections, requiring IVIG therapy. In a follow-up study of this patient, Day et al. [102] reported that booster immunizations with diphtheria/tetanus toxoid, pneumococcal polysaccharide and bacteriophage øX174 led to an impaired antibody response with low titers that were not sustained, suggesting an association of IRAK-4 deficiency with impaired antibody response and failure to sustain it. Thus, it appears that if IRAK-4 is mutated, thereby blocking the TLR pathway, B lymphocytes fail to be properly activated, resulting in inadequate maturation to antibody-secreting plasma cells. Alternatively, the mutation of IRAK-4 may lead to decreased co-stimulatory molecule expression and, thus, improper T-lymphocyte activation, resulting in insufficient T-helper function.

Our studies have focused on a now 23-year old patient with a history of repeated bacterial infections, without fever [101], who failed to become febrile or produce cytokines in response to LPS challenge *in vivo*. *In vitro*, patient's PBMC and neutrophils showed hyporesponsiveness to LPS and IL-1 stimulation, as evidenced by suppressed NF-κB activation and p38 phosphorylation, yet TNF-α responsiveness remained intact [87, 88, 101]. Subsequent cloning and sequencing revealed that the patient expresses a compound heterozygous genotype (i.e., a point mutation on one allele, "M1", and a two nucleotide deletion, "M2", on the other allele of the *IRAK-4* gene) ([87]; Fig. 3), in contrast to autosomal recessive genotypes expressed by all but one of the other patients who have been genotyped to date. The *IRAK-4* mutations expressed by our patient were inherited in a strictly Mendelian fashion: the two nucleotide deletion mutation was carried heterozygously by the maternal grandfather, mother, and only sibling, while the point mutation was carried heterozygotically by the paternal grandmother and father. Our recent studies have underscored a failure of LPS to upregulate expression of a number of co-stimulatory molecules, including CD18 and CD67, in patients' polymorphonuclear cells (PMNs), in contrast to strong responses observed in patient's parents and an unrelated control volunteer (Fig. 4). Again, stimulation of patient's PMNs with a non-TLR-specific stimulus, f-Met-Leu-Phe (fMLP), induced an increase in CD18 and CD67 expression that was similar to responses observed in her parents or a normal healthy volunteer (Fig. 4 and data not shown). Interestingly, our patient also had an abnormal inflammatory response to a non-microbial stimulus *in vivo*, e.g., blister formation, manifested by depressed influx of neutrophils into the blister base, and diminished cytokine and chemokine production in the region of the blister when compared to controls. Yet, under these conditions, complement activation was normal [87]. Thus, even a non-microbial stimulus revealed the deficiency in IRAK-4 in this patient, supporting previous observations that endogenous proteins (e.g., heat shock proteins, fibrinogen, etc.) may serve as TLR agonists (reviewed in [22]). Most interestingly, the point mutation expressed by our patient (Q293X) has been observed in five unrelated families ([103], and D. Speert, personal communication at the time of this report). Although a founder effect has not been formally excluded

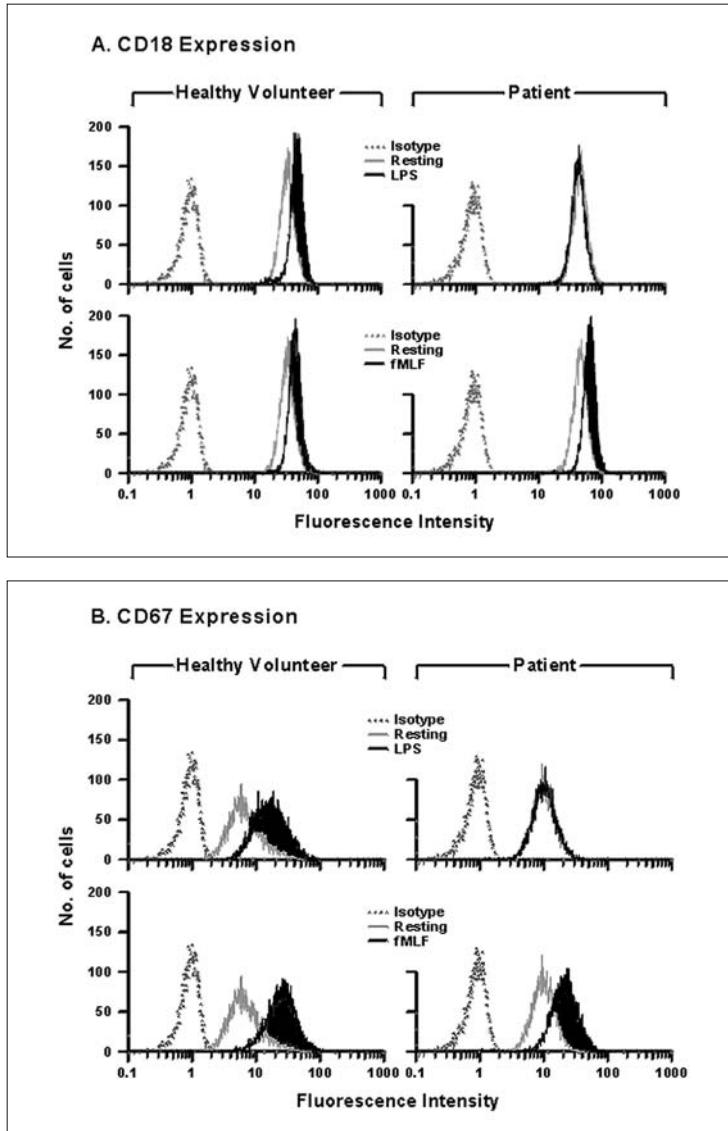


Figure 4

LPS-mediated regulation of expression of CD18 and CD67 in PMNs obtained from IRAK-4-deficient patient versus a normal volunteer

PMNs obtained from IRAK-4-deficient patient or normal volunteer were left unstimulated (resting) or treated with LPS (top panels) or fMLF (bottom panels). Cells were subjected to FACS analysis with either isotype control Abs (dotted lanes) or anti-CD18 (A) and anti-CD67 Abs (B) (depicted in bold lanes).

ed, these patients are from eight different countries and differ in their ethnicities. However, the sequence surrounding the C877T point mutation does not, from inspection, appear to be a typical hypermutable sequence or “hot spot” [104]. Studies are currently in progress to evaluate these possibilities.

The point mutation and the small, two nucleotide deletion expressed heterozygously on distinct alleles by our patient were predicted to encode truncated forms of the IRAK-4 protein, with both truncations occurring within the kinase domain. Unfortunately, we were unable to detect IRAK-4 protein (either WT or mutant) by Western analysis using a polyclonal antiserum obtained from Tularik. Endogenous IRAK-4 protein is expressed at extremely low levels and requires a very large number of cells ($\sim 200 \times 10^6$ cells per treatment; e.g., see [55]), and, in all probability, even more cells would be required to detect this protein in primary PBMC, the main source of cells from our patient. Unfortunately, this large number of cells was not available from our patient. Despite extensive efforts to detect WT and mutant forms of endogenous IRAK-4 in PBMC cell lysates and IRAK-1 immune complexes obtained from our patient *versus* healthy volunteers, the amount of endogenous IRAK-4 protein was below the level of detection by Western analysis. However, when we introduced these two mutations into the pRK7 IRAK-4 expression vector encoding epitope (flag)-tagged WT IRAK-4 and transfected them in HEK293 cells, the predicted sizes for the truncated forms were readily observed by Western analysis using anti-flag antibody [87].

Importantly, when overexpressed in HEK293 cells, both mutant forms act as dominant negative inhibitors of WT, endogenous IRAK-4, as evidenced by suppression of IL-1-induced IRAK-1 kinase activity, with the highest level of inhibition achieved upon transfection of the vector encoding the C877T point mutation [87]. Analysis of the molecular mechanisms by which these kinase-defective, short forms of IRAK-4 block signaling has demonstrated that mutations in the kinase domain of IRAK-4 impair its IL-1-inducible association with the IL-1RI and IRAK-1 [88]. In addition, we have shown that overexpressed mutant IRAK-4 proteins failed to be recruited to TLR4 upon stimulation of HEK293/TLR4/MD-2 cells with LPS (Fig. 5). We have also found that overexpression of truncated IRAK-4 variants inhibited recruitment of endogenous IRAK-1 and MyD88 to the IL-1RI in response to IL-1 stimulation, and result in constitutive association of kinase-defective IRAK-4 with endogenous or overexpressed cytoplasmic MyD88 [88]. Since the heterozygous parents, grandparents, and sibling of this patient are phenotypically normal with respect to LPS sensitivity and exhibit normal resistance to infection, we can only surmise that insufficient amounts of the “truncated” forms of IRAK-4 are produced by these individuals to block the activity of the normal IRAK-4 protein. In other words, only a single copy of wild-type IRAK-4 is necessary to render an individual phenotypically “normal.” In contrast, upon overexpression, a large proportion of kinase-defective IRAK-4 variants are likely to be present in the cells relative to the amount of WT IRAK-4, leading to an inhibitory effect. These results demon-

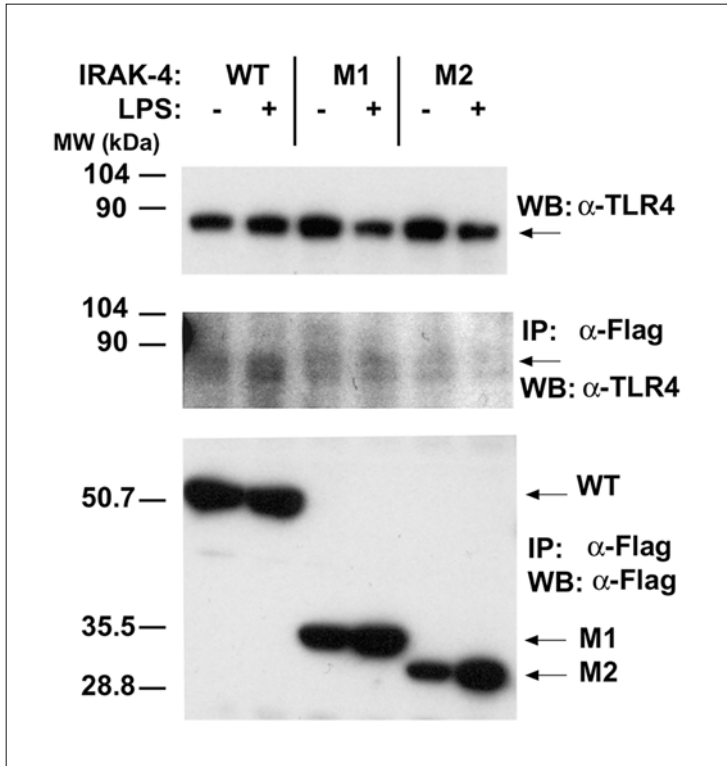


Figure 5

Mutations in the kinase domain impair LPS-inducible recruitment of IRAK-4 mutant proteins to TLR4

HEK293/TLR4 cells were transfected with pRK7 expression vectors encoding Flag-tagged WT, M1, or M2 IRAK-4. After recovery for 48 h, cells were treated for 15 min with medium or 100 ng/ml LPS. The top panel shows total expression of TLR4 detected by Western analysis of cell lysates with anti-TLR4 Ab prior to immunoprecipitation. Cell lysates were immunoprecipitated with anti-Flag Ab, and immune complexes subjected to Western analysis with anti-TLR4 Ab (middle panel, detection of TLR4 association with IRAK-4) or anti-Flag Ab (bottom panel; total expression of transfected IRAK-4 species).

strate that hyporesponsive phenotype of our IRAK-4-defective patient may result from the failure of mutant IRAK-4 species to form functional signaling complexes with components of the IL-1R/TLR4 pathways in response to stimulation with IL-1 and LPS. They also suggest that small molecular weight mimetic compounds may be designed to diminish IRAK-4-dependent signaling in people with hyperinflammatory syndromes [88].

Conclusions

Extreme complexity and a potential for cross-talk within the TLR signaling pathways suggest that mutations in almost any receptors or intracellular signaling components would result in severe innate immune deficiency. However, to date, relatively few mutations have been described that underlie deficient TLR4 signaling and increased susceptibility to infectious disease. These include mutations in TLR4, the IRAK-4 enzyme, and in NEMO (IKK γ) and I κ B α components of the IKK signaling complex. Due to the central role for IRAK-4 in mediating TLR-mediated signal transduction, IRAK-4 mutations severely affect antibacterial immune defense mechanisms. This defect is associated with mutations within the kinase domain of IRAK-4 that seem to inhibit the formation of functional signaling complexes with receptor and intracellular components of the IL-1R/TLR4 pathway. This property of truncated IRAK-4 molecules also raises the possibility for therapeutic intervention in hyperinflammatory states by using IRAK-4 mimetics to inhibit signaling. Future studies will likely reveal the feasibility of such an approach, and delineate how IRAK-4 deficiency affects other antibacterial immune defense mechanisms, including development of adaptive immunity.

Acknowledgements

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Endogenous regulation of toll-like receptor signalling

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Introduction

As discussed in all chapters in this book, individual Toll-like receptors (TLRs) recognise distinct pathogen associated molecular patterns (PAMPs) that have been evolutionarily conserved in certain classes of microbes including bacteria [1–3], viruses [4–7], fungi [8] and parasites [9]. Since the importance of TLRs in the innate immune response was first realised much work has been carried out to elucidate the series of events triggered by ligand binding to TLRs which eventually result in proinflammatory cytokine production and also the importance of TLRs in the pathogenesis of various disease states. It has become clear that TLRs are, on one hand, essential regulators of both innate and adaptive immune responses and, on the other, have been implicated in the development of autoimmune, chronic inflammatory and infectious diseases. One of the best characterised diseases to be implicated with TLR signalling is sepsis and its most severe form, septic shock, a syndrome associated with bacterial infection. More than 50% of the infections are associated with Gram-negative bacteria [10] and therefore with lipopolysaccharide (LPS) a ligand for TLR4 [1]. Signalling through TLR9 has been implicated in the production of autoantibodies recognising self-DNA, a detrimental state seen in patients with systemic lupus erythematosus (SLE) [11, 12]. People carrying the well studied D299G polymorphism of TLR4 show a reduced risk of carotid artery atherosclerosis [13] and acute coronary events [14]. Patients with mutations in downstream components of the signalling pathway such as NEMO and IRAK4 showed increased risk of bacterial and viral infections [15]. The importance of the link between TLRs and various disease states is evident and most of the aforementioned diseases and others are discussed elsewhere in this book. It now seems possible that most immune and inflammatory diseases will have a TLR component at some level.

The immune response needs to constantly strike a balance between activation and inhibition to avoid detrimental inflammatory responses. TLR signalling and functions are therefore, of necessity, under tight regulation. Many of the aforementioned disease states may arise through a lack of regulation of TLR signalling, either

from uncontrolled activation of TLRs or from a dysregulation of endogenous TLR-signalling processes. Although the mechanism of activation of TLRs and their downstream signalling pathways has now been well characterized, the extent of the endogenous regulation of the pathways has only recently come to light. Over the past few years many negative regulatory mechanisms of TLRs have been identified and it is clear that endogenous regulation of TLRs occurs at every possible level of the pathway. Some regulators are present at a constitutive level to control TLR activation, whereas others are upregulated by TLR signalling to attenuate the TLR response in a negative feedback loop indicating the very delicate nature of these control processes. Here, we discuss the various known negative regulatory mechanisms that have evolved to attenuate TLR signalling in order to maintain an immunological balance. Understanding how TLR signalling is regulated will aid the development of new strategies to control TLR-mediated inflammatory diseases.

Extracellular regulators

The importance of regulation of signalling pathways at the extracellular level has been exemplified by the IL-1 signalling pathway where several mechanisms of control are known to exist. The IL1 receptor antagonist is capable of binding the type 1 IL-1 receptor with near equal affinity to both IL-1 α and IL-1 β . As such this molecule is a true antagonist, which acts to competitively block IL-1 from its receptor [16]. The type II IL-1 receptor (IL1RII) also acts as a negative regulator of IL-1 signalling and is termed a 'decoy receptor' as it has an extracellular domain highly similar to that of IL1RI and binds IL-1 but is not capable of signalling as it possesses a truncated extracellular tail that lacks a TIR domain [17]. A soluble form of the IL1 receptor accessory protein, which arises from alternative splicing of the IL1 receptor accessory protein gene has recently been shown to increase the binding affinity of IL-1 α and β for IL1RII by nearly 100-fold, while not affecting the low binding affinity of IL1ra [18], suggesting that this soluble receptor contributes to the negative regulation of IL-1. It is, therefore, not surprising, given the similarities between the IL-1R and TLR signalling pathways, that similar mechanisms of control have been identified for the TLRs. Such mechanisms could provide the most direct attenuation of acute host inflammatory responses to pathogenic ligands and stress proteins.

In mammalian hosts, although there is only a single copy of the TLR4 gene, multiple mRNA products have been detected [19], suggesting the presence of TLR4 isoforms. A cDNA encoding a soluble form of TLR4 was identified by screening a murine macrophage cDNA library [20]. It comprises a protein of 122 amino acids of which 86 are identical to those of the extracellular domain of TLR4, while the remaining 36 amino acids share 70% homology with the N-terminal end of mouse phosphatidylinositol 3-kinase (PI3-kinase). Recombinant sTLR4 inhibited LPS-induced NF- κ B activation and TNF- α production by macrophages *in vitro*. Multi-

ple TLR4 mRNAs were also detected in humans, indicating that sTLR4 may also functionally exist in humans. Although the mechanism by which sTLR4 attenuates TLR4 function is currently unclear, one possibility is that it may block the interaction between TLR4 and other co-receptor complexes, especially MD2 and CD14 and hence terminate TLR4 signalling [20].

A soluble form of human TLR2 has also been identified. LeBouder et al. found that blood monocytes released a constant amount of sTLR2 that might result from the post-translational modification of the transmembrane receptor protein [21]. Six sTLR2 isoforms are found to be naturally present in human milk and plasma with molecular weights of 20–85 kDa. sTLR2 inhibited IL-8 and TNF- α production by monocytes induced by bacterial lipopeptide (BLP), an agonist of TLR2. sTLR2 has been shown to interact with sCD14, a TLR2 co-receptor, and it is possible that this interaction sequesters sCD14 from TLR2, thereby limiting TLR2 activation [22]. The importance of the high number of isoforms is as yet unknown but it seems that, certainly for both TLR2 and TLR4, the existence of these soluble receptors provides the first level of regulation in terms of limiting activation of the receptor by interfering with either ligand or co-receptor binding to the receptor.

Membrane bound regulators

There are now several known membrane bound regulators that have been implicated in negative regulation of TLR signalling. The first of these to be identified was Single Ig IL-1R-related molecule (SIGIRR), otherwise known as TIR8, which is extensively discussed in a chapter by Garlanda et al. in this book. Here we will briefly summarise the negative regulation function of SIGIRR. SIGIRR, although a member of the IL-1 receptor family, only possesses one immunoglobulin domain extracellularly and does not bind to IL-1 or activate TLR/IL1R signalling pathways [23] SIGIRR is expressed strongly in the kidney and moderately expressed in the small intestine, lung, spleen and liver. Wald et al. showed that overexpression of SIGIRR inhibited IL-1, IL-18 and LPS induced NF- κ B activation. SIGIRR-deficient mice showed a more potent inflammatory response than WT, following intraperitoneal injection with LPS and exhibited a considerably reduced threshold to lethal endotoxin challenge. *In vitro* experiments showed that splenocytes from these mice were hyper-responsive to IL-1 and the Toll ligands LPS and CpG. These authors also demonstrated that SIGIRR was able to interact with components of the TLR signalling pathway including both TLR4 and the IL-1 receptor, and the downstream signalling components IRAK and TRAF6 [24]. A second group to make a SIGIRR knockout detected expression of SIGIRR on DCs which had been previously unreported as expression was thought to be primarily confined to epithelial cells. DCs but not macrophages from their knockout mouse were also hyper-responsive to LPS and CpG in terms of production of cytokines and chemokines. The mice were also

more susceptible to intestinal inflammation, having increased severity of colitis induced by dextran sulphate sodium thereby indicating the potential importance of this modulator in the control of inflammatory responses in the gut [25].

The second membrane bound regulator to be identified was ST2, also known as T1, Fit1 and DER4. ST2 is also a member of the IL-1 receptor family possessing three immunoglobulin domains extracellularly [26–28]. Although ST2 is a member of the IL-1R family it does not bind to IL-1 α , IL-1 β or the IL-1R antagonist [29, 30]. It is an orphan receptor with no known functional ligand. In both mouse and humans differential mRNA processing of the ST2 gene gives rise to two main forms; a soluble (sST2) form predominately expressed by fibroblasts [31] and also a membrane bound form of ST2 (ST2L) which possesses a TIR domain and is expressed primarily on haematopoietic cells but also on immune cells such as basophiles, mast cells and selectively on Th2, not Th1, cells. ST2L has been implicated in Th2 cell function as treatment of mice with an antibody to ST2 enhanced Th1 cell responses in mice [32] and neutralization of ST2 inhibits allergic airway inflammation [33]. The soluble form but not the transmembrane form of ST2 is upregulated by treatment with proinflammatory cytokines such as IL-1 and TNF [34]. More recently an inhibitory role for ST2 with respect to TLR signalling has been identified. The first indication that ST2 may be inhibitory arose from work done by Sweet et al. where they showed that treatment of bone marrow derived macrophages (BMMs) with the soluble form of ST2 resulted in the downregulation of TLR4 and also TLR1. Treatment of BMMs with sST2 also resulted in a decrease in the level of proinflammatory cytokines produced by these cells [35]. Interestingly the importance of sST2 as a regulator is highlighted by the fact that levels of sST2 are elevated in plasma from patients with asthma, SLE, idiopathic pulmonary fibrosis, lung tumours and cardiac infarct [36–39]. It was subsequently shown that over expression of ST2L was able to inhibit NF- κ B activation by TLR4 and also by IL1R1 [40]. Overexpression of ST2L does not inhibit TLR3 induced activation of either NF- κ B or IRF3. As ST2L binds to the common adaptor MyD88 and also to the TLR2/4 adaptor Mal but not to the TLR3 adaptor Trif, it seems possible that the mechanism of inhibition of ST2L may occur by sequestration of these downstream adaptors. The authors also showed that the level of proinflammatory cytokines produced in the ST2 knockout mouse in response to LPS, IL-1, CpG and BLP is significantly elevated compared to WT. This increase is not observed in response to the TLR3 ligand Poly(I:C). The ST2 knockout mice also failed to develop endotoxin tolerance *in vitro* and *in vivo*. Therefore, ST2L performs an effective negative-feedback role in certain TLR signalling pathways including contributing to endotoxin tolerance and this role also provides a molecular explanation for the role of ST2L in Th2 responses as inhibition of TLRs would promote a Th2 response.

More recently two other membrane bound regulators, TRAIL-R and RP105, have been identified. Although the ability of TRAIL (TNF-related-apoptosis-inducing-ligand) to induce apoptosis in transformed cells has been well studied [41, 42],

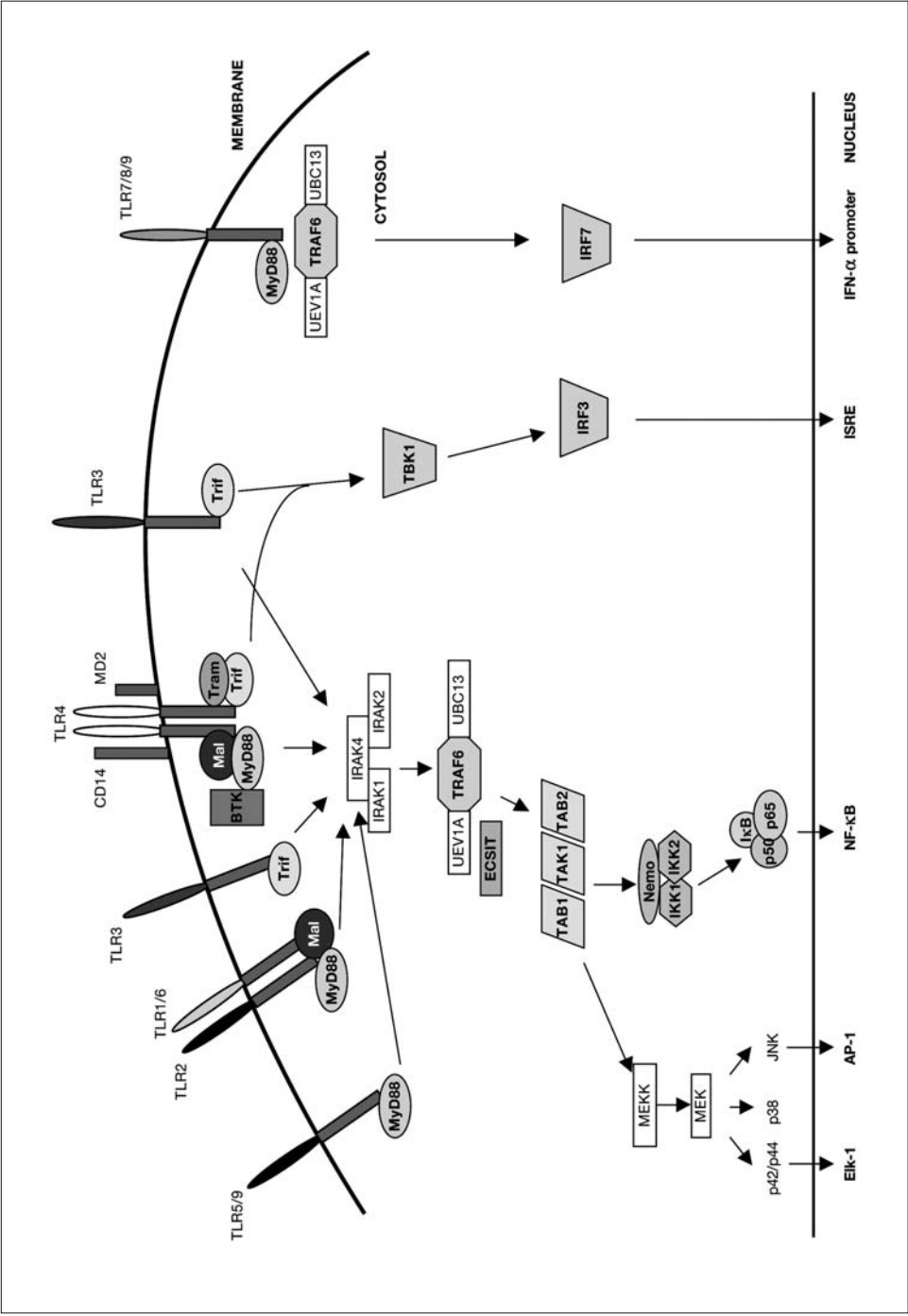
the significance of this cytokine and its ligand TRAIL-R in normal physiology has not been characterised. Generation of the TRAIL-R knockout mouse by Diehl et al. has aided this characterisation and pointed to a role for TRAIL-R as a negative regulator of immune responses. WT macrophages upregulate TRAIL-R following stimulation with LPS, LTA and polyI:C. Enhanced cytokine secretion from TRAIL-R^{-/-} macrophages compared to WT was observed following stimulation with ligands for TLR2, 3 and 4 but not 9 [43]. RP105 is a TLR homolog as it possess leucine-rich repeats extracellularly and a TLR-like pattern of juxta-membrane cysteines [44], but has no TIR domain, only a short cytoplasmic tail. Divanovic et al. have shown that expression of RP105 mirrors that of TLR4 on APCs and that overexpression of RP105 in HEK293 cells inhibits LPS-induced IL-8 production and that this inhibition is reliant on the extracellular region of RP105. RP105 seems to be able to interact directly with TLR4, inhibiting its ability to bind to microbial ligand. These data indicate that RP105 is a specific inhibitor of TLR4 signalling [45].

Intracellular regulators

The downstream signalling cascades from TLRs to the nucleus have been well characterised over the last few years and it is known that following ligand binding all TLRs form either homo- or hetero-dimers allowing recruitment of either one or more of the adaptor proteins MyD88, Mal, Trif and Tram, depending on which TLR is activated. These adaptors also possess the Toll/IL-1R receptor (TIR) domain and so can interact directly with the TLR itself. Binding of the adaptors then allows the recruitment and activation of a family of kinases known as the IL-1R-associated kinases (IRAKs) and the adaptor molecule TRAF6, which eventually leads to the activation of four protein kinase cascades, culminating in the activation of NF- κ B and the mitogen-activated protein (MAP) kinases p38, JNK, ERK1/2 [46] (also see Fig. 1). The next obvious line of defence concerning regulation of TLR signalling, are these processes which occur intracellularly following ligand binding and receptor activation. Recently several key regulatory proteins have been identified, some inducible and some constitutively expressed, whose role is to control these intracellular signalling cascades. For ease of identification, in this section we will review these intracellular inhibitors, not in the order they were first identified but by progressing from the receptor level to the membrane.

MyD88s

The most critical adaptor in IL-1R/TLR signalling is MyD88. It is the only adaptor used by the IL-1R, IL-18R, TLRs 5, 7, 8 and 9 and while TLRs 2 and 4 uses this adaptor in combination with others to signal, inhibition of MyD88 causes abroga-



tion of NF- κ B signalling, that is only partially compensated at a later time by other adaptors. MyD88 is composed of three main regions coded for by 5 exons [47]. The first exon codes for the death domain (DD) component of MyD88 which mediates downstream interactions with the IRAK family of kinases. Exon 2 encodes the inter-domain (ID), while the last three exons encode the TIR domain which mediates the interaction of MyD88 with the IL-1R/TLR complex. RT-PCR for MyD88 on RNA isolated from the murine macrophage cell line Mf4/4 identified two cDNA species of 890 and 747 bp. The larger isoform was identified as the full length form of MyD88 while the shorter version was a splice variant of MyD88, now designated as MyD88s, which lacks the ID. Although MyD88 is ubiquitously expressed, expression of MyD88s was only detected in the spleen and, less strongly, in the brain. Its expression was however found to be upregulated in the human monocytic cell line THP-1 following a 16h stimulation of these cells with LPS, indicating that this splice variant can be induced by proinflammatory stimuli. Overexpression of MyD88s inhibits IL-1 and LPS but not TNF induced NF- κ B activation [48]. The mechanism of inhibition has been elucidated and is determined by altered interactions in the downstream signalling pathway. Burns et al. showed that the ID component of MyD88 is essential for recruitment of IRAK4 to the signalling complex and as MyD88s lacks this domain, IRAK-4 cannot be recruited and therefore no phosphorylation of IRAK1 occurs [49]. Interestingly, however, MyD88s is still able to drive AP-1 activation, indicating that this protein is multi-functional [50].

SOCS-1

The suppressor of cytokine signalling (SOCS) family comprises proteins induced on cytokine stimulation which then block further signalling in a classical negative feedback manner. This protein family consists of eight members and these members have

Figure 1

TLR signalling involves four adapters

MyD88 is used by all TLRs apart from TLR3, and engages with the IRAK family, leading to engagement with Traf6, which ultimately leads to activation of NF- κ B and MAP kinases such as p42/p44 MAP kinase and JNK. These pathways lead to the induction of cytokines such as TNF and other proinflammatory proteins. MyD88 has recently been shown to have an additional role in the activation of IRF7 leading to induction of interferon- α . TLR2 and TLR4 signalling specifically recruits the second adapter, Mal, whose main function so far appears to be to act as a bridging adapter for MyD88 recruitment. TLR3 signals via Trif, which can specifically engage with the kinase TBK-1 leading to IRF3 activation. Target genes here include interferons. Finally, the fourth adapter, Tram, appears to be a bridging adapter for Trif recruitment specifically for TLR4.

been traditionally characterised to be inhibitors of the JAK-STAT signalling pathway [51]. It has been suggested that SOCS family members attenuate cytokine signal transduction by binding to phosphorylated tyrosine residues on signalling intermediates such as receptor chains and JAKs, thereby targeting these for degradation [52]. However, it seems that SOCS1 in particular may also play a role in the regulation of the innate immune response as expression of SOCS1 is induced by the TLR ligands LPS and CpGDNA [53, 54]. Macrophages from SOCS-1 deficient mice show hypersensitivity to LPS, leading to an increase in TNF- α and IL-12 production and also that SOCS1^{-/-} mice are more sensitive to endotoxin shock than wild-type littermates [55]. SOCS-1 has been suggested to target p65/RelA for degradation, giving a possible explanation for its effects on LPS signalling [56]. However, two reports have recently indicated that the inhibitory effect of SOCS1 on TLR signalling is indirect and occurs by inhibiting the type I interferon signalling pathway following IFN- β upregulation by TLRs and not by inhibiting the NF- κ B pathway [57, 58]. The actual role of SOCS-1 as a negative regulator of TLR signalling remains, therefore, to be confirmed.

PI3 kinase

The phosphoinositide 3-kinases (PI3K) are a conserved family of signal transduction enzymes that are involved in regulating cell growth and proliferation. Work done on the role of PI3K in innate immunity has implicated a role for this enzyme in negative feedback regulation in response to pathogens. The PI3K pathway has been shown to negatively regulate NF- κ B and the expression of inflammatory genes. Wortmannin, a specific inhibitor of PI3K, enhanced LPS-induced nitric oxide synthase in murine peritoneal macrophages and activation of PI3K-Akt suppressed LPS-induced lipoprotein lipase expression in J774 macrophages [59]. Recent studies done on both splenic and bone-marrow derived dendritic cells (DCs) from WT and PI3K^{-/-} mice have shown that PI3-K is an endogenous suppressor of IL-12 as the level of IL-12 production in the knockout cells was increased compared to wild type in response to LPS, PGN and CpGDNA [60]. Treatment of wild type BMDCs with Wortmannin also increased IL-12 production in response to LPS and PGN. A similar inhibitory role was identified in the human monocytic cell line THP-1, where the PI3-K/Akt pathway was inhibited using a dominant negative form of AKT and the PI3K inhibitor LY294002. These investigators reported that inhibition of PI3-K, enhanced LPS-induced MAPkinase activation and also enhanced LPS-induced nuclear translocation of NF- κ B [61]. Interestingly, it was previously shown that a signalling cascade from TLR2 involving the PI3-K/Akt pathway was necessary for p65 induced transactivation in THP-1 cells [62]. A previous report also indicated a proinflammatory role for PI3K in terms of IL-1 induced NF- κ B activation [63]. This conflicting evidence may reflect alternative roles for PI3-K with respect to cell types and the agonists used.

IRAKM

IRAK-M was originally identified by Wesche et al. and shows between 30–40% homology to other IRAK family members. The expression pattern of IRAK-M is, however, more restricted than that of the other IRAKs being expressed predominantly in peripheral blood leukocytes and only weakly expressed in other tissues. IRAK-M was found to be strongly upregulated in the human monocyte cell line THP-1 following treatment of the cells with TPA and ionomycin. This treatment causes differentiation of THP-1 to more mature macrophages indicating a possible role for IRAK-M in immune effector functions [64]. The IRAK-M knockout mouse confirmed a negative regulatory role for IRAK-M as macrophages from these mice are hyper-responsive to all TLR ligands tested including ligands for TLRs 2, 4 and 9 in terms of the level of proinflammatory cytokines produced by the cells. These macrophages showed increased NF- κ B and MAP kinase activation in response to LPS and CpG DNA stimulation [65]. Furthermore, IRAK-M knockout mice showed an increased inflammatory response to challenge with the bacteria *Salmonella typhimurium*. The site of colonisation of *Salmonella* in the intestinal tract is Peyer's patches. The Peyer's patches in the IRAK-M^{-/-} infected mice were increased in number and grossly enlarged compared to WT. As overexpression of IRAK-M inhibits the formation of IRAK/TRAF6 complexes these authors suggest that IRAK-M may operate by inhibiting dissociation of IRAK1 and IRAK-4 from the TLR complex by either inhibiting phosphorylation of IRAK1 and IRAK-4 or stabilising the TLR/MyD88/IRAK-4 complex [65].

IRAK-M also seems to play a role in endotoxin tolerance as the cytokine levels produced by IRAK-M^{-/-} macrophages upon LPS restimulation following priming with a non-lethal dose of LPS were not decreased as much as in restimulated wild-type macrophages. After 24 h of incubation, however, IRAK-M macrophages showed equivalently reduced levels of IL-6, TNF- α and IL-12 production indicating a possible second mechanism to mediate endotoxin tolerance. Human monocytes and monocytes isolated from septic patients show a more rapid upregulation of IRAK-M following a second LPS challenge [66]. IRAK-M has also been shown to be important in peptidoglycan induced tolerance [67].

Tollip

Tollip (Toll-interacting protein) was originally shown to interact with both the type 1 IL-1 receptor and also the IL-1R accessory protein following IL-1 stimulation. Tollip is able to block IL-1 signalling by forming a complex with IRAK1 and inhibiting IRAK1 phosphorylation [68]. It was subsequently shown that Tollip can also interact with several TLRs including TLR2 and TLR4 and that overexpression of Tollip inhibited TLR-mediated NF- κ B activation [69]. This effect was confirmed by Zhang

et al. who also showed that Tollip and unphosphorylated IRAK1 interact in the resting state. Upon stimulation, however, IRAK1 phosphorylates Tollip and this may facilitate its dissociation from IRAK1 and subsequent degradation. They hypothesise that Tollip is, therefore, an endogenous inhibitor whose role is somewhat similar to that of I κ B, i.e., to keep IRAK1 in an inactive resting state in the cytoplasm [70]. Additional evidence to point to a negative regulatory role for Tollip comes from data showing that in intestinal epithelial cells, which are hyporesponsive to TLR ligands, the level of Tollip is elevated compared with other cell types [71]. A possible mechanism of action of inhibition has been proposed by Li et al. who have recently identified a unique C2-like domain in the N-terminus of Tollip which may be involved in its inhibitory role. C2 domains are involved in binding of various phospholipids. These authors showed that Tollip preferentially binds to phosphatidylinositol-3-phosphate (PtdIns(3)P) and phosphatidylinositol-3,4,5-phosphate (PtdIns(345)P). A mutation of a critical lysine residue (K150) to glutamic acid (Tollip(KE)) within the C2 domain abolishes this binding. The Tollip(KE) mutant is unable to inhibit LPS-induced NF- κ B activation indicating that its lipid binding capability is in some way, as yet unknown, connected to the inhibitory role of Tollip [72].

Dok-1/Dok-2

Dok-1 and Dok-2 are adaptor proteins that negatively regulate Ras-Erk signalling downstream of protein tyrosine kinases (PTKs). Both Dok-1 and Dok-2 are tyrosine phosphorylated by PTKs and recruit multiple SH2-containing proteins such as p120, rasGAP and Nck [73]. Experiments using Dok1 and Dok2 knockout mice showed a critical role for these adaptors in the negative regulation of the Map kinase ERK in various haematopoietic cells [74]. As LPS has been shown to activate cytoplasmic PTKs including Lyn [75] and Btk [76], Shinohara et al. investigated the role of Dok1/2 in TLR signalling and showed that both adaptors are rapidly phosphorylated upon LPS stimulation. Macrophages from mice deficient in these adaptors showed elevated ERK activation upon LPS stimulation, but no elevated activation of the other MAP kinases or of NF- κ B. Overexpression of either adaptor, but not of Tyr mutant forms of the adaptors, inhibited LPS-induced Erk activation in macrophages. This effect was not seen with any other TLR ligands including CpG DNA, PolyI:C or Pam3Cys identifying both of these adaptors as constitutively expressed inhibitors of LPS [77].

Ubiquitination: A means of regulating TLR signalling

A general mechanism by which plasma membrane receptors and signalling intermediates may be regulated involves protein degradation mediated by a ubiquitination

pathway. In this pathway ubiquitin molecules are covalently attached to proteins in a three step process by three separate enzymes. The first enzyme is a ubiquitin activating enzyme termed E1, the second enzyme E2 is a ubiquitin conjugating enzyme and the third E3 is a ubiquitinating protein ligase that is essential for target protein recognition and catalyses the formation of an isopeptide bond between the ubiquitin chain and a lysine on the target protein. E3 ligases contain several domains including a RING finger domain [78]. Several components of the TLR signalling pathway have long been known to be ubiquitinated and degraded as a means of control of the pathway, with one of the best characterised being the inhibitory protein I κ B which initially becomes phosphorylated by the upstream kinases IKK α / β , causing NF- κ B to be released, and is then degraded [79]. Recently a novel RING finger containing protein termed Triad3A was identified through a hybrid yeast-2-screen using TLR9 and TLR4 as bait. Triad3A does not interact with TLR2 and in subsequent cell based assays it was shown that overexpression of Triad3A promoted degradation of TLR4/9 with a resulting decrease in signalling from these receptors but had no effect on TLR2. Ubiquitination of specific TLRs by Triad3A, therefore, represents another mechanism by which the intensity and duration of the TLR signal is controlled [80].

Conversely, it has also been shown that ubiquitylation may also be a mechanism for activation. In the TLR signalling pathway, the adaptor molecule TRAF6 must be ubiquitinated in order to activate the TAK1/TAB complex, to allow NF- κ B activation [81]. An additional level of control of this pathway is shown by the zinc-finger containing protein A20. A20 was originally identified as an inhibitor of NF- κ B activation induced by TNF- α receptor activation [82] and also inhibits IL-1 [83] and LPS [84] induced NF- κ B activation. A20 knockout mice develop severe organ inflammation due to their inability to terminate TNF and LPS induced NF- κ B activation [85] and macrophages from A20^{-/-} mice show elevated levels of proinflammatory cytokine production following stimulation with TLR2/3 and 9 ligands as well as LPS. A20 seems to control this pathway by cleaving the ubiquitin chain of TRAF6 and thereby causing its dissociation from the TAK/TAB complex [86]. A20 has also been shown to interact with IKK β and to be phosphorylated upon overexpression of IKK α , suggesting that A20 may also regulate TLR signalling at the level of the signalosome [87]. As several mammalian orthologues of A20 have been identified, these may also prove to play a role in the regulation of TLR signalling [88].

Conclusions

Activation of TLRs results in a proinflammatory response needed for the host to combat infection. Thus, limiting TLR signalling is essential for preventing what is meant to be a protective response from causing injury to the host and provoking disease states. This chapter has outlined the rapidly expanding area of known endoge-

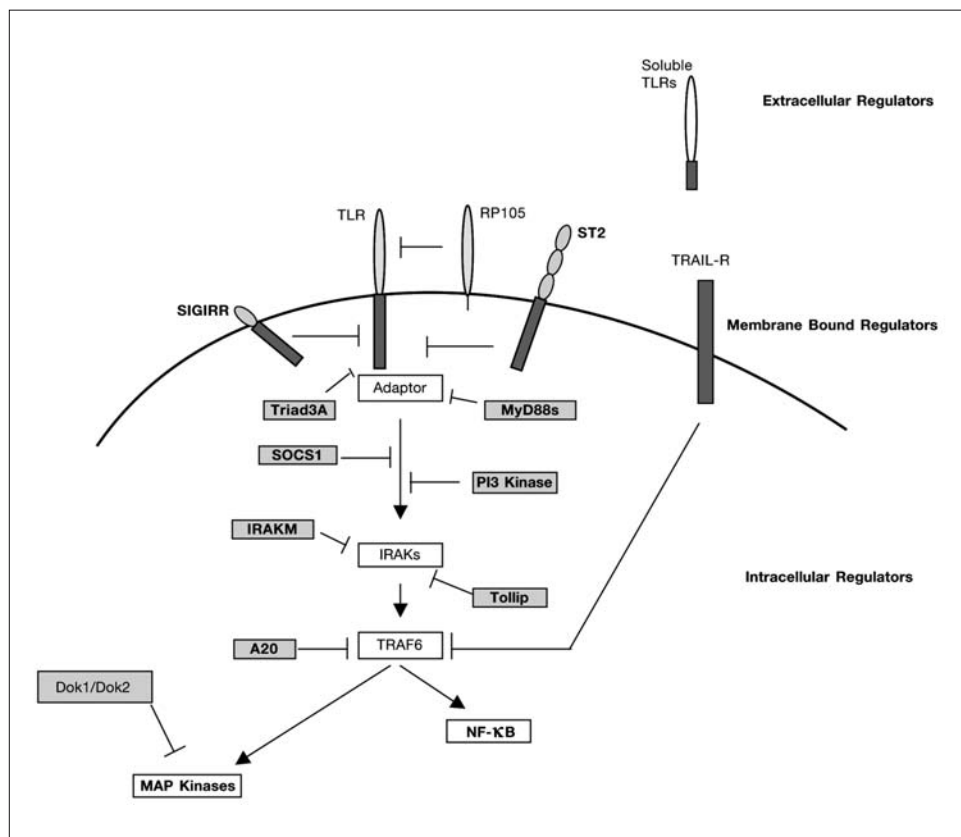


Figure 2

Multiple endogenous inhibitors of TLR action have been identified

Soluble forms of the ectodomains of TLRs have been described which presumably sequester ligands or complex with membrane-associated ectodomains. Another level of control is exerted by membrane bound inhibitory receptors such as ST2, SIGIRR, RP105 and TRAIL-R. Intracellular inhibitory proteins such as MyD88s, SOCS1, IRAK-M and Tollip also block signalling pathways. PI3-kinase negatively regulates some TLR responses through an as-yet unknown mechanism. Triad3A will promote ubiquitination and degradation of certain TLRs while A20 will have the same overall effect on Traf6.

nous regulators of TLR signalling. These regulatory mechanisms seem to be present at every possible level of the pathway from decoy receptors extracellularly, to membrane bound negative regulators to a whole host of intracellular proteins whose role is to continuously dampen-down the potentially detrimental signalling pathways activated by TLRs. All known inhibitory mechanisms are shown in Figure 2. Sever-

al other mechanisms including downregulation of receptors and also, finally, receptor induced apoptosis are also employed to ensure that TLR signalling is maintained under the strictest possible control. Of the receptors, TLR4 seems to be the most heavily regulated, highlighting its importance in the immune response. Most regulatory mechanisms seem to be present in order to control the activation of NF- κ B which given the importance of this transcription factor and the number of genes it can turn on, is not surprising. As discussed above, the only known inhibitors of the TLR induced MAP kinase pathway is the recently identified Dok1/2 proteins and interestingly, to date, no inhibitors of the TLR induced interferon pathway have been identified. Given the rapidly expanding nature of this field, however, it is likely that it is only a matter of time until endogenous regulators of this arm of the TLR pathway are identified.

Identifying the importance of these negative regulators in disease and inflammation is of fundamental importance. Clearly, any defect in the ability of these to perform their role in controlling the pathway could result in uncontrolled inflammation. It is possible that polymorphic variations of these proteins would cause an increased susceptibility to disease and strategies to upregulate expression of these proteins may prove effective in treating both chronic and acute inflammatory disease.

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Tuning of inflammatory cytokines and toll-like receptors by TIR8/SIGIRR, a member of the IL-1 receptor family with unique structure and regulation

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Introduction

Interleukin-1 receptors (IL-1Rs) and Toll like receptors (TLRs) are members of a large superfamily of phylogenetically conserved proteins involved in innate immunity and inflammation [1–4]. The common characteristics of the members of this family is the presence in their cytoplasmic region of a conserved sequence, called the Toll/IL-1R (TIR) domain, which is involved in the activation of a stereotypical signalling pathway leading to translocation of nuclear factor kappa B (NF- κ B) to the nucleus and activation of protein kinases such as p38 mitogen-activated protein kinase and JNK [5]. The family is divided in two subfamilies, depending on the structure of the extracellular region: TLRs bear leucine-rich repeats in the extracellular domain, whereas IL-1Rs bear Ig-like domains. TLRs act as sensors for the presence of microorganisms through the recognition of well identified and specific pathogen associated molecular patterns [2] and activate a complex, multifaceted cellular response. The IL-1R subfamily includes both receptors and accessory proteins for IL-1 and IL-18, which are involved in the initiation of an amplification cascade of innate resistance, contribute to the activation and orientation of adaptive immunity and play a key role in inflammatory conditions [6]. In both subfamilies several members remain orphan receptors with still unknown ligands and functions. The orphan receptor T1/ST2 is a member of the IL-1R family, preferentially expressed on T helper type 2 cells, that appears to be involved in the regulation of Th2 cell function [7, 8]. Recent results indicate that it plays a role in endotoxin tolerance, acting as an inhibitor of signalling by sequestration of the adaptors MyD88 and Mal [9]. Among mammalian TLRs, TLR10 has no characterised ligand so far [4].

The activation of the signalling cascade leading to the production of proteins related to inflammation and immunity by the IL-1R/TLR family members is tightly regulated. An uncontrolled or deregulated activation of these receptors can be detrimental as they mediate potentially devastating local and systemic inflammatory reactions. For the IL-1 system, which has served as a paradigm for the definition of signalling and regulatory mechanisms, the control is exerted at different levels, extracellularly and intracellularly [10]. IL-1R antagonists are pure polypeptide antagonists [11, 12] and the Type II IL-1R, which lacks a signalling domain, binds IL-1 preventing its interaction with a signalling receptor complex, both in membrane-bound or secreted form. In addition, it forms a non signalling complex with the accessory protein (AcP), thereby sequestering the AcP, rendering it unable to complex with IL1RI [13–15]. Finally, recent data suggest that in neutrophils IL-1RII also acts as a scavenger [16].

Cellular activation by members of the IL-1R/TLR receptor family is also regulated intracellularly by inhibitors of signalling. A negative regulation of signal transduction is exerted by IRAK-M [17], one of the members of the IL-1 receptor associated kinase (IRAK) family, which lacks kinase activity and regulates the dissociation of IRAK-1 and IRAK-4 from the adaptor protein MyD88, and by MyD88s [18], a spliced form of MyD88 which prevents IRAK-4 recruitment.

As discussed in other chapters of this book, TLRs are clearly implicated in the pathogenesis of a range of disease states. Disease might result from non-activation of TLRs or from a defect in negative regulators of the TLR signalling pathway. Recent evidence suggests that the IL-1R family member TIR8 (also known as SIGIRR) is an intracellular inhibitor of IL-1R/TLR signalling (Fig. 1). Mice lacking TIR8 develop colitis, attesting to the importance of negative regulators of TLRs in the gut epithelium in the limitation of inflammation. In this review, we summarise the current understanding of the structure and function of TIR8. Available information *in vitro* and *in vivo* is consistent with hypothesis that TIR8 negatively regulates IL-1R/TLR signalling by acting as a decoy for key components of the signalling cascade, playing a non-redundant and selective regulatory role in the control of inflammation in epithelial tissues and mucosal sites.

TIR8 gene and protein organisation

TIR8 was identified by our group in 1998 (Accession number: AF113795) and by others [19], by searching in EST databases TIR domain containing sequences.

The human TIR8 gene is localised on human chromosome 11, band p15.5 and is organised in 10 exons spanning about 11700 bp. Its overall structure suggests that TIR8 and IL-1RI evolved from a common ancestor. The murine TIR8 gene is localised on chromosome 7, band F4, and is organised in 9 exons spanning about

Negative pathways of regulation of IL-1 receptor (R) and Toll-like receptor (TLR) signalling
Modified from [10]. LPS, lipopolysaccharide; AcP, accessory protein; SIGIRR, single immunoglobulin IL-1-related receptor; TIR8, Toll/IL-1 receptor-8; IRAK, IL-1R-associated kinase; TRAF, tumour necrosis factor receptor associated factor; MAL, MyD88-adaptor-like; TIRAP, Toll/IL-1R domain-containing adapter protein; TRIF, Toll/IL-1R domain-containing adapter-inducing interferon (IFN)- β ; TOLLIP, Toll-interacting protein; SOCS, suppressor of cytokine signalling; JNK, c-jun NH2-terminal kinase; NF- κ B, nuclear factor- κ B; sIL-1ra, soluble IL-1R antagonist; iCL-1ra, intracellular IL-1ra.

Human TIR8 full length cDNA predicts a protein of 410 amino acids with unique and interesting characteristics: TIR8 is composed by a single Ig domain in its extracellular region (amino acids 17–112), contrasting with other IL-1R members, all of which contain three Ig domains; a transmembrane domain (amino acids 117–139); an intracellular conserved TIR domain (amino acids 166–305) which interestingly lacks two conserved residues, Ser447 and Tyr536, shown to be essential for the signalling of IL-1RI. Finally, TIR8 has a characteristic 95 amino-acid long tail. Among the IL1R/TLR family members, only *Drosophila* Toll is also

known to possess a 98 amino-acids long tail with inhibitory properties. Protein sequences of human and mouse TIR8 are 82% identical and show a 23% overall identity to IL-1RI.

There are five potential N-glycosylation sites in the extracellular region of human TIR8 and four in murine TIR8 and Western blotting analysis indicates that TIR8 is highly glycosylated [19].

TIR8 mRNA expression and regulation

Tissue expression array hybridisation and Northern blot analysis revealed that both human and murine TIR8 have ubiquitous expression in tissues, but that expression is particularly high in organs with an epithelial component such as the digestive tract, the kidney, the liver, the lung and also in lymphoid organs [19, 20]. However, cell-type expression is more specific being particularly high in epithelial cell lines and low or undetectable in leukocytes, fibroblasts and endothelial cell lines. When the high expression in the kidney was further dissected, TIR8 mRNA was found to be localised in the epithelial component and in particular in the proximal tubular cells but not in mesangial cells [20]. When TIR8 expression in leukocytes was examined in detail [20], resting and stimulated T and B lymphocytes and mononuclear phagocytes generally had low levels of TIR8 mRNA. Monocytes also expressed low levels of the transcript that were further reduced upon maturation to macrophages. Unexpectedly, immature DC derived from monocytes or bone marrow precursors, which have a definite relationship to the myelomonocytic differentiation pathway [21], expressed appreciable levels of TIR8 [22] while mature DC, obtained by exposure to LPS, expressed lower levels of the transcript. It remains to be established whether expression of TIR8 is shared by diverse DC populations, for instance plasmacytoid DC [21]. Finally, fresh and cultured NK cells expressed particularly high levels of the messenger RNA [20].

A variety of pro- and anti-inflammatory cytokines, microbial moieties and heat inactivated microbes failed to induce TIR8 expression in mononuclear phagocytes [20]. However, TIR8 transcripts were downregulated by *in vitro* or *in vivo* administration of LPS [20]. Other members of the IL-1R family are regulated by pro- and anti-inflammatory signals [13, 15, 23–25]. For instance the orphan receptor T1/ST2 [7] is upregulated by LPS [23]. Thus, the tissue and cellular expression of TIR8 as well as its regulation are distinct from those of other members of the IL-1R family.

TIR8 exerts a negative role in IL-1/TLR-induced NF- κ B activation

Ligands for TIR8 have not yet been identified. In particular, binding studies by surface plasmon resonance have demonstrated that TIR8 does not bind IL-1 α , IL-1 β or

IL-1 receptor antagonist, consistently with the fact that its extracellular domain is too short to fold around in an IL-1-like ligand [19].

Different studies using NF- κ B driven reporter systems have been performed to investigate the potential role of TIR8 in activating NF- κ B or in acting as an accessory protein [19, 20]. In contrast with what has been observed with other IL-1R family members as IL-1RI or AcP [26], forced overexpression of constructs containing the full length or truncated versions of the molecule did not activate NF- κ B expression, nor did it modulate IL-1RI-induced NF- κ B activation. In contrast, when cell were treated with IL-1, full length TIR8 inhibited IL-1RI-induced NF- κ B activation. Interestingly the inhibitory effect was lost when truncated TIR8 molecules were co-transfected with IL-1RI [20].

To study the role played by the extracellular and cytoplasmic domains of TIR8, chimeric molecules expressing the extracellular and transmembrane domains of the murine IL1RAcP and the full length or truncated versions of TIR8 were generated. In the presence of IL-1, the chimeric molecule containing the full length intracellular domain of TIR8 inhibited NF- κ B activation induced by the IL-1R complex. The blocking effect was lost when chimeric molecules containing truncations of the TIR8 intracellular domains were used [20].

TIR8 activity seems to be specific for IL-1R/TLR members, as it exerts its negative regulatory function on IL-1, IL-18 and several TLR ligand-dependent NF- κ B activation (see below), but not on IFN- γ -dependent STAT1 activation [27].

After IL-1 stimulation, receptor-proximal signalling components, including IRAK and TRAF6, are recruited to the IL-1R to form a receptor complex. After their appropriate activation at the receptor complex, these signalling molecules are released from the receptor to interact with and activate downstream components. TIR8 exerts its negative regulatory function in TLR-IL-1R-mediated pathways through its direct effect on the immediate signalling events, including NF- κ B and JNK activation. As suggested by co-immunoprecipitation studies, TIR8 interacts with IL-1R1, IRAK and TRAF6 after IL-1 treatment [27]. Thus, TIR8 may negatively regulate the IL-1 pathway through its interaction with the IL-1R complex by interfering with the appropriate recruitment and activation of the receptor-proximal signalling components, such as IRAK and TRAF6, key elements in the signalling cascade [26, 28–30]. Alternatively, it may attenuate the dissociation of the activated signalling components from the receptor, inhibiting the activation of downstream signalling events.

Collectively, these results suggest that TIR8 does not activate NF- κ B either alone or in concert with IL-1R1, as searches for an accessory function in signalling complexes have yielded negative results [19]. In contrast, TIR8 inhibits signalling from the IL-1R/TLR complexes. Inhibition is ligand-dependent, requires the intracellular portion of TIR8, while the extracellular domain is dispensable for blocking activity. Finally, TIR8 inhibits IL-1R and TLR signalling possibly by trapping IRAK-1 and TRAF-6.

TIR8-mediated negative control is cell-type specific

Involvement of TIR8 in immediate signalling events through the IL-1R/TLR family was investigated in primary cell types obtained from TIR8 deficient mice [22, 27]. Enhanced responses to IL-1 and LPS but not to TNF were observed in kidney cells and splenocytes [27], but not in bone marrow derived macrophages, as expected on the basis of undetectable TIR8 expression in this cell type. Immature DC, which among the myelomonocytic lineage are the cells that selectively express TIR8, were investigated in detail. TIR8-deficient DC, but not macrophages, showed increased responsiveness to LPS and CpG oligodeoxynucleotides in terms of production of cytokines and chemokines (IL-6, CXCL10, IL-12, IL-10) [22]. LPS and CpG interact with signalling receptor complexes which include TLR4 and TLR9, respectively [4]. The finding that TIR8-deficient DC show increased responsiveness to TLR agonists is consistent with its pattern of expression and its proposed function as a negative regulator of IL-1R/TLR signalling. Moreover, given the sentinel function of DC and their localisation at epithelial surfaces, the expression of TIR8 in this cell type is consistent with the view that this molecule has a regulatory role in epithelial tissues and at mucosal sites (see below). Therefore, the negative regulatory function of TIR8 is likely to be cell-type specific, given its differential expression in different cell types.

Relevance of TIR8 inhibitory effects in inflammatory conditions *in vivo*

Gene-targeted mice demonstrate that TIR8 acts as a non-redundant negative regulator *in vivo* in particular inflammatory conditions.

Susceptibility to LPS-induced inflammation was investigated with different models. Survival after endotoxin challenge was reduced in TIR8 deficient mice on a BALB/c background [27]. TIR8 deficient mice on a C57BL/6 $\times$ 129/Sv background showed normal systemic or local inflammatory reactions to LPS in terms of mortality, systemic cytokine production and leukocyte recruitment [22]. The apparent discrepancy between these results on the susceptibility to LPS is likely due to the different genetic background. The results may also reflect a differential involvement in the systemic toxicity of LPS to cellular components other than myelomonocytic cells and endothelial cells, which generally do not express TIR8 [20, 27] and are credited to play a central role in endotoxic shock [31, 32].

Given the expression of TIR8 in the intestinal tract and in DC, the role played *in vivo* by TIR8 in intestinal inflammation has been investigated. Dextran sodium sulfate (DSS)-induced colitis is due to a toxic effect of DSS on colon epithelial cells followed by phagocytosis by lamina propria cells and production of proinflammatory cytokines such as TNF- α , IL-6 and IL-1 [33, 34]. In the chronic phase, the slow regeneration after DSS damage to the colon epithelial barrier causes further perpet-

uation of intestinal inflammation by bacterial products from the lumen, activation of DC and of a T cell mediated colitis [35]. Recognition of microbial moieties of the enteric flora and production of inflammatory cytokines, in particular IL-1 α and IL-18, play a key role in intestinal inflammation both in experimental systems and in humans [36–39]. TIR8 deficient mice showed increased severity of colitis induced by DSS, in terms of local tissue damage and mortality ([22] and X. Li, unpublished). The observation of increased severity of colitis in TIR8 deficient mice is consistent with a non-redundant regulatory role of this molecule in the gastrointestinal mucosa. It is conceivable that increased production of inflammatory cytokines in response to tissue damage and exposure to microbial molecules by DC in the lamina propria and possibly by epithelial cells is responsible for a more severe colon inflammation in Tir8^{-/-} mice.

The restricted pattern of expression of TIR8 and the selectivity of the inflammatory phenotype of deficient mice are consistent with a selective regulatory role of TIR8 in epithelial tissues and mucosal surfaces.

Concluding remarks

Thus, TIR8 represents a negative pathway of regulation of the IL-1R/TLR system, with a unique pattern of expression in epithelial cells and DC, crucial for tuning inflammation at mucosal surfaces, in particular in the gastrointestinal tract. Further research is needed to explore the role of TIR8 in controlling inflammation at other mucosal tissues and to investigate its expression and involvement in the pathogenesis of human diseases, in particular in inflammatory bowel disease.

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Toll-like receptors as pharmacological targets

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The toll-like receptors (TLRs) are the first responders in the major pathway by which the immune system detects infection or damaged tissue. Through the recognition of microbial products and endogenous molecules released from injured tissue, TLRs provide a critical link between the innate and the adaptive immunity [1]. Since the first human TLR was identified in 1997 [2], ten additional TLRs have been described in mammals [3, 4]. Furthermore, more than 30 molecules from *Xenopus*, *Drosophila* and plants were added to what is now collectively known as the interleukin 1 receptor (IL-1R)/TLR superfamily [5]. Considerable information has been collected on the structure, function and signaling of the TLRs. The biological function of these receptors as sensors of infection and tissue damage makes them attractive drug targets for designing vaccine adjuvants and for the treatment of immune related disorders including inflammation, infections, autoimmunity, allergies and cancer.

In the present chapter we address the rationale for the exploitation of TLRs as drug targets based on the existence of naturally occurring agonists and antagonists. In addition, existing synthetic molecules described to interact with the receptors leading to enhanced or blocked signaling are reviewed. Finally we describe the design of a novel, low molecular weight, systemically active inhibitor of IL-1R signaling. The pharmacological effects of this molecule are described as an example of a rational drug design to block the intracellular signaling of a specific member of the IL1R/TLR family. The structural and biochemical aspects of TLRs will be summarized as necessary only to improve clarity of the concepts exposed. Detailed and comprehensive reviews of such aspects are covered by other contributors to this volume and within the published literature over the past eight years.

Introduction

TLRs are homologues of Toll, a protein responsible for the establishment of dorso-ventral polarity in the developing embryo of *Drosophila melanogaster* [6]. TLRs are

members of the IL-1R/TLR superfamily which includes the IL-1R and the adaptor myeloid differentiation primary-response protein 88 (MyD88) [5] containing a cytosolic conserved domain known as Toll/IL-1R (TIR) domain. To date, 11 mammalian TLRs (TLR1-11) have been identified. They are cell surface receptors (TLR1, TLR2, TLR4, TLR5, TLR6, TLR10, TLR11) or endosomally expressed receptors (TLR7 and TLR9). Each of these TLRs recognizes a different class and in few cases a single element of bacterial or viral components.

Tissue damage attributed to infection or sterile inflammation is believed to participate in the initiation and progression of numerous pathologies including cardiovascular and neurodegenerative diseases. Microbial products or host-derived molecules are released from infected or injured tissue and cells, and the recognition of these products leads to receptor activation. Inflammation is sustained by proinflammatory cytokines whose synthesis and release are initiated by signaling through the TLRs. In addition, some inflammatory cytokines including interleukin-1 β (IL-1 β), act directly through a receptor belonging to the IL-1R/TLR superfamily.

Targeting of the IL1R/TLR family therapeutically has already proven to be important in some applications, such as the clinical use of the interleukin 1 receptor antagonist (IL-1ra) (KineretTM) for the treatment of rheumatoid arthritis (RA). A major area of interest for the development of therapeutics acting on the TLRs is the treatment of bacterial and viral infections. Sepsis is a severe illness caused by overwhelming infection of the bloodstream by toxin-producing bacteria and is a major cause of mortality following systemic bacterial infection. Original studies have shown that mice deficient in the adaptor proteins involved in TLR signaling: MyD88 [7] or signaling kinases such as the IL-1 receptor associated kinase 4 (IRAK4) [8] are resistant to microbial and the lipopolysaccharide (LPS) induced sepsis. Furthermore, the LPS receptor TLR4 is regarded as the most promising drug target for treatment of sepsis caused by Gram-negative bacteria [9–11]. Viral DNA is predominantly recognized by TLR9 and studies have demonstrated that its stimulation may enhance antiviral immunity. In addition to TLR9, the receptors TLR3, TLR4, TLR7 and TLR8 were also demonstrated to be of importance in achieving antiviral effects [12–18].

In development of vaccines and in cancer immunotherapy, the elaboration of protective immunity requires the participation of components of innate and adaptive immunity. As a critical link between these two components of the immune response, TLRs represent an attractive target for development of vaccine adjuvants. As the mechanism of action of several presently used adjuvants was never clearly elucidated, it remained difficult to develop more potent alternatives. With the discovery of TLRs it is becoming clear that adjuvants work via specific activation of one or more of the TLR receptors. For example, the bacillus Calmette-Guerin (BCG) cell wall adjuvant activates TLR2/4 on dendritic cells enhancing their antigen presentation. Adjuvants based on dsRNA have been shown to activate TLR3 on dendritic cells and induce type 1 interferon secretion leading to tumor cell apoptosis and

NK-cell mediated cytotoxicity [19]. In a study that measured the efficacy of targeting specific TLRs to stimulate cytotoxic T lymphocytes in response to viral particles, the best adjuvant effects were obtained by stimulating TLR9 while effects were moderate with stimulation of TLR3, TLR5 and TLR7 and absent with activation of TLR2 and TLR4 [20]. Studies identifying key TLRs necessary for the recognition of viral particles suggest that designing small molecules to target specific TLRs can lead to new adjuvants [21]. The TLRs have also been implicated in modulating the balance between cellular and humoral immunity and may thus also be useful in the treatment of allergic diseases.

TLRs as drug targets

Stimulation of TLRs leads to interaction with MyD88 or other members of the MyD88 family of adaptors. This family includes the TIR containing adaptor protein (TIRAP or MAL), the TIR-domain-containing adaptor protein inducing interferon- β (TRIF), the TRIF-related adaptor molecule (TRAM) and the sterile α - and armadillo motif-containing protein (SARM). The MyD88 transduction pathway ultimately leads to the activation of the nuclear factor κ B (NF- κ B) via the sequential recruitment/activation of the IL-1 associated kinase (IRAK), the tumor necrosis factor receptor associated factor 6 (TRAF6) and the I κ B kinase (IKK) (see [4] for a comprehensive review). Each of these adaptors, including IKK β are well studied drug targets on their own. This review will concentrate on the first few steps of this signaling, namely: (A) TLR extracellular ligand interaction, and (B) TLR-adaptor protein interaction (Fig. 1).

Specificity in the targeting of TLRs can be achieved by agonists/antagonists that act at the extracellular ligand binding domains, or by designing molecules that interfere with the protein-protein interaction of the adaptors initiating the signaling cascade. Examples of naturally occurring molecules or therapeutic applications that targets TLRs at both the extracellular and the signaling cascade exist for the IL-1 system.

For instance, blocking IL-1R or TLRs could be achieved with specific antibodies. Another strategy is blocking the receptor with an antagonist. A receptor antagonist must bind the receptor with both high specificity and high affinity (affinity at least comparable to that of the agonists) and preferably have a slow dissociation rate from the receptor. A *bona fide* endogenously occurring receptor antagonist for IL-1 β , the IL-1ra, exists for the interleukin receptor. In many systems it was shown that IL-1ra competes with IL-1 binding to IL-1RI but does not induce IL-1 signaling [22]. Human recombinant IL-1ra is already used as a therapeutic agent to treat RA, and is sold under the trade name KineretTM (Anakinra) [23].

Blocking the receptor signaling pathway represents another avenue for designing drugs to TLRs. Lessons can be learned from naturally occurring proteins that inter-

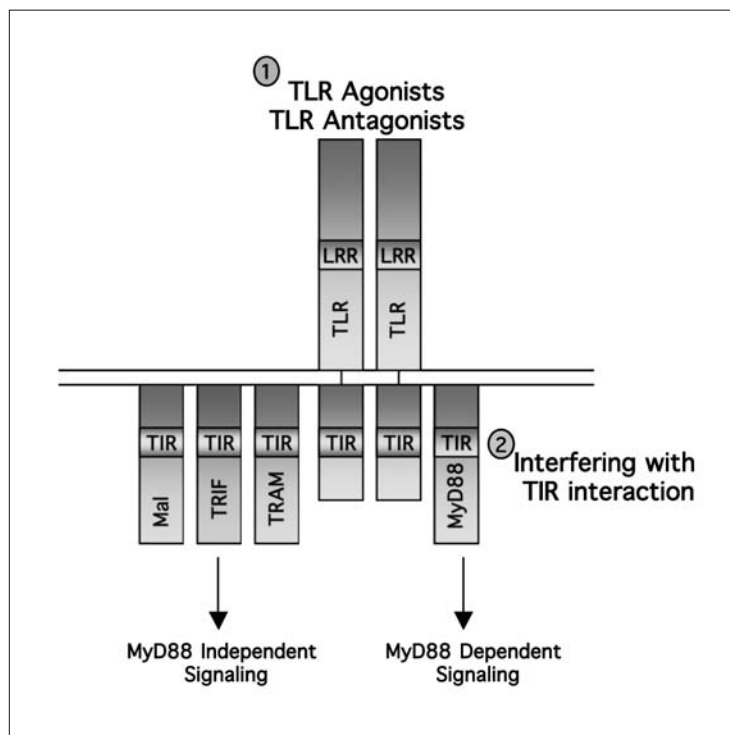


Figure 1

Schematic representation of the Toll-like receptor and the adaptor molecules

Two main sites of action of molecules that can specifically target the TLR activation or signaling are indicated. (1) TLRs can be targeted specifically with synthetic agonists or antagonist extracellularly. (2) TLR signaling can be modulated by small molecules interfering with the protein–protein interaction at the TIR domain between TLRs and adaptor molecules. Abbreviations: TLR, Toll-like receptor; TIR, Toll/IL-1R (TIR) domain; LRR, leucine-rich repeat (LRR) domain; MyD88, myeloid differentiation primary-response protein 88; TIRAP, TIR containing adaptor protein; TRIF, TIR-domain-containing adaptor protein inducing interferon- β ; TRAM, TRIF-related adaptor molecule

fere with TLR signaling. The Toll/IL-1R 8 (TIR8), also known as single Ig IL-1-related receptor (SIGIRR) [24] is an orphan receptor member of the IL-1R family with a single extracellular Ig domain and an intracellular TIR domain. The SIGIRR receptor has been shown to inhibit NF- κ B activation by interacting with TRAF6 and IRAK1, representing a negative pathway of regulation of the IL-1R/TLR system [25]. As SIGIRR is a transmembrane protein it may also interact with the extracellular domains of other TLRs and modify their signaling. Another example is the

Toll-interacting protein (Tollip). Tollip forms a complex with IRAK and inhibit its phosphorylation blocking IL-1 as well as TLR2 and TLR4-induced signaling [26, 27].

Targeting TLRs with specific ligands

Several synthetic TLRs ligands are available. Most of them are specific for one TLR and are being tested in different applications (Fig. 2).

TLR3: dsRNA

Novel therapeutic vaccinations are being developed to activate tumor antigen-specific T cells and prolong their activity in the host. Adjuvants based on dsRNA directly stimulate TLR3, present on myeloid dendritic cells and T cells in humans [28]. Poly I:C was the first dsRNA to be used clinically in leukemia and HIV patients for its ability to stimulate type I interferon [29]. However, it produced toxic side effects in many patients. Modification of the poly I:C structure by the introduction of unpaired uracil and guanine bases resulted in a unique dsRNA, poly I:C12U, which is associated with reduced toxicity in humans [30]. Recently a vaccination protocol was described and tested in patients with ovarian cancer, where the TLR3 agonist was used as an adjuvant with conventional chemotherapy to counter immunosuppressive effects generated by the tumor and to exploit tumor-derived exosomes as a source of cancerous antigens to generate antigen-specific T cell immunity. This polymer (AmpligenTM) is currently produced for intravenous administration by Bioclones PTY in South Africa. Methods for the production and purification of clinical GMP-grade exosomes have recently been developed, and the Phase I clinical trial is pending to test the potential value of tumor-derived exosomes for immunotherapy [31].

TLR4: Lipid A mimetics

Eritoran (E5564) is a synthetic lipid A analogue (α -D-glucopyranose) that has been shown to antagonize the effects of LPS through its interactions with TLR4 [32, 33]. Clinically, Eritoran is being investigated for the treatment of severe sepsis, septic shock, and other endotoxin-mediated indications. Results from *in vitro* studies have found Eritoran to be a highly active antagonist of the action of LPS on responsive cells. However, low amounts of Eritoran (300–500 μ g) injected into animals display a relatively short pharmacodynamic half-life in blood or plasma that is observable in the absence of clearance or measurable metabolism. *In vivo* studies

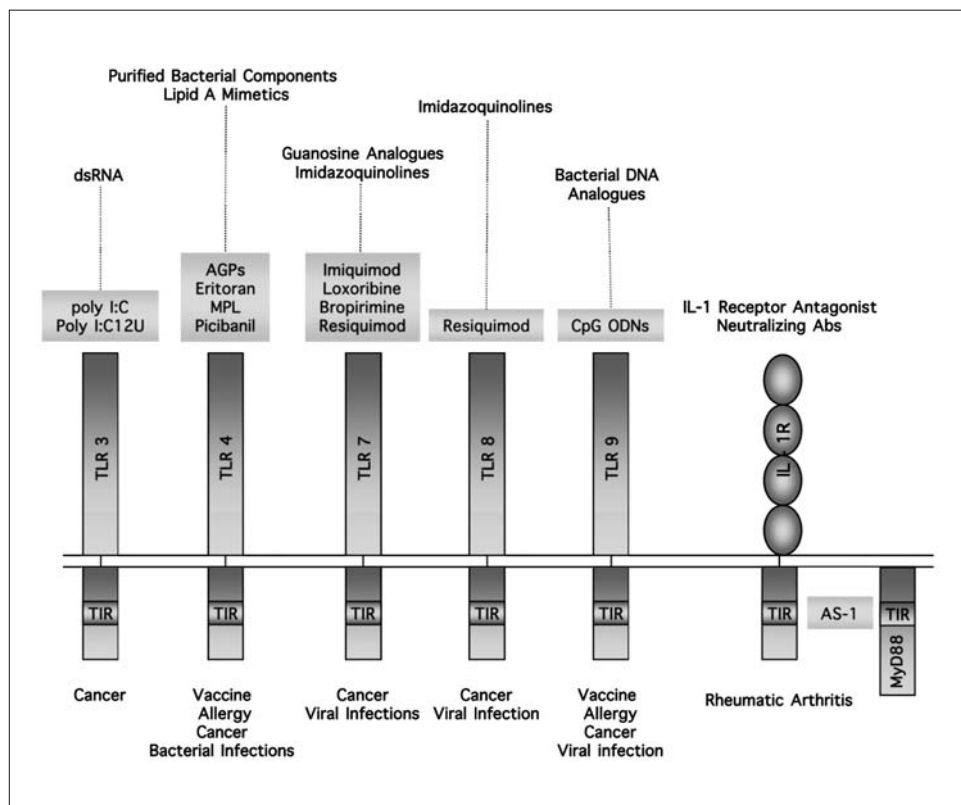


Figure 2

Schematic representation of the synthetic molecules described in the text to stimulate or inhibit specific Toll-like receptors

Each compound is assigned to the specific TLR it targets and to the therapeutic application which is believed to be applicable or is being tested for. The name compounds are given in the shaded boxes above the respective targets and the class of molecules they belong to is provided above. Refer to the text for detailed explanation.

of low doses administered as short infusions into humans further support this observation by demonstrating that Eritoran is extremely active when co-administered with LPS, but its activity decreases shortly after ending infusion [34]. Higher doses of Eritoran (12–252 mg) infused intravenously over 72 h demonstrated extended pharmacodynamic activity by blocking the effects of LPS in a human model of clinical sepsis [32]. Dosing of Eritoran by continuous infusion or intermittent dosing even at the highest doses has been shown to be safe and effective. A very recent study has shown that long-term infusions of high doses of Eritoran

(2000–3500 µg/h for 72 h) provide *ex vivo* LPS antagonist activity that persists for at least 72 h after infusion, indicating that *in vivo* protection against LPS may be maintained after the infusion has been discontinued [35]. It was further demonstrated that Eritoran displayed LPS-antagonist activity using a human endotoxemia model [36]. Combined results from these studies indicate that Eritoran is an effective *in vivo* antagonist of LPS, and may prove to be of benefit in a variety of endotoxin-mediated diseases.

A class of synthetic lipid A mimetics, the aminoalkyl glucosaminide 4-phosphates (AGPs), have been engineered specifically to target human TLR4 and are showing promise as vaccine adjuvants, and as therapeutic agents capable of inducing protection against a wide range of infectious pathogens. It has been demonstrated that intranasal immunization with pathogens mixed with the synthetic adjuvant RC529 in aqueous form induces higher titers of serum and mucosal antibodies against the pathogens in mice [37]. Baldrige et al. recently showed that two AGPs, RC524 and RC529, can induce a protective innate immune response that involves activation of TLR4 as well as stimulating the release of proinflammatory cytokines [38].

Picibanil (OK-432) is a lyophilized penicillin-inactivated preparation of a low-virulence strain of *Streptococcus pyogenes*. It has been demonstrated that TLR4 signaling is involved in regulating anticancer immunity in mice [39], and that oral cancer patients who lacked expression or displayed reduced expression of TLR4 or MD-2 gene, did not achieve a therapeutic response to Picibanil [40]. Clinical Phase I and Phase II studies have demonstrated therapeutic effects in patients receiving intra-tumoral administration of dendritic cells in combination with Picibanil and a therapeutic effect was obtained in oral cancer patients [41, 42].

TLR7 and TLR8: Imidazoquinolines, loxoribine and bropirimine

Synthetic low molecular weight compounds of the imidazoquinoline family, imiquimod (Aldara, R-837, S-26308), resiquimod (R-848), S-27609, and guanosine analogues such as loxoribine and bropirimine have been shown to activate TLR7. Both imiquimod and resiquimod were unable to induce dendritic cell maturation or TNF- α , IL-12, or IFN- γ production in TLR7-deficient mice [43]. It was further demonstrated that imiquimod and resiquimod induce NF- κ B activation in HEK293 cells transfected with human or mouse TLR7 [44]. However, resiquimod only activated NF- κ B in HEK293 cells transfected with human TLR8 and not in those transfected with mouse TLR8 [45]. Many studies have indicated that the imidazoquinoline family compounds have potent antiviral and antitumor properties in multiple animal models of infection. Imiquimod was also shown to be effective against arbovirus and cytomegalovirus in humans [46]. The activity of imiquimod is mediated predominantly through the induction of cytokines includ-

ing IFN- γ and IL-12. Topical imiquimod therapy is used for the treatment of external genital and perianal warts caused by Papilloma virus infection [17]. The FDA has recently approved imiquimod for the treatment of actinic keratoses, and there is mounting evidence that imiquimod is an effective treatment of certain types of skin cancer [47, 48]. Resiquimod is a more potent analogue of imiquimod, and trials are under way to assess its use in treatment of genital herpes and hepatitis C virus [49].

Loxoribine is a very powerful stimulator of the immune system producing its effects through the enhancement of natural killer (NK) cell activity, B lymphocyte proliferation, and by stimulating the production of interferons [50]. Bropiramine is an orally active immunostimulant that increases endogenous IFN- γ and other cytokines, and is used clinically against carcinoma of the bladder and upper urinary tract [51, 52]. Gorden and colleagues at 3M Pharmaceuticals recently described novel synthetic selective agonists for TLR7 (3M-001) and TLR8 (3M-002). The two compounds were used to provide evidence that TLR7 and TLR8 are functionally distinct in human innate immune cells [53]. The data suggest that TLR8 agonists may be effective at driving Th1-like immune responses requiring myeloid dendritic cell activation, whereas TLR7 agonists may be important in driving Ig production. The recent development of TLR7 and TLR8-selective agonists could be used to define the roles of these two receptors in both the innate and adaptive immune response.

TLR9 ligands: Bacterial DNA analogues

Synthetic analogues of bacterial DNA, termed CpG oligodeoxynucleotides (ODN) have shown a great promise in mobilization of protective immunity against pathogens through their general ability to stimulate B cells, NK cells, DC, and monocytes/macrophages [54]. Immune activation by CpG ODN depends on the presence of TLR9, as mice genetically deficient in this receptor show no CpG-induced activation of B cells, DC, or NK cells [55]. Transfection of TLR9 in HEK293 cells causes these cells to become CpG-responsive, as cells expressing mouse TLR9 become responsive to the preferred mouse CpG motif, and cells expressing human TLR9 become responsive to the preferred human CpG motif [56, 57].

Studies indicate that CpG DNA can stimulate the innate immune response, improving resistance to infection induced by various pathogens [58]. By slowing the early growth and spread of these pathogens, CpG treatment increases the ability of the host to mount an adaptive immune response. In addition to the immunostimulatory effects, CpG DNA promotes the initiation of Th1-type immune responses by increasing the production of proinflammatory cytokines and inducing the maturation/activation of professional antigen presenting cells. When CpG ODNs are used as vaccine adjuvants, they promote the immunogenicity of co-administered anti-

gens. Also, CpG ODNs either administered alone or mixed with allergen are able to reduce susceptibility to allergic diseases.

Multiple Phase I human clinical trials have been designed to explore the safety and immunostimulatory properties of CpG ODNs administered alone, or in combination with vaccines, antibodies or allergens. Several Phase II studies are also underway to evaluate the therapeutic potential of CpG ODNs in the treatment of cancer, allergy and asthma, or as vaccine adjuvants. Studies have investigated the use of CpG ODNs to reduce allergic rhinitis and immunization of allergen mixed with CpG ODN, allergen-CpG ODN conjugates, and CpG ODN alone have proved effective in the reduction of the allergic phenotype in mice [59]. Preliminary results using vaccines containing allergen-CpG ODN conjugates in human patients show that this combination reduces allergic symptoms with relatively few adverse reactions [60]. Clinical trials have used CpG ODNs as vaccine adjuvants co-administered with the Engerix B hepatitis B vaccine and the Fluarix influenza vaccine [61, 62]. Healthy adult volunteers were immunized with the vaccine plus CpG ODNs. The immunized subjects responded favorably to the treatment, showing increased antibody titers and increased release of interferons. All groups of subjects however reported short lived adverse reactions such as injection site reactions and flu-like symptoms.

The administration of CpG ODNs could have therapeutic potential for the treatment of HSV-2, as it was demonstrated that HSV-2 DNA directly stimulates TLR9 [63]. Recent studies in animal models of genital herpes have established that local-vaginal delivery of immunostimulatory CpG ODNs is effective against both primary and recurrent genital herpes infection and disease [64] (see [65] for further review in CpG ODNs applications).

Purified bacterial components

Monophosphoryl lipid A (MPL) is a purified lipopolysaccharide extracted from the cell walls of *Salmonella minnesota* developed by Corixa Corporation (Seattle, Washington, USA). MPL has been used as a potent adjuvant in allergy vaccines, promoting the development of Th1-type immune response. Antigen presenting cells are stimulated by MPL both *in vivo* and *in vitro* leading to the production of IL-12, IL-1, TNF- α and granulocyte-macrophage colony stimulating factor [66]. In humans, MPL activates both human dendritic cells and T cells and promotes the production of IL-10, IL-12 and TNF- α from monocytes and peripheral blood mononuclear cells [67]. A recent review by Francis and Durham identifies cases in which MPL has proven to be safe and effective as a vaccine adjuvant in over 120,000 human doses [68]. In addition to its use as a vaccine adjuvant, therapeutic uses for MPL are being explored in seasonal allergies. It was shown that MPL was effective in preventing seasonal allergies in children and adults when administered over a period of 4 weeks [69, 70].

Targeting TLRs-adaptor protein interactions

AS-1: Rational design of drugs interfering with the interaction between MyD88 and TLR

A pharmacological alternative to targeting TLRs using extracellular ligands is interfering with their intracellular signaling. This modulation can be achieved by targeting the protein–protein interaction between the TLRs and the adaptor proteins that couple the ligand binding to the intracellular signaling cascades. During infection, rapid microbial replication can result in a large excess of agonist that can be counteracted only with large doses or very selective antagonists binding with high affinity. Targeting of adaptor molecules would block signaling regardless of increasing amounts of agonist and may represent an attractive alternative to the use of ligands or antagonists to treat inflammation.

Blocking the TLR receptor(s) interaction with MyD88 has proven to be an evolutionary selected strategy to evade the immune system in vaccinia virus (VV) [71–73]. At least two VV proteins have evolved for viral immune evasion by interfering with TLRs. The viral protein A52R can block the activation of NF- κ B by multiple TLRs by interacting with IRAK2 and TRAF6 [71, 72]. The TIR domain-containing VV protein A46R inhibits TLR-induced signaling by associating with the TIR-domain containing adaptor molecules MyD88, Mal, TRIF and TRAM [72, 73]. In addition, dominant negative forms and null mutations of MyD88 have recently been shown to preclude bacterial product or IL-1-mediated activation of NF- κ B pathways, demonstrating that MyD88 is an essential component of TLR signaling [74].

The structural basis for TIR-mediated homotypic interactions were resolved [75], making possible the rational design of drugs inhibiting the interaction of MyD88 and TLR. This interaction involves five stranded parallel β -sheets and five surrounding α -helices interconnected by loops in a large conserved surface with consensus sequences (F/Y)-(V/L/I)-(P/G) in multiple Toll receptors and MyD88 homologs [75]. On this basis, a low molecular weight analog of the central 3-aa sequence of the BB-loop, hydrocinnamoyl-L-valyl pyrrolidine (AS-1), was synthesized [76] (Fig. 3). AS-1 is a very small molecule of only 340 Da, far smaller in size than the average drug (ca. 600 Da). The size of the AS-1 molecule allows for the possibility of generating derivatives with increased affinity and selectivity by attaching additional groups to it. Such work is in progress in multiple companies. One aspect of these studies involves the engineering of specificity into the blockade of MyD88/TLR interaction. While the BB loops are well conserved in the TIR domains of the different TLRs, they are not 100% homologous giving a possibility for a TLR specific MyD88-mimic design. AS-1 itself does not block several TLR-mediated signaling events in the range of concentrations shown to block IL-1RI signaling. In addition, AS-1 analogs do not block IL-1RI mediated signaling but block other

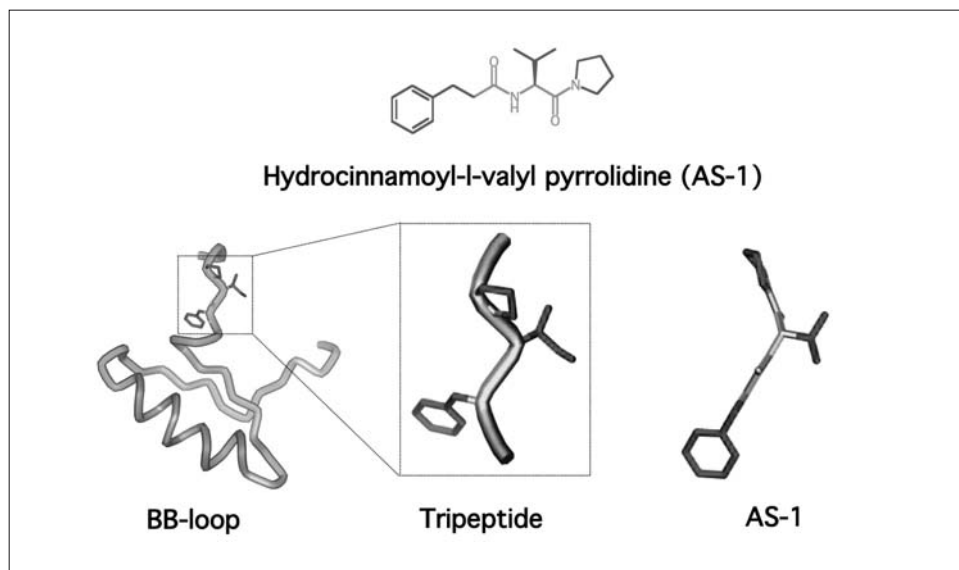


Figure 3

Structure and schematic representation of the rational design of hydrocinnamoyl-L-valyl pyrrolidine (AS-1)

AS-1 was designed to mimic the tripeptide sequence of the BB-loop [(F/Y)-(V/L/I)-(P/G)] of the TIR domain based on the crystal structure of TLR2.

TLRs ([76]; personal communication from Novartis). Several experiments demonstrated the ability of AS-1 to interfere with the interactions between mouse MyD88 and IL-1RI at the TIR domains [76]. AS-1 inhibited IL-1 β -induced, but not LPS-induced phosphorylation of the mitogen-activated protein kinase p38 in EL4 cells and in freshly isolated murine lymphocytes in a concentration-dependent manner. Furthermore, AS-1 inhibited the co-immunoprecipitation of IL-1RI and MyD88, but not the association of TLR4 and MyD88, suggesting that its site of action is indeed at the interface of IL-1RI and MyD88 [76] (Fig. 4).

The febrile response represents a simple measurement of one of the numerous *in vivo* effects of IL- β . Intraperitoneal injection of AS-1 significantly attenuated IL-1 β -induced fever response *in vivo* at a dose of 200 mg/kg. AS-1 did not block LPS-induced fever, again suggesting that AS-1 may be specific for IL-1R signaling (the pharmacokinetics of the AS-1 has not been optimized, thus it was not possible to demonstrate whether full blockade could be achieved at other doses and time points). It is clear that AS-1 is systemically active and that it crosses the blood brain barrier to block IL-1 receptors in anterior hypothalamus, the proposed site of IL-1 and PGE2 pyrogenic action. Taken together these results indicate that it is possible to generate

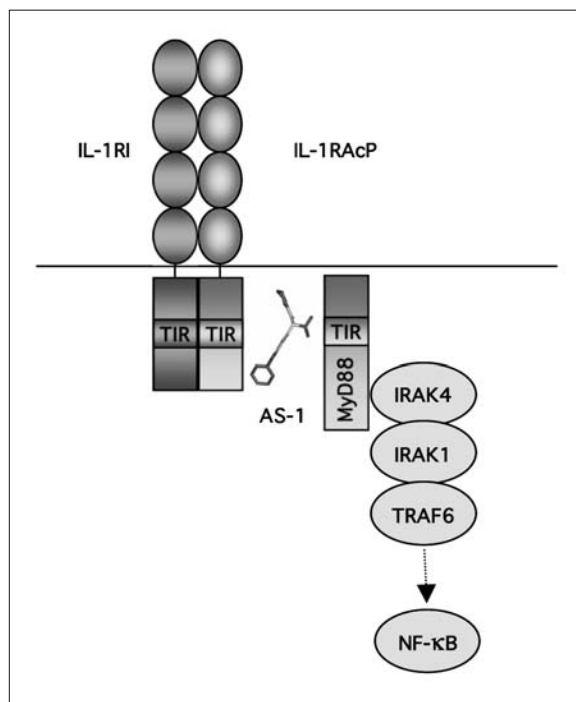


Figure 4

Schematic representation of the proposed disruption by AS-1 of the association between MyD88 and the IL-1R/RACp complex

As a result, AS-1 inhibits IRAK/TRAF signal transduction and the activation of NF-κB.

small molecules to mimic the *in vitro* and *in vivo* effects of the dominant negative form of MyD88 or the effects of the vaccinia proteins A46R, and A52R. The results also suggest an intracellular site for anti-inflammatory drug action and represent a proof of principle for the development of small molecules to interrupt protein–protein interactions in the cell. Thus, AS-1 may prove to be of pharmacological importance since blockade of IL-1 signaling has therapeutic effects in sepsis as shown by the recently introduced IL-1R antagonist (KineretTM, Anakinra) in clinical practice.

Conclusions

TLRs represent attractive drug targets for the modulation of the immune response and hold promising applications for the treatment of infection and inflammation as well as for the development of vaccine adjuvants. Two main strategies for targeting

TLRs seem to be promising for drug development: targeting TLRs with synthetic agonists or antagonists and targeting the protein–protein interaction involved in the TLR activated signaling cascade. Specifically, blockade of TIR/TIR interaction between TLRs and adapter proteins by low molecular weight is particularly encouraging for the development of orally available agents.

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