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THE ROLE OF TOLL LIKE RECEPTORS IN INNATE IMMUNITY

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ABSTRACT

Toll-like receptors (TLRs) are the best-characterized membrane-bound receptors in innate immune cells, including macrophages and dendritic cells. These evolutionary conserved receptors, homologues of the Drosophila Toll gene, recognize highly conserved structural motifs only expressed by microbial pathogens, called pathogen-associated microbial patterns (PAMPs). PAMPs include various bacterial cell-wall components such as lipopolysaccharides, peptidoglycans and lipopeptides, as well as flagellin, bacterial DNA and viral double-stranded RNA. Stimulation of TLRs by PAMPs initiates a signalling cascade that involves a number of proteins. This leads to the activation of the transcription factor NF-kB, which induces the secretion of pro-inflammatory cytokines and effector cytokines that direct the adaptive immune response. TLRs are predominantly expressed in tissues involved in immune function, such as spleen and peripheral blood leukocytes, as well as those exposed to the external environment such as lung and the gastrointestinal tract. Thirteen TLRs (named simply TLR1 to TLR13) have been identified in humans and mice together, and equivalent forms of many of these have been found in other mammalian species. In many cases, TLRs need the presence of co-receptors to initiate the signalling cascade, like CD14. Studies on TLRs indicate that these receptors are essential elements in host defence against pathogens by activating the innate immunity, a prerequisite to induction of adaptive immunity. The detailed study of TLRs will bring us closer to understanding the role of TLR mediated responses and increase our range to treat infectious and immune diseases.

KEYWORDS: TLRs, PAMPs, lipopeptides, flagellin, bacterial DNA, viral double-stranded RNA.

1. INTRODUCTION

The mammalian innate immune system acts as a sentinel by facilitating the efficient recognition of infectious microbes and providing protective mechanisms that eradicate microbial infections. In this context, Toll-like receptors (TLRs) are the best-characterized innate receptors, can be rapidly activated, and consist of functional modules that provide crucial host defense during microbial infection (1). Different TLRs sense the unique molecular signatures of microbes and trigger the innate immune system. Pathogens possess several components that are not found in the host, and have been referred to as pathogen associated molecular patterns (PAMPs). These molecules elicit strong responses from the innate immune system and are quite common among a broad range of pathogens. PAMPs are the molecular targets of the innate immune response. Three main features of PAMPs are: (i) they are usually expressed by microbes and not by host cells, (ii) they show little variation among microorganisms of a given class, and (iii) their expression is essential for the survival of the microbes. Whereas the first two characteristics allow class recognition of microbes and not of the host cells, the latter prevents the development of mutants which escape recognition by the host immune system. Microbial carbohydrates like lipopolysaccharides (LPS) are a good example of PAMP, which are common structures present on the cell walls of many species of bacteria. In addition, it is now clear that a range of other bacterial molecules, such as CpG DNA, lipoteichoic acid, peptidoglycan, lipoarabinomannans, lipopeptide and choline-containing phosphoglycolipids can indeed interact with the innate immune system of mammals. PRRs present on immune cells bind to PAMPs and discriminate between self and nonself. This is the basic concept of innate immunity (Hoffmann *et al*., 1999) . The list of molecules in mammals that can act as receptors, i.e. PRRs of PAMPs is expanding and includes secreted PRRs (such as LPS binding protein), cell-surface PRRs (such as CD14, the macrophage scavenger receptor and the mannose receptor), and intracellular PRRs (such as double-stranded, RNA-activated protein kinase). However, recent knowledge of the important role of Toll-like receptors (TLRs) in microbial recognition has raised a renewed interest in this field. TLRs are conserved molecules, cloned initially in Drosophila and shown to discriminate between different pathogens and induce an appropriate antimicrobial response5. Activation of TLRs on the surface of the immune and epithelial cells is accompanied by their enhanced ability to express co-stimulatory molecules, present antigens, secrete pro-inflammatory cytokines, and mediate microbial killing. In this review we discuss TLRs, their ligands and their signalling mechanism.

1.1 Drosophila Toll members

The prototypic Toll protein of Drosophila melanogaster is a plasma-membrane receptor characterized by a single transmembrane domain and a series of leucine-rich ectodomain repeats. Toll was originally described in Drosophila as a type-I transmembrane receptor that controls dorsal–ventral polarity during embryogenesis. Nüesslein- Volhard and Wieschaus discovered the first Toll mutants in fruit-fly embryos. Wieschaus noted that the Toll mutant embryos failed to hatch and developed no ventral or lateral cell types. When Nüesslein-Volhard saw the particular embryos lacking the entire mesoderm and nervous system she exclaimed, 'Toll!' (German for jazzy or cool). The new gene was thus given its name. To date, nine toll like proteins have been identified in Drosophila (Tauszig *et al* ., 2000). The extracellular regions of Toll and 18W (another Toll-like receptor) contain multiple leucine-rich repeats and carboxyl- terminal cysteine-rich domains (Gay and Keith 1991). The role of Toll signalling in innate immunity in the fly was initially studied in the setting of antifungal responses to the pathogen, *Aspergillus fumigatus* (Lemaitre *et al* ., 1996). Adult flies carrying a Toll mutation failed to induce expression of the antifungal peptide drosomycin when infected with Aspergillus. Toll mutant flies showed reduced survival because of overwhelming fungal infection. Interestingly, these flies were not susceptible to bacterial infections and expression of antibacterial gene was not reduced, indicating that different pathways are involved for the activation of antibacterial and antifungal activity.

2. Mammalian TLRs

Following the identification of Toll as an essential receptor in the innate immune recognition in Drosophila, a homology search of databases led to the discovery of a homologue of Toll in humans (Medzhitov *et al*., 1997). The human homologue of Toll, now designated TLR4, was shown to be involved in the gene expression of inflammatory cytokines and costimulatory molecules (Medzhitov *et al*.,1998) . Subsequent studies identified several proteins that are structurally related to TLR4. The TLR family now consists of at least ten members (TLR1–TLR10), and is set to expand (Medzhitov *et al*., 1997, Rock *et al*., 1998, Du *et al*., 2000). TLRs belong to a family of type-I transmembrane receptors characterized by an extracellular amino terminus. They have an amino-terminal leucine-rich repeat (LRR) domain and a carboxy-terminal intracellular tail containing a conserved region called the Toll/interleukin-1 receptor (TIR) homology domain. The cytoplasmic portion (intracelluler domain) of TLRs shows a high similarity with that of the interleukin-1 (IL-1) receptor family, and is now called the TIR domain. In spite of this similarity, the extracellular portions of both receptors are

structurally unrelated. TLR is characterized by the presence of LRRs in the extracellular domain which are presumably involved in ligand-binding, but may also be necessary for TLR dimerization. The extracellular domain of TLR4 is highly polymorphic compared with the transmembrane and proximal cytoplasmic domains of the protein (Smirnova *et al*., 2000). In addition, the extracellular domain of TLR4 contains an 82 amino-acid region that is highly variable and contributes to species-specific differences in recognition of LPS. The intracellular TIR domain region spans over 200 amino acids and itself contains three highly conserved regions (Neil *et al*., 2000). The TIR domain mediates protein–protein interactions between the TLRs and signal-transduction components.

2.1 TLRs and their ligands

TLRs recognize the specific microbial patterns. Since the last decade there has been a steady increase in the number of TLR family members and their ligands. Till now, thirteen TLRs have been identified and ligands have been known now for many of them. Most of the ligand studies are based on the knockout mice. Different TLRs seem to play crucial roles in the activation of the immune response to PAMPs. In spite of this specificity for the receptor ligand-binding, the studies indicate that the overall innate immune response is the sum of signals generated by the interaction of multiple TLRs and other cooperating receptor molecules. For example, different TLRs can interact with the complex surface of the bacterium. Here we discuss different TLRs and the interaction with their ligands.

2.1.1 TLR1

TLR1, the first member of the TLR family, was identified by the presence of a domain homology found in both Drosophila Toll and human IL-1 receptors. TLR1 is expressed at higher levels in the spleen and peripheral blood cells (Zarember *et al*., 2002). No direct ligands have been identified so far for TLR1, and its function remains unclear. TLR1 seems to act as a co-receptor. TLR1 was shown to associate with TLR2 in response to triacylated lipopeptides, but not diacylated lipopeptide. These observations indicate that TLR1 is able to discriminate among lipoproteins by recognizing the lipid configuration.

2.1.2 TLR2

TLR2 is involved in the recognition of multiple products of Gram-positive bacteria, mycobacteria and yeast. Earlier studies reported that TLR2 mediates LPS response, but later several studies indicated that TLR4 is the principal receptor for LPS. The cell wall of Gram-positive bacteria contains a thick layer of peptidoglycan (PGN) within which lipoproteins and

lipoteichoic acids are embedded, which can provoke immune responses similar to those generated by LPS. Analysis of TLR2-deficient mice demonstrated clearly that TLR2 is essential for the response to PGN24. Mycoplasma lacks a cell wall, but its cytoplasmic membrane contains various lipoproteins or lipopeptides that can also cause inflammatory responses. One of the Mycoplasma lipopeptides, the 2 kDa macrophage-activating lipopeptide-2 (MALP-2), was shown to utilize TLR2 as its signal transducer25. TLR2 has been found to interact with lipoarabinomannan, which is a major cell wall associated glycolipid derived from Mycobacterium tuberculosis26. TLR2 is known to heterodimerize with other TLRs, which may be a possible explanation for the wide range of PAMPs that TLR2 can recognize. TLR2 cooperates with TLR6 in the response to PGN27 and diacylated mycoplasmal lipopeptide, and associates with TLR1 to recognize triacylated lipopeptides.

2.1.3 TLR3

TLR3 recognizes double-stranded RNA (dsRNA), a molecular pattern associated with viral infection. Viral replication within infected cells results in the generation of dsRNA that can initiate antiviral defence; thus dsRNA can act as PAMPs. Stimulation with polyinosine–polycytidylic acid (poly(I :C)), a synthetic analogue of dsRNA, was shown to induce hyporesponsiveness in TLR3-deficient mice and marked responsiveness only in cells expressing TLR3, suggesting a specific recognition to poly(I :C) by TLR3. Furthermore, TLR3 signalling is not elicited by either single-stranded RNA (ssRNA) or dsDNA. TLR3 activation induces cytokine production through a signalling pathway dependent on MyD88.

2.1.4 TLR4

TLR4 is the principal LPS receptor. LPS, a major component of the outer membrane of Gram-negative bacteria is composed of polysaccharides extending outward from the bacterial cell surface and a lipid portion, lipid A, which is embedded in the cell surface. LPS can provoke a variety of immune stimulatory responses; for example, production of proinflammatory cytokines such as IL-12 and inflammatory effector substances such as nitric oxide. Lipid A portion of LPS is mainly responsible for its biological activities. LPS can cause a clinically life threatening condition called endotoxin shock. In addition to TLR4, a glycosyl phosphatidylinositol anchoring protein, CD14, has been identified that facilitates LPS action by binding and retaining LPS on the cell surface. Studies using TLR4 knockout mice confirmed that TLR4 is critical for LPS signalling (Hoshino *et al*., 2001). Later studies in humans also suggested a similar role of TLR4 in human; TLR4 mutations are associated

with impaired responsiveness to LPS (Arbour *et al*, 2000). It is generally accepted that LPS from Gram-negative bacteria stimulate inflammatory responses through TLR4. Although certain species of LPS, derived from *Leptospira* or *Porphyromonas*, have subsequently been shown to act through TLR2, they are structurally different from the typical *Escherichia coli* or *Salmonella* LPS.

2.1.5 TLR5

TLR5 recognizes flagellin from both Gram-positive and Gram-negative bacteria (Gewirtz *et al*., 2001). Flagellin is the monomeric subunit of bacterial flagella. Flagellin shows potent proinflammatory activity by inducing expression of IL-8. TLR5 was identified by the presence of the TIR domain and is expressed in the spleen, peripheral blood leukocytes and epithelial cells. It has been found that the culture supernatants of the Gram-positive and Gram-negative bacteria have the ability to stimulate the Chinese hamster ovary cells expressing the human TLR5. It has also been confirmed that flagellated bacteria and not the nonflagellated ones activated TLR5, indicating that flagellin is the specific ligand for TLR5.

2.1.6 TLR6

TLR6 is expressed in the spleen and peripheral blood leukocytes and, like TLR1, acts as a co-receptor. Studies have shown that TLR6 cooperates with TLR2 to recognize PGN and the yeast cell-wall particle, zymosan (Ozinsky *et al*., 2000). Furthermore, TLR6 and TLR2-deficient mice were reported to be hyporesponsive to mycoplasma MALP-2, a diacylated lipoprotein, suggesting that TLR2 and TLR coordinate the response to this ligand.

2.1.7 TLR7

TLR7 is abundantly expressed in the lung, placenta, spleen and peripheral blood leukocytes (Zarember and Godowski 2002). TLR7 is phylogenetically close to TLR8 and TLR9, and has a higher molecular weight compared with hTLR1-6, largely as a result of a longer ectodomain (Chuang *et al*.,2000). The natural ligand for TLR7 has not yet been identified. However, studies with TLR7-deficient mice have shown that TLR7 recognizes imidazoquinoline compounds such as R848, a small synthetic antiviral molecule.

2.1.8 TLR8

TLR8 was identified together with TLR7 and TLR9, and is expressed more abundantly in the peripheral blood leukocytes and the lung (Chuang *et al*.,2000). The natural ligand for TLR8 is still unknown. Recently, human TLR8 and TLR7 were reported to independently confer responsiveness to R848, an imidazoquinoline compound with antiviral activity.

2.1.9 TLR9

TLR9, which is localized intracellularly, is involved in the recognition of specific unmethylated CpG-ODN sequences, that distinguishes bacterial DNA from mammalian DNA. Bacterial DNA can stimulate immune cells. This activity is mainly because of the unmethylated CpG motifs, which are rarely detected in vertebrate DNA and, if present, are highly methylated. This stimulation leads to the production of Th1 (T helper 1) cytokines and costimulatory molecule upregulation. This feature of these CpG motifs makes them PAMPs. Analysis of TLR9- deficient mice has indicated that TLR9 is involved in recognizing this bacterial DNA as PAMP. All CpG DNA induced effects, including cytokine production, B-cell proliferation, dendritic cell maturation, and induction of systemic shock were completely abolished in TLR9- deficient cells and mice. Bacterial DNA should be exposed to the cell through digestion of bacteria in the phagoendosome before being recognized by the TLR9. This gives the endosome as a possible location for TLR9.

2.1.10 TLR10

TLR10 is the last human member of the TLR family discovered so far, and its function and direct ligand are still unknown. Human TLR10 (hTLR10) is preferentially expressed on immune cells present in lymphoid tissues such as the spleen, lymph node, thymus, and tonsil (Chaung 2001). Phylogenetic analysis indicates that among all the human TLRs, hTLR10 is most closely related to hTLR1 and hTLR6; the overall amino acid identity is 50% and 49%, respectively. These observations suggest that hTLR10 is involved in the immune response like other known TLRs, and might act as a co-receptor similar to TLR1 and TLR6.

3. TLR SIGNALLING PATHWAY

TLRs are believed to function as dimers. Though most TLRs appear to function as homodimers, TLR2 forms heterodimers with TLR1 or TLR6, each dimer having a different ligand specificity. TLRs may also depend on other co-receptors for full ligand sensitivity, such as in the case of TLR4's recognition of LPS, which requires MD-2. CD14 and LPS-Binding Protein (LBP) are known to facilitate the presentation of LPS to MD-2. A set of endosomal TLRs comprising TLR3, TLR7, TLR8 and TLR9 recognize nucleic acid derived from viruses as well as endogenous nucleic acids in context of pathogenic events. Activation of these receptor leads to production of inflammatory cytokines as well as type I interferons (interferon type I) to help fighting viral infection.

The adapter proteins and kinases that mediate TLR signaling have also been targeted. In addition, random germline mutagenesis with ENU has been used to decipher the TLR signaling pathways. When activated, TLRs recruit adapter molecules within the cytoplasm of cells in order to propagate a signal. Four adapter molecules are known to be involved in signaling. These proteins are known as MyD88, Tirap (also called Mal), Trif, and Tram (toll-like receptor 4 adaptor protein). (Yamamoto *et al*., 2003).

TLR signaling is divided into two distinct signaling pathways, the MyD88-dependent and TRIF-dependent pathway.

3.1 MyD88-dependent pathway

The MyD88-dependent response occurs on dimerization of the TLR receptor, and is utilized by every TLR except TLR3. Its primary effect is activation of NFκB and Mitogen-activated protein kinase. Ligand binding and conformational change that occurs in the receptor recruits the adaptor protein MyD88, a member of the TIR family. MyD88 then recruits IRAK 4, IRAK1 and IRAK2. IRAK kinases then phosphorylate and activate the protein TRAF6, which in turn polyubiquinates the protein TAK1, as well as itself in order to facilitate binding to IKKβ. On binding, TAK1 phosphorylates IKKβ, which then phosphorylates IκB causing its degradation and allowing NFκB to diffuse into the cell nucleus and activate transcription and consequent induction of inflammatory cytokine (Kawai *et al*., 2010).

3.1.1 TRIF-dependent pathway

Additionally, TLR3 uses TRIF to activate interferonregulated factor 3 (IRF3) through a MyD88-independent and TRIF-dependent pathway. TLR4 is the only TLR that uses MyD88 and TRIF-dependent pathways. The TRIF pathway has been reported to induce interferon (IFN)-β production through the activation of IRF3 (Yamamoto *et al*., 2003). TRIF associates with TRAF6 and TANK-binding kinase (TBK)-1 in an independent manner. A novel inhibitory role for MyD88 in TLR3-TRIF signaling was also reported wherein MyD88 prevents activation of the TRIF pathway upon TLR3 stimulation through inhibition of c-Jun N-terminal kinase (JNK) phosphorylation. Further, other adaptors including Mal, TRAM, and sterile α and HEAT/armadillo (ARM) motif protein (SARM) have been demonstrated to play an essential role in the regulation of TLR-dependent signaling pathways as bridging adapters (Sheedy *et al*.,2007). Mal recruits MyD88 to TLR2 and TLR4, whereas TRAM recruits TRIF to TLR4 (Sheedy *et al*.,2007). Although Mal is required for TLR2 and 4 signaling, it also inhibits TLR3 signaling to the JNK pathway and IL-6 induction. There are

four groups of mammalian MAPKs: extracellular signal-regulated kinase 1/2 (ERK1/2), p38 proteins (p38α/β/γ/δ), JNKs and ERK5 (Chang *et al*., 2001). The MAPK pathways regulate numerous cellular events, including cellular proliferation, survival, and inflammatory responses. Regarding TLR activation, TAK1 is a member of the MAP kinase kinase kinase (MAPK3K) family and phosphorylates MKK3 and MKK6, which subsequently activates the MAPK pathways JNK and p38 MAPK. All three MAPKs (ERK1/2, p38, and JNK) are activated by various TLR agonistic ligands including LPS, peptidoglycan, polyI:C, and unmethylated CpG DNA. Many of the roles of TLRs cell proliferation and/or apoptosis might be associated with the signaling components of the MAPK cascades and their crosstalk with PI3K and other signaling molecules.

3.1.2 Negative regulators in TLR signaling

TLR signaling pathways are tightly controlled to prevent excessive and uncontrolled inflammatory responses that often lead to deleterious pathogenesis with an increased mortality rate. In TLR signaling, several negative regulators that function through the prevention of ligand-receptor binding, degradation of the target protein, and inhibition of recruitment or transcription of intermediates, have been identified.

Soluble receptors play a central role in the regulation of inflammation in various conditions. Earlier studies demonstrated that the soluble forms of TLRs (sTLRs), includingTLR2 and TLR4, function as a feedback mechanism for the inhibition of excessive TLR activation. The soluble form of TLR4 (sTLR4) significantly abrogates LPS-mediated TNF production and NF-κB activation via blockade of TLR4- MD2 interactions in mouse macrophage(Iwami *et al.*, 2000). The soluble form of TLR2 (sTLR2), which is naturally expressed in breast milk and plasma, is produced by post-translational modification of the TLR2 protein(LeBouder *et al*., 2003). sTLR2 is not constitutively released in the resting state, but is upon cell activation, and inhibits IL-8 and TNF production by stimulation of bacterial lipopeptide (TLR2 ligand) . These findings suggest that soluble forms of TLR2 and TLR4 function as a critical first-line negative regulator in TLR signaling.

The transmembrane receptors ST2 and single immunoglobulin interleukin-1 receptor-related protein (SIGIRR) are involved in the negative regulation of TLR signaling . ST2 (also known as T1, Fit-1 or DER4) is an orphan receptor that has two main forms, ST2L and sST2. ST2L belongs to the IL-1 receptor family, which comprises three extracellular immunoglobulin-like domains and an intracellular TIR domain (Bergers *et al*., 1994). ST2L

overexpression was shown to attenuate NF-κB activation induced by IL-1 and LPS, but not poly I:C. Macrophages from ST2L-deficient mice enhanced the production of proinflammatory cytokines in response to IL-1 receptor and TLR4, but not TLR3. In addition, ST2L interacts with the essential TLR adaptors MyD88 and Mal, but not TRIF or IRAK, through proline 431 in box2 of the TIR domain. These data indicate that ST2L can inhibit IL-1 and TLR signaling through sequestration of the TLR proximal signaling components MyD88 and Mal. SIGIRR is also an orphan receptor of the IL-1 receptor family, and contains a single immunoglobulin domain and a conserved TIR domain. SIGIRR-deficient bone marrow-derived dendritic cells, but not macrophages, had higher proinflammatory cytokine and chemokine production in response to LPS and CpG ODN. SIGIRR deficient mice are more susceptible to intestinal inflammation, but not to systemic inflammation, such as endotoxic shock. SIGRR can inhibit TLR responses through binding to TLR4, IRAK, and TRAF6 in a ligand-dependent pathway.

Other intracellular proteins, such as MyD88 short protein (MyD88s), interleukin-1 receptor-associated kinase-M (IRAKM), and A20, have been shown to negatively regulate TLR signaling. MyD88s, an alternatively spliced form of MyD88, can inhibit IL-1- and LPS-, but not TNF-, induced NF-κB activation. MyD88s-MyD88 heterodimers are more often recruited to the IL-1R complex than MyD88 homodimers, and fail to activate IRAK phosphorylation, although they still bind IL-1R and IRAK. Among the IRAK family members, IRAKM, which lacks intrinsic kinase activity, is mainly expressed in peripheral blood leukocytes and its expression is increased by TLR stimulation. In one study, IRAKM-deficient macrophages markedly enhanced the production of inflammatory responses to bacterial infection and reduced tolerance in response to endotox. The mechanisms by which IRAKM regulates TLR signaling are involved in the dissociation of the IRAK1 and IRAK4 complex from MyD88, thereby preventing formation of the IRAK1-TRAF6 complex. A20 is one of the best-characterized negative regulators of TLR signaling. A20 was initially reported to be a TNF-induced novel zinc-finger protein that inhibits TNF-induced NF-Kb activation. Further research revealed that A20 is an inducible cysteine protease de-ubiquitinylating enzyme that removes ubiquitin moieties from TRAF6 to terminate TLR signaling in both the MyD88-dependent and MyD88- independent TLR-signaling pathway. A20 regulates the production of inflammatory cytokines in response to TLR2, 3, and 9 ligands, and modulates the development of endotoxin-induced lethal shock. Recent studies have added an orphan nuclear receptor, small heterodimer partner (SHP), to the known negative regulators of TLR

signaling. SHP contributes to the transcriptional regulation of various metabolic pathways, including cholesterol homeostasis, duodenal expression of secretin, and hepatic glucose homeostasis (Lee *et al*., 2007). SHP-deficient mice exhibited increased susceptibility to endotoxin- or zymosan-induced sepsis in vivo. SHP-deficient myeloid lineage cells secreted larger amounts of proinflammatory cytokines and chemokines in response to various TLR or non-TLR ligands, with the exception of Dectin-1, when compared to wild-type cells. Dual mechanisms were determined to be involved in SHP inhibition of TLR signaling. In resting cells, SHP inhibits NF-κBdependent signaling through interaction with NF-κB p65. In addition, after TLR stimulation, SHP attenuates K63- linked polyubiqutination of TRAF6 through interaction with TRAF6 via its RING-domain (Yuk *et al*., 2011). These findings demonstrate a novel negative role for SHP in the modulation of TLR-dependent signaling.

4. The roles of TLR signaling in innate immunity: animal studies

The roles of TLRs in innate immunity have been characterized in mice deficient in individual TLRs. TLR4 and 2 are sequentially involved in the innate immune responses to the Gram-negative bacterial pathogen Salmonella. TLR2-TLR4 double-deficient mice were more susceptible than TLR4-deficient mice, although MyD88-deficient mice were the most susceptible, when challenged with Salmonella typhimurium (Weiss *et al*., 2004). Other studies have shown that MyD88- deficient mice have an increased susceptibility to, and decreased cytokine responses upon acute infection with, Trypanosoma cruzi; however, TLR2-deficient mice had no major defect in parasite control. In a mouse model of Clostridium difficile infection, MyD88-deficient mice had severe and often fatal intestinal disease. Moreover, TLR5 ligation using flagellin enhances host resistance to *C. difficile* infection in vivo. Earlier studies reported that TLR2- and MyD88-deficient mice exhibit an increased susceptibility and bacterial burden in the kidneys and blood after systemic infection with *Staphylococcus aureus* (*S. aureus*). MyD88-deficient macrophages did not produce cytokines in response to *S. aureus*, although TLR2-deficient macrophages produced detectable cytokine levels. Both TLR2 and IL-1 are required for host protection from systemic and cutaneous *S. aureus* infection. Generally, the phenotype of MyD88-deficient mice is more severe than that of TLR2-deficient mice. In nasal, cutaneous, and corneal infection models, TLR2 deficiency is associated with higher bacterial loads and an increased disease severity. However, TLR9-deficient mice did not show an impaired response to S. aureus corneal infection. MyD88- and IL-1R-deficient mice were more susceptible to S.

aureus infection than TLR2-deficient mice, suggesting that other family members contribute to IL-1R/TLR signaling.

TLR5 has a dual role in host defense against microbial infection in terms of infection route and infectious dose. TLR5-deficient mice exhibited increased susceptibility to urinary tract infection with *Escherichia coli*, and increased inflammation in the bladder and kidneys. TLR5 contributes to protection after systemic infection with *S. typhimurium* and intranasal infection with *Pseudomonas aeruginosa*, although this can be masked by TLR4 and other flagellin-sensing pathways (Feuillet *et al*., 2006). In contrast, a deleterious role for TLR5, which is mainly expressed on intestinal CD11c+ lamina propria cells, was reported in mice orally infected with *S. typhimurium*. In this study, susceptibility and survival were dependent on the transport of pathogens from the intestinal tract to the mesenchymal lymph nodes, and TLR5-deficient mice showed improved protection against *S. typhimurium* . Recent studies by Arpaia, *et al*. suggested a role for TLR signaling in the induction of signals for bacterial expression of virulence genes. Mice deficient in both TLR2 and TLR4 are highly susceptible to oral infection with *S. typhimurium*, as well as depressed innate responses. However, TLR2-TLR4-TLR9 triple-knockout mice were less susceptible to infection than TLR2-TLR4 double-knockout mice, although they showed a marked reduction in cytokine production (Arpaia *et al*., 2011). Interestingly, induction of Salmonella pathogenicity island 2 (SPI-2) genes that encode proteins for survival within the Salmonella-containing vacuole (SCV) was absent in cells from TLR2-TLR4-TLR9 triple-knockout mice, thereby inhibiting intracellular replication (Arpaia *et al*., 2011).

4.1 The roles of TLR signaling in innate immunity: clinical evidence

Microbial infection initiates TLR responses, and this interaction between TLRs and pathogen-associated molecular patterns (PAMPs) results in the induction of an array of antimicrobial immune responses. Various cytokines and chemokines, including TNF-α, cytokines of the IL-1 family (IL-1β, IL-18), IL-12, and IFN-γ, can be induced by the recognition of PAMPs by TLRs. The appropriate activation of these inflammatory cytokines and antimicrobial proteins is required for the induction of host defense against diverse microbial infection (Kumar *et al*., 2011).

Evidence for the essential role of human TLRs in host defense was obtained in patients with germline mutations or variations in TLR and TLR signaling proteins. Human primary immunodeficiencies, such as anhidrotic ectodermal dysplasia with immunodeficiency (EDA-

ID) that carry either X-linked recessive hypomorphic mutations in NEMO or autosomal dominant hypermorphic mutations in IKBA, have a cellular defect in NF-κB activation (degradation of NF-κB inhibitor α) and exhibit diminished responses to TLR stimulation. Most patients with IRAK-4 deficiency have invasive pyogenic bacterial diseases and/or peripheral infections, particularly those caused by Streptococcus pneumonia . Previous studies have shown that patients with defects in UNC-93B, TLR3 or TRAF3 adaptor molecule, suffer from herpes simplex encephalitis. TLR3 signaling is associated with mutation of UNC-93B, a protein present in the endoplasmic reticulum and known to interact with TLR3, 7, 8, or 9. Fibroblasts with an autosomal dominant TLR3 deficiency infected with herpes simplex virus 1 exhibited impaired IFN-β and –λ production, suggesting that TLR3-mediated immunity is essential for protection against HSV-1 in the central nervous system during primary infection in childhood.

5. TLRs and tolerance

The phenomenon of tolerance has been studied most extensively with LPS stimulation. It has been shown that pre-exposure to LPS induces suppression of a variety of cytokines when a second LPS stimulation is performed61. Experimentally, cross-tolerance can be induced when primary and secondary stimuli are directed through different TLRs. Consistent with the above hypothesis, patients suffering from sepsis display a tolerant phenotype that might be induced by IL-10 production. It has also been suggested that tolerance can be explained on the basis of changes in the expression of TLRs. In addition, downstream signalling molecules can be affected by the first microbial challenges (Medvedev *et al*., 2000). Relevance of tolerance phenomenon in terms of clinical impact is still not clear. For example, in secondary infections an uncontrolled immune response can lead to a lethal effect, while in another case it may be useful to some extent.

5.1 TLRs and heat shock proteins

Heat shock proteins (HSPs), also called stress proteins, are a group of proteins that are present in both prokaryotic and eukaryotic cells. Their highly conserved structure suggests that they play a role in fundamental cellular processes. As the name suggests, HSPs are induced in cells exposed to sublethal heat shock. HSPs were discovered in Drosophila salivary gland cells which were exposed to a temperature 37°C for 30 min and then returned to their normal temperature of 25°C for recovery. A ‘puffing’ of genes was found to have occurred in the chromosome in the recovering cells, accompanied by an increase in the

expression of proteins with molecular masses of 70 and 26 kDa72. These proteins were named 'heat shock proteins'.

Under conditions of stress, HSPs constitute as much as 15% of prokaryotic cellular proteins, while in eukaryotic cells stress increases expression of HSPs more modestly. Most HSPs function as chaperones, i.e. HSPs participate in folding, assembly and disassembly of protein complexes and may also assist in translocation of proteins from one compartment to another. In fact, accumulation of unfolded or misfolded proteins is a form of stress that induces expression of HSPs. Heat shock is not the only stimulus that can induce and increase synthesis of HSPs. Exposure of cells to heavy metals, protein kinase C stimulators, Ca2+-increasing agents, ischemia, sodium arsenite, microbial infections, nitric oxide, hormones and antibiotics also induce the expression of HSPs. The group of HSPs is immense with regard to their number in both prokaryotes and eukaroytes. Most of them have nothing in common except for the name. Original classification of HSPs was based on their molecular weight. HSPs are present in the cytosol, mitochondria, endoplasmic reticulum and nucleus.

The immunological functions of HSPs began to emerge in the 1980s, when it was observed that homogeneous preparations of certain HSPs that were isolated from cancer cells elicited immunity to cancers, whereas corresponding preparations from normal tissues did not. The earlier studies were carried out with the HSP gp96 (Vabulas 2001), but similar results were later obtained with HSP70, HSP90, calreticulin HSP110 and GRP170.

There is now enough evidence from experiments inTLR4-deficient mice, that hsp60 requires TLR4 to elicit responses, and the same signalling molecules downstreamof TLR4 (MyD88, TRAF6). These molecules are critical in LPS signalling. It has also been suggested that TLR2 can also mediate responses to human HSP60 (Randow *et al*., 2001); this indicates a possible association between HSP60 and two different TLRs. The eukaryotic endoplasmic reticulum chaperone gp96 is required for the maturation of certain oligomeric protein complexes88. Absence of gp96 is compatible with cellular survival even under stress conditions and causes a defect in the formation of only a small subset of cell-surface receptors. TLRs are retained intracellularly in the absence of gp96, explaining the unresponsiveness of the mutant to microbial stimuli.

6. TLR-Targeted Therapeutics

Significant progress has been made over the past years in the understanding of TLR function. TLRs are essential receptors in host defense against pathogens by activating the innate

immune system, a prerequisite to the induction of adaptive immune responses. Although TLR-mediated signaling is paramount in eradicating microbial infections and promoting tissue repair, the regulation must be tight. TLRs are implicated in a number of inflammatory and immune disorders and play a role in cancer (Rakoff-Nahoum and Medzhitov 2009).

Many single nucleotide polymorphisms have been identified in various TLR genes and are associated with particular diseases. Several therapeutic agents targeting the TLRs are now under pre-clinical and clinical evaluation. However, the complexity lies in that TLRs act as double-edged swords either promoting or inhibiting disease progression. Furthermore, therapeutic agents targeting the TLRs must be able to antagonize the harmful effects resulting without affecting host defense functions. Nonetheless, the potential of harnessing and directing the innate immune system with drugs targeting TLRs, to prevent or treat human inflammatory and autoimmune diseases as well as cancer, appears to be promising.

7. CONCLUSION

The roles of TLRs in innate immunity and inflammation have been well-characterized. Upon stimulation, TLRs initiate intracellular signaling cascades to activate proinflammatory and innate immune responses. Each TLR recognizes distinct PAMPs to produce unique outcomes. TLR signalling pathways are activated by several intracellular adaptors and kinases and are associated with the signaling components of MAPK pathways. NF-κB-mediated transcription is required for the induction of the proinflammatory cytokines and mediators that contribute to innate and adaptive immunity. The diverse signaling pathways that cross-talk with TLRs and NF-κB are being progressively unraveled. A number of animal and clinical studies have revealed that TLR signalling pathways play a key role in innate immunity and host defense against pathogenic microbes. Recent insights into the function of several molecules involved in the negative regulation of TLR signaling have extended our understanding of the inhibitory feedback mechanisms through which a variety of extracellular and intracellular decoys fine-tune the activation of innate immune responses. TLR signaling plays a role in the pathogenesis of numerous human diseases; thus, therapies targeting TLR signaling are being developed. Understanding the roles of TLRs and their regulators in animals and humans will facilitate the development of novel therapeutics for TLR-mediated diseases.

REFERENCE

1. Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M and Frees K. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nature Genet*, 2000; 25: 187–191.
2. Arpaia N, Godec J, Lau L, Sivick KE, McLaughlin LM and Jones MB. TLR signaling is required for Salmonella typhimurium virulence. Cell, 2000; 144: 675 -88.
3. Bergers G, Reikerstorfer A, Braselmann S, Graninger P and Busslingere M. Alternative promoter usage of the Fosresponsive gene Fit-1 generates mRNA isoforms coding for either secreted or membrane-bound proteins related to the IL-1 receptor. *EMBO J*, 1994; 13: 1176-88.
4. Chang L and Karin M. Mammalian MAP kinase signalling cascades. *Nature*, 2001; 410: 37-40.
5. Chuang TH, and Ulevitch RJ. Toll like receptor a mediate cpG-DNA signalling. *Eur. Cytokine Netw*, 2000; 11: 372–378.
6. Chuang TS, and Ulevitch RJ. Identification of hTLR10: a novel human Toll-like receptor preferentially expressed in immune cells. *Biochim. Biophys. Acta*, 2001; 1518: 157–161.
7. Du X, Poltorak A, Wei Y and Beutler B. Three novel mammalian toll-like receptors: gene structure, expression, and evolution. *Eur. Cytokine Netw*, 2000; 11: 362–371.
8. Gay NJ and Keith FJ. Drosophila Toll and IL-1 receptor. *Nature*, 1991; 351: 355–356.
9. Gewirtz AT, Navas TA, Lyons S, Godowski PJ and Madara JL. Bacterial flagellin activates basolateraly expressed TLR-5 to induce epitheial proinfalmmatory gene expression. *J. Immunol*, 2001; 167: 1882–1885.
10. Hoffmann JA, Kafatos FC, Janeway CA and Ezekowitz. Phylogenetic perspectives in innate immunity. *R. A. B., Science*, 1999; 284: 1313–1318.
11. Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K and Akira S. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J. Immunol*, 2000; 162: 3749–3752.
12. Iwami KI, Matsuguchi T, Masuda A, Kikuchi T, Musikacharoen T, Yoshikai Y. Cutting edge: naturally occurring soluble form of mouse Toll-like receptor 4 inhibits lipopolysaccharide signaling. *J Immunol* 2000; 165: 6682-6.
13. Kawai T and Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nature Immunology*, 2010; 11: 5.

14. Kumar H, Kawai T and Akira S. Pathogen recognition by the innate immune system. *Int Rev Immunol*, 2011; 30: 16-34.
15. Lemaitre B, Nicolas E, Michaut L, Reichhart JM. and Hoffmann JA. The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell, 1996; 86: 973–983.
16. LeBouder E, Rey-Nores JE, Rushmere NK, Grigorov M, Lawn SD, Affolter M. Soluble forms of Toll-like receptor (TLR)2 capable of modulating TLR2 signaling are present in human plasma and breast milk. *J Immunol*, 2003; 171: 6680-9.
17. McKnight AJ, and Gordon. Forum in immunology: Innate recognition systems – Introduction. *S., Microb. Infect*, 2000; 3: 241**.**
18. Medvedev AE, Kopydlowski KM and Vogel SN. Inhibition of lipopolysaccharide-induced signal transduction in endotoxin-tolerized mouse macrophages: dysregulation of cytokine, chemokine, and toll-like receptor 2 and 4 gene expression. *J. Immunol*, 2000; 164: 5564–5574.
19. Medzhitov R, Preston-Hurlburt P and Janeway CA. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature*, 1997; 388: 394–397.
20. Medzhitov R, Preston-Hurlburt and Kopp E. myd88 in adaptor protein in the htoll/IL-1 receptor family signalling pathway. *Mol. Cell*, 1998; 2: 253–258.
21. O'Neill LA and Dinarello CA. The IL-1 receptor/toll like receptor superfamily –cricial receptor for inflammation and host defense. *Immunol. Today*, 2000; 21: 206–209.
22. Ozinsky A, Underhill DM, Fontenot JD and Hajjar AM. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. Proc. *Natl. Acad. Sci. USA*, 2000; 97: 13766– 13771.
23. Rakoff-Nahoum S and Medzhitov R. Toll-like receptors and cancer. *Nat Revs Cancer, 2009;* 9: 57- 63**.**
24. Randow F and Seed B. Endoplasmic reticulum chaperons gp96 is required for innate immunity but not cell availability. *Nature Cell Biol*, 2001; 10: 891–896.
25. Rock FL, Hardiman G, Timans JC, Kastelein RA and Bazan JF. A family of human receptors structurally related to Drosophila Toll. *Proc. Natl. Acad. Sci. USA*, 1998; 95: 588–593.
26. Sheedy FJ and O'Neill L. The Troll in Toll: Mal and Tram as bridges for TLR2 and TLR4 signaling. *J Leukoc Bio*, 2007; 82: 196-203.

27. Smirnova I, Poltorak A, Chan EK, McBride C and Beutler B. Phylogenetic variation and polymorphism at the toll-like receptor 4 locus (TLR4). *Genome Biol*, 2000; 1: 1–10.
28. Tauszig S, Jouanguy E, Hoffmann JA and Imler JL. Toll related receptors and the control of antimicrobial peptide expression in Drosophila. *Proc. Natl. Acad. Sci. USA*, 2000; 97: 10520–10525.
29. Vabulas RM, Ahmad-Nejad P, Da Costa C, Miethke T, Kirschning CJ, Hacker H and Wagner H. Endocytosed HSP60 use TLR-2 and TLR-4 to activate the toll/interleukin receptor signalling pathway in innate immune cells. *J. Biol. Chem*, 2001; 276: 31332–31339.
30. Weiss DS, Raupach B, Takeda K, Akira S and Zychlinsky A. Toll-like receptors are temporally involved in host defense. *J Immunol*, 2004; 172: 4463-9.
31. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T and Sanjo H. Role of adaptor TRIF in the MyD88- independent toll-like receptor signaling pathway. *Science*, 2003; 301:640.
32. Zarember KA and Godowski PJ. Tissue expression of human toll like receptor and differential regulation of toll like receptor mRNA in leucocyte in response to microbes, their products and cytokines. *J.* Immunol, 2002; 168: 554–561.