Toll-like receptors and their role in innate immunity

Bhanu P. Singh[†,#], R. S. Chauhan* and Lokesh K. Singhal*

[†]Department of Pathology, Faculty of Veterinary Medicine, 3508 TD Utrecht, The Netherlands
*Department of Pathology, College of Veterinary Sciences, G.B. Pant University of Agriculture and Technology, Pantnagar 263 145, India

The innate immune system is an ancient mechanism of host defence found in essentially every multicellular organism, from plants to humans. In invertebrates, it is the only mechanism of defence. Vertebrates also developed an adaptive immune response; however, the innate immune system is essential for instructing the cells of the adaptive system (T and B cells). Toll-like receptors (TLRs) play an important role in innate immunity to invading pathogens by sensing microorganisms. 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-κB, 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. Ten human and nine murine TLRs have been characterized and for many of them, ligands have been identified. 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.

THE ability of the host organism to discriminate between infectious nonself and self is essential for identification and for the fight against invading pathogens, thus allowing it to survive in an environment heavily populated with infectious agents. Mammals have developed two main systems that act in cooperation as safeguards against infection: (i) an innate nonclonal system that promptly recognizes conserved molecular patterns on pathogens, and (ii) an adaptive system that relies on gene rearrangement and clonal expansion upon detection of specific antigens of the invading pathogen. Unlike adaptive immunity, which takes weeks to generate, innate immune responses are mobilized within hours of exposure to a pathogen. This is accomplished by germline-encoded receptors on the surface of cells of the innate immune system. These receptors are known as pattern-recognition receptors (PRRs), which identify molecules produced exclusively by bacteria and other pathogens. In the last few years, there is a lot more focus on such host molecules that have the distinct ability to recognize pathogens in a nonclonal but specific way, which also leads the adaptive immune response towards protection[1,2].

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[3]. The list of molecules in mammals that can act as receptors, i.e. PRRs of PAMPs is expanding[4] 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 know-

[#]For correspondence. (e-mail: singhbps2001@rediffmail.com)

ledge 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 response[5]. 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 legends and their signalling mechanism.

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[6]. Nüessleln-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*[7]. The extracellular regions of Toll and 18W (another Toll-like receptor) contain multiple leucine-rich repeats and carboxyl-terminal cysteine-rich domains[8].

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*[9]. 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.

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[10]. The human homologue of Toll, now designated TLR4, was shown to be involved in the gene expression of inflammatory cytokines and co-stimulatory molecules[11]. 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[10,12–15].

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[16]. 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 prototypic TLR4 ligand[17]. The intracellular TIR domain region spans over 200 amino acids and itself contains three highly conserved regions[18]. The TIR domain mediates protein–protein interactions between the TLRs and signal-transduction components.

Evolution and organization of Toll gene

Drosophila genome contains nine genes that encode Toll and related receptors (dToll1–dToll9), whereas ten TLR genes have been identified in mice and humans[12]. The human and murine TLR2 genes and the murine TLR4 gene have two 5′ non-coding exons followed by a third coding exon. In contrast, the human TLR4 gene has an additional 5′ non-coding exon[19]. Gene-mapping studies have revealed that TLR genes are dispersed throughout the mammalian genome. Specifically, human TLR genes reside on chromosomes 4 (TLR1, TLR2, TLR3, TLR6 and TLR10), 9 (TLR4), 1 (TLR5), 10 (TLR7 and TLR8) and 3 (TLR9). DNA sequence comparisons of genes encoding Toll-related proteins in *Drosophila*, reptiles, birds and mammals have revealed that the genes are well-conserved and have evolved independently from a common ancestor gene[20].

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, ten TLRs have been identified and ligands have been known now for many of them (Table 1). 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

Table 1. Toll-like receptors and their ligands

Toll-like receptors	Ligands	Pathogens
TLR1	? Cofactor for TLR2	Gram-positive, Gram-negative bacteria, mycobacteria, spirochetes, mycoplasma
TLR2	Lipoteichoic acid, glycopeptides, peptidoglycans, zymosan, lipoarabinomannan, soluble tuberculosis factor, LPS (some species), heat shock protein 60 (HSP60)	Gram-positive, Gram-negative bacteria, mycobacteria, spirochetes, mycoplasma
TLR3	Poly I : C (double-stranded DNA)	Virus
TLR4	LPS (lipid A), acyclic synthetic lipid A analogue, respiratory syncitial virus (RSV) fusion protein, HSP 60, fibronectin A domain, fibrinogen, saturated fatty acids, taxol	Gram-negative bacteria, RSV, plant
TLR5	Flagellin	Flagella of Gram-positive and Gram-negative bacteria
TLR6	Cofactor of TLR2	Gram-positive, Gram-negative bacteria, mycobacteria, spirochetes, mycoplasma
TLR7	Imidazoquinoline	
TLR8	?	?
TLR9	Unmethylated CpG motifs (bacterial DNA)	Bacteria
TLR10	?	?

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.

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[21]. 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[22], but not diacylated lipopeptides[23]. These observations indicate that TLR1 is able to discriminate among lipoproteins by recognizing the lipid configuration.

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 PGN[24].

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 transducer[25]. TLR2 has been found to interact with lipoarabinomannan, which is a major cell wall-associated glycolipid derived from *Mycobacterium tuberculosis*[26]. 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 PGN[27] and diacylated mycoplasmal lipopeptide, and associates with TLR1 to recognize triacylated lipopeptides[22].

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 (ref. 28), suggesting a specific recognition to poly(I : C) by TLR3. Furthermore, TLR3 signalling is not elicited by either single-stranded RNA (ssRNA) or dsDNA[29]. TLR3 activation induces cytokine production through a signalling pathway dependent on MyD88.

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 immunostimulatory 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 glycosylphosphatidylinositol 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[30]. Later studies in humans also suggested a similar role of TLR4 in human; TLR4 mutations are associated with impaired responsiveness to LPS[31]. 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[32].

TLR5

TLR5 recognizes flagellin from both Gram-positive and Gram-negative bacteria[33]. 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 non-flagellated ones activated TLR5, indicating that flagellin is the specific ligand for TLR5 (ref. 34).

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[27]. Furthermore, TLR6 and TLR2-deficient mice were reported to be hyporesponsive to mycoplasma MALP-2, a diacylated lipoprotein, suggesting that TLR2 and TLR6 coordinate the response to this ligand.

TLR7

TLR7 is abundantly expressed in the lung, placenta, spleen and peripheral blood leukocytes[21]. 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[13]. 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[35].

TLR8

TLR8 was identified together with TLR7 and TLR9, and is expressed more abundantly in the peripheral blood leukocytes and the lung[13]. 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[36].

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 co-stimulatory molecule upregulation[37]. 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[38]. 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.

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[39]. 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.

TLR signalling pathway

The role of TLR is mainly to alert the immune system to the presence of microorganisms. Engagement of TLRs

with their ligands leads to the production of various pro-inflammatory cytokines, chemokines and effector molecules, depending on the cell type[32,40,41]. Studies on TLR knockout mice have suggested their importance *in vivo*. For example, TLR2-deficient mice are highly susceptible to septic shock caused by *Staphylococcus aureus*[42]. In another study, TLR4-deficient mice have been shown to have persistent infection with *Haemophilus influenza* or with respiratory syncitial virus (RSV), which indicates involvement of TLR4 in defence against such infections[43].

Both the TLRs and IL-1 receptor share a homologous cytoplasmic signalling domain known as TIR domain, and the downstream signalling for both IL-1 receptor and TLRs is the same. For example, the adapter molecule myeloid differentiation protein (MyD88) interacts with both TLRs and IL-1 receptor through its TIR domain (Figure 1). Activation of signalling through TIR domain results in recruitment of the cytoplasmic adopter protein, MyD88 (ref. 44). The IL-1 receptor associated kinases (IRAK-1) interact with the death domain of MyD88, a motif found in many apoptosis-inducing signalling molecules, and are recruited to the TLR complex. Another protein termed as TOLLIP (Toll-interacting protein) is also important for the recruitment of IRAK-1 to the TIR domain. Phosphorylation of IRAK-1 then leads to the recruitment of TNF-receptor associated factor 6 (TRAF6)[45,46]. The formation of this complex phosphorylates the NF-κB (nuclear factor κB) inhibitor, I-κB leading the NF-κB to enter the nucleus, which then leads to gene transcription. Homologues of many molecules in

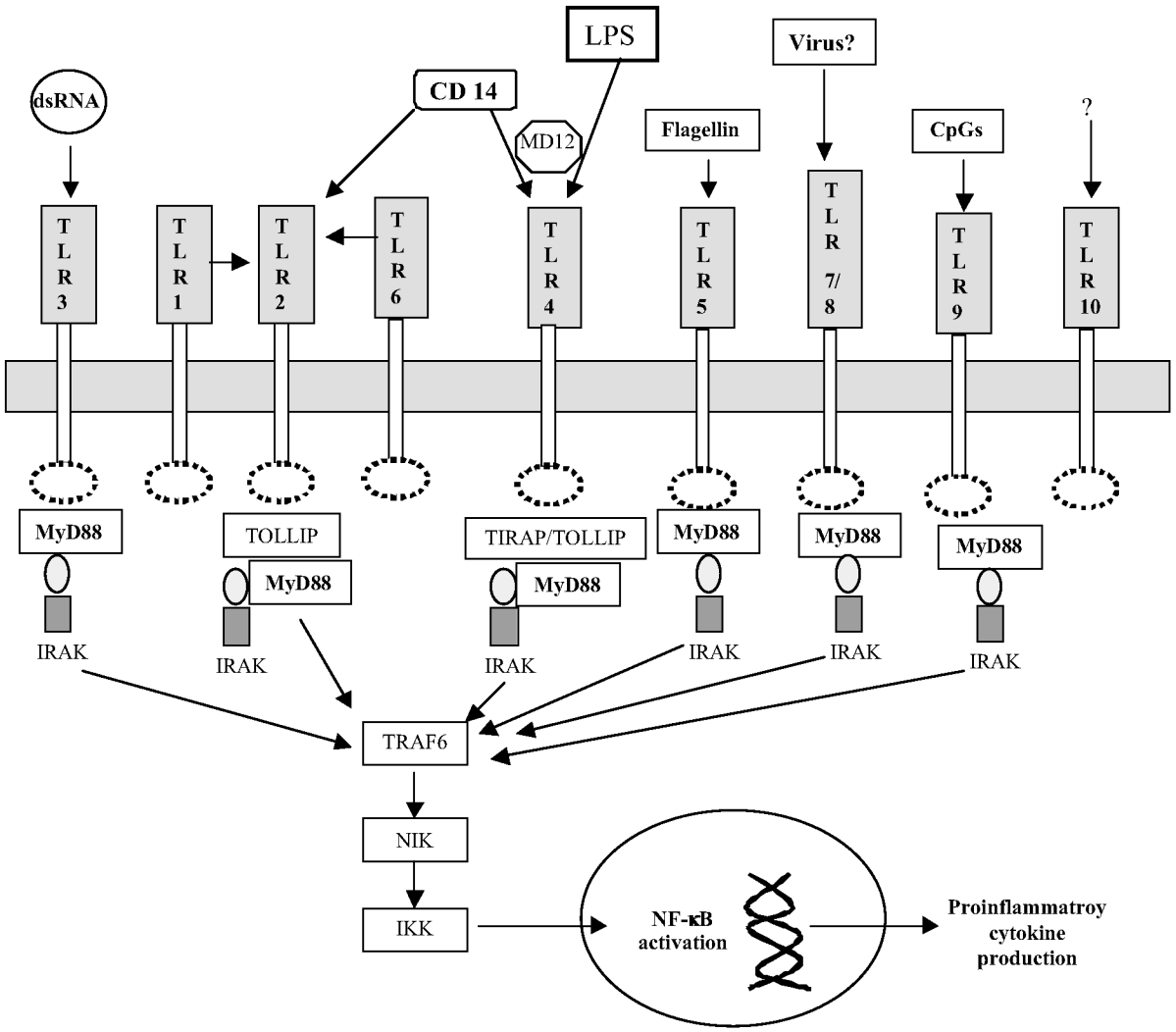

Figure 1. TLR signalling pathway: Upon activation by different ligands, TLRs induce the recruitment of MyD88 via its TIR domain which activates the IL-1 receptor associated kinase (IRAK) by phosphorylation. Activated IRAK combines with TRAF6 and induces downstream signalling leading to the activation of NF-κB, which in turn induces the production of the proinflammatory cytokines. This is a rather simple picture but there are many other molecules involved in this pathway. For example, a molecule homologue to MyD88 (termed as TIRAP or MAL) has been reported, which interacts with TLRs and leads to downstream signalling in an MyD88-independent manner.

volved in TLR signalling in mammals have been reported in *Drosophila*.

An additional TLR4 signalling pathway was revealed through the observation that certain LPS-induced responses did not require MyD88 (ref. 47). It indicates that other signalling pathways exist upstream from NF-κB for TLR4. A molecule termed as 'TIR domain containing adapter protein' (TIRAP) or 'MyD88 adapter-like' (Mal) has been found, which interacts with TLR4 and mediates MyD88-independent TLR4-signalling[48,49]. Recently, interaction of TLR4 with its ligand was shown to induce the secretion of antiviral interferon-β (IFN-β) via a TIRAP/Mal-dependent, but MyD88-independent pathway[50]. The downstream component of this MyD88 pathway remains to be elucidated.

TLRs in cross-talking between innate and adaptive immunity

Specificity of the TLRs for products of microbial origin allows them to detect the presence of infection and to induce activation of inflammatory and antimicrobial innate immune responses. Dendritic cells (DCs) have a key role in interacting the innate and adaptive immune-recognition systems. Immature DCs are located in peripheral tissues, including the potential pathogen-entry sites, where they can detect and capture microbial invaders[51]. Immature DCs express a full set of TLRs, which, on recognition of their ligands, induce DC maturation. Mature DCs express high levels of MHC and co-stimulatory molecules (CD80 and CD86) and migrate to draining lymph nodes where they present pathogen-derived antigens to naive T cells[51]. TLRs also induce expression by DCs of various cytokines, including IL-12, which directs T_H cell-differentiation into T_H1 effector cells. Studies indicate that TLR-mediated recognition is vital for the generation of T_H1, but not T_H2 effector responses[52]. One possible explanation for these observations is that all the known TLR ligands are products of either prokaryotic, viral or protozoan metabolism, and T_H1 responses are required for protection against pathogens of these classes. T_H2 responses, by contrast, are protective against multicellular eukaryotic parasites such as helminths. These pathogens might not produce any ligands for Tolls, and perhaps are recognized by a distinct set of PRRs that could be specific for glycoproteins and glycolipids produced by worms, but not by the host or prokaryotic pathogens. Allergens also lack PAMPs that are recognized by TLRs and might initiate adaptive immune responses by a TLR-independent mechanism. It is also possible that T_H2 responses might be TLR-dependent, but MyD88-independent[53]. This is less likely, however, as MyD88 is expressed constitutively in most cell types.

It is established that CD4 T cells (T regulatory cells or Treg cells) control inflammatory responses to commensal bacteria and pathogens[54]. Treg cells protect from disease by inhibiting both the protective and inflammatory responses, leading to the notion of quality control of the immune response[55]. Evidence exists for rapid Treg migration to inflammatory sites, likely as a result of the constitutive expression of chemokine receptors and high sensitivity to inflammatory chemokines[56]. It remained unclear for a long time whether activation of Treg function is triggered by components of the host inflammatory response or by direct recognition of microbial products. In either case, engagement of TLRs was an attractive possibility. The first evidence for selective expression of TLR by Treg cells came by the study of Caramalho and coworkers[57]. They showed that TLR4 plays a crucial role in the physiology of this cell subpopulation. LPS promotes Treg survival/proliferation and enhances their suppressive functions. This indicates that T cells involved in the control of inflammation directly respond to proinflammatory microbial products. Thus, this study provides a link between innate and adaptive immunity, and reveals a novel mechanism for the control of immune responses. Four TLR genes (TLR4, 5, 7, and 8) are selectively expressed in this particular $CD4^+$ subset. TLR5 expression appeared the most selective for Treg cells. In line with the present observations on LPS activation of Treg cells through TLR4, it is striking that the TLR5 receptor binds flagellin, another bacterial product. Natural ligands for TLR7 and TLR8 are not yet identified, but the finding regarding expression of TLR molecules by Treg cells suggests that a rather large universe of inflammation-related endogenous and pathogen-associated molecules might directly modulate their activities.

B lymphocytes play an essential role in adaptive immune response, but also primarily participate in innate immunity. LPS is a well-known strong stimulant of B cells. LPS stimulation activates B cells, leading to proliferation and IgM secretion. B cells utilize two known receptor-signalling systems[58] for LPS stimuli: RP105 (CD 180) and TLR4. RP105 is a type-I transmembrane protein of 105 kDa with a LRR motif in the extracellular domain, but without a TIR domain. RP105-mediated pathway is different from TLR4-mediated pathway. RP105 pathway is independent of MyD88 expression, which is essential to all the other known mammalian TLRs. RP105 receptor signalling pathway activates pathways similar to B cell-receptor (BCR) signalling, such as the Lyn/CD19/Vav complex and the PI 3-kinase and signalosome molecules. Thus, LPS signalling in B cells consists of two independent pathways, RP105-mediated pathway and TLR4-mediated pathway, and the B cell-specific LPS receptor RP105 shares signalling molecules with BCR to induce cellular activation[59]. This may explain why LPS-induced activation is impaired in B cells lacking various signalling molecules that are apparently not involved in the common TLR signalling pathway. This unique signalling system may be characteristic of B cells, which link innate

immunity and adaptive immunity. Furthermore, since a recent study has shown that TLRs can drive autoantibody production[60], the disruption of these pathways may also contribute to the development of autoimmunity.

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 performed[61]. Experimentally, cross-tolerance can be induced when primary and secondary stimuli are directed through different TLRs[62,63]. Consistent with the above hypothesis, patients suffering from sepsis display a tolerant phenotype[64] that might be induced by IL-10 production[65]. It has also been suggested that tolerance can be explained on the basis of changes in the expression of TLRs[66–68]. In addition, downstream signalling molecules can be affected by the first microbial challenges[69,70]. 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.

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[71], accompanied by an increase in the expression of proteins with molecular masses of 70 and 26 kDa[72]. 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[73–75]. 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[76], protein kinase C stimulators[77], Ca^{2+}-increasing agents[77], ischemia, sodium arsenite[78], 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[79]. The earlier studies were carried out with the HSP gp96 (refs 80–82), but similar results were later obtained with HSP70, HSP90, calreticulin HSP110 and GRP170 (refs 83–86).

There is now enough evidence from experiments in TLR4-deficient mice, that hsp60 requires TLR4 to elicit responses, and the same signalling molecules downstream of TLR4 (MyD88, TRAF6). These molecules are critical in LPS signalling. It has also been suggested that TLR2 can also mediate responses to human HSP60 (ref. 87); 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 complexes[88]. 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.

Future prospects in TLR research

The discovery of TLRs provided us an important clue to understand the mechanism of innate immunity. The innate immune system has to recognize a large number of pathogen-associated molecular patterns and to discriminate among pathogens. There is little doubt that the list of ligands for TLRs will increase over the years. It is interesting that the TLR family members not only interact with lipids and polysaccharides (such as PGN and LPS), but also with DNA, RNA and proteins of both bacterial and viral origin.

There is need for more work to define *in vivo*, the contribution of TLRs upon challenge with bacterial compounds or entire microorganisms. Studies with TLR2 and TLR4 have given promising results in models of endotoxemia. TLR4-deficient mice can tolerate a large dose of LPS (1-mg LPS from *E. coli* 055.B5), whereas mice disrupted for the TLR2 gene responded to LPS to the same extent as wild type mice[24], confirming *in vitro* observations attributing a central role for TLR4 in the response to LPS. Further studies are required in this context.

It would be interesting to study the role played by the various bacterial components in connection with TLRs in

the initiation of the inflammatory response. The mechanism underlying the recognition of TLRs to their ligands is not fully known. How LPS, CD14, MD-2 and TLR4 are intimately associated within a single complex, is not known. Crystal structures of such complexes might help elucidate these types of interaction. Basic studies are needed to identify more ligands for various TLRs.

Another challenge is to define receptors for downstream signalling pathway leading to cell activation. TLRs are known to activate cells via the MyD88/TRAF pathway. Yet, alternative signalling pathways are likely. The similarities between human and *Drosophila* signalling cascades open up a new model system, a study of which may provide insights into the regulation and organization of the innate immune system. It seems that the immune system of *Drosophila* is more sophisticated than the mammalian innate immune system. The possible reason may be the existence of acquired immune system in mammals. Basic understanding of TLR signalling will help in the development of new therapies of sepsis/endotoxemia. Also, if we are able to describe the genetic basis of susceptibility to infectious challenges, then tools may become available to identify patients who are at risk for septic shock.

1. Fearon, D. T. and Locksley, R. M., *Science*, 1996, **272**, 50–53.
2. Janeway, C. A., *Proc. Natl. Acad. Sci. USA*, 2001, **98**, 7461–7468.
3. Hoffmann, J. A., Kafatos, F. C., Janeway, C. A. and Ezekowitz, R. A. B., *Science*, 1999, **284**, 1313–1318.
4. McKnight, A. J. and Gordon, S., *Microb. Infect.*, 2000, **3**, 241.
5. Lemaitre, B., Reichhart, J. M. and Hoffman, J. A., *Proc. Natl. Acad. Sci. USA*, 1997, **94**, 14614–14619.
6. Stein, D., Roth, S., Vogelsang, E. and Nüesslein-Volhard, C., *Cell*, 1991, **65**, 725–735.
7. Tauszig, S., Jouanguy, E., Hoffmann, J. A. and Imler, J. L., *Proc. Natl. Acad. Sci. USA*, 2000, **97**, 10520–10525.
8. Gay, N. J. and Keith, F. J., *Nature*, 1991, **351**, 355–356.
9. Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M. and Hoffmann, J. A., *Cell*, 1996, **86**, 973–983.
10. Medzhitov, R., Preston-Hurlburt, P. and Janeway, C. A., *Nature*, 1997, **388**, 394–397.
11. Medzhitov, R., Preston-Hurlburt, P. and Kopp, E., *Mol. Cell*, 1998, **2**, 253–258.
12. Rock, F. L., Hardiman, G., Timans, J. C., Kastelein, R. A. and Bazan, J. F., *Proc. Natl. Acad. Sci. USA*, 1998, **95**, 588–593.
13. Chuang, T. H. and Ulevitch, R. J., *Eur. Cytokine Netw.*, 2000, **11**, 372–378.
14. Chuang, T. S. and Ulevitch, R. J., *Biochim. Biophys. Acta*, 2001, **1518**, 157–161.
15. Du, X., Poltorak, A., Wei, Y. and Beutler, B., *Eur. Cytokine Netw.* 2000, **11**, 362–371.
16. Smirnova, I., Poltorak, A., Chan, E. K., McBride, C. and Beutler, B., *Genome Biol.*, 2000, **1**, 1–10.
17. Hajjar, A. M., Ernst, R. K., Tsai, J. H., Wilson, C. B. and Miller, S. I., *Nature Immunol.*, 2002, **3**, 354–359.
18. O'Neill, L. A. and Dinarello, C. A., *Immunol. Today*, 2000, **21**, 206–209.
19. Rehli, M., Poltorak, A., Schwarzfischer, L., Krause, S. W., Andreesen, R. and Beutler, B., *J. Biol. Chem.*, 2000, **275**, 9773–9781.
20. Kimbrell, D. A. and Beutler, B., *Nature Rev. Genet.*, 2001, **2**, 256–267.
21. Zarember, K. A. and Godowski, P. J., *J. Immunol.*, 2002, **168**, 554–561.
22. Yamamoto, M., Sato, S., Mori, K., Hoshino, K., Takeuchi, O., Takeda, K. and Akira, S., *J. Immunol.*, 2002, **169**, 6668–6672.
23. Takeuchi, O. *et al.*, *Int. Immunol.*, 2001, **13**, 933–940.
24. Takeuchi, O. *et al.*, *Immunity*, 1999, **11**, 443–451.
25. Takeuchi, O. *et al.*, *J. Immunol.*, 2000, **164**, 554–557.
26. Glickman, M. S. and Jacobs, W. R., *Cell*, 2001, **104**, 477–485.
27. Ozinsky, A. *et al.*, *Proc. Natl. Acad. Sci. USA*, 2000, **97**, 13766–13771.
28. Alexopoulou, L., Holt, A. C., Medzhitov, R. and Flavell, R. A., *Nature*, 2001, **413**, 732–738.
29. Matsumoto, M., Kikkawa, S., Kohase, M., Miyake, K. and Seya, T., *Biochem. Biophys. Res. Commun.*, 2002, **293**, 1364–1369.
30. Hoshino, K. *et al.*, *J. Immunol.*, **162**, 3749–3752.
31. Arbour, N. C. *et al.*, *Nature Genet.*, 2000, **25**, 187–191.
32. Hirschfeld, M. *et al.*, *Infect. Immunol.*, 2001, **69**, 1477–1482.
33. Gewirtz, A. T., Navas, T. A., Lyons, S., Godowski, P. J. and Madara, J. L., *J. Immunol.*, 2001, **167**, 1882–1885.
34. Hayashi, F. *et al.*, *Nature*, 2001, **410**, 1099–1103.
35. Hemmi, H. *et al.*, *Nature Immunol.*, 2002, **3**, 196–200.
36. Jurk, M. *et al.*, *Nature Immunol.*, 2002, **3**, 499.
37. Shirota, H. *et al.*, *J. Immunol.*, 2001, **167**, 66–74.
38. Hemmi, H. *et al.*, *Nature*, 2000, **408**, 740–745.
39. Chuang, T., *Biochim. Biophys. Acta*, 2001, **1517**, 161–167.
40. Jones, B. W., Heldwein, K. A., Means, T. K., Saukkonen, J. J. and Fenton, M. J., *Ann. Rheum. Dis.*, 2001, **60**, 1112–1116.
41. Birchler, T. *et al.*, *Eur. J. Immunol.*, 2001, **31**, 3131–3137.
42. Takeuchi, O., Hoshino, K. and Akira, S., *J. Immunol.*, 2000, **165**, 5392–5396.
43. Wang, X., Moser, C., Louboutin, J. P., Lysenko, E. S., Weiner, D. J., Weiser, J. N. and Wilson, J. M., *J. Immunol.*, 2002, **168**, 810–815.
44. Akira, S., *Adv. Immunol.*, 2001, **78**, 1–56.
45. Cao, Z., Henzel, W. J. and Gao, X., *Science*, 1996, **271**, 1128–1131.
46. Cao, Z., Xiong, J., Takeuchi, M., Kurama, T. and Goeddel, D. V., *Nature*, 1996, **383**, 443–446.
47. Kaisho, T., Takeuchi, O. and Kawai, T., Hoshino, K. and Akira, S., *J. Immunol.*, 2001, **166**, 5688–5694.
48. Horng, T., Barton, G. M., Medzhitov, R., *Nature Immunol.*, 2001, **2**, 835–841.
49. Fitzgerald, K. A. *et al.*, *Nature*, 2001, **413**, 78–83.
50. Toshchakov, V. *et al.*, *Nature Immunol.*, 2002, **3**, 392–398.
51. Banchereau, J. and Steinman, R. M., *Nature*, 1998, **392**, 245–252.
52. Schnare, M., Barton, G. M., Holt, A. C., Takeda, K., Akira, S. and Medzhitov, R., *Nature Immunol.*, 2001, **2**, 947–950.
53. Medzhitov, R., *Nature Rev. Immunol.*, 2001, **1**, 135–145.
54. Singh, B. *et al.*, *Immunol. Rev.*, 2001, **182**, 190–200.
55. Coutinho, A., Hori, S., Carvalho, T., Caramalho, I. and Demengeot, J., *Immunol. Rev.*, 2001, **182**, 89–98.
56. Bystry, R. S., Aluvihare, V., Welch, K. A., Kallikourdis, M. and Betz, A. G., *Nature Immunol.*, 2001, **2**, 1126–1132.
57. Caramalho, I., Carvalho, T., Ostler, D., Zelenay, S., Haury, M. and Demengeot, J., *J. Exp. Med.*, 2003, **197**, 403–411.
58. Ogata, H. *et al.*, *J. Exp. Med.*, 2000, **192**, 23–29.
59. Yazawa, N. *et al.*, *Blood*, 2003, **11**, 3573.
60. Leadbetter, E. A., Rifkin, I. R., Hohlbaum, A. M., Beaudette, B. C., Shlomchik, M. J. and Marshak-Rothstein, A., *Nature*, 2002, **416**, 603–607.
61. Schade, F. U., Flash, R. and Flohe, S., In *Endotoxin in Health and Disease* (eds Brade, H. *et al.*), Marcel Dekker, New York, 1999, pp. 751–768.
62. Medvedev, A. E. *et al.*, *J. Immunol.*, 2001, **167**, 2257–2267.