Role of the innate immune system in host defence against bacterial infections: focus on the Toll like receptors

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Introduction

The human body is constantly exposed to microbes that usually only colonize the host harmlessly, but that may cause infectious diseases, sometimes leading to fatal outcomes. To control the resident colonizing microflora, as well as to fight pathogens, the human body has developed a variety of host defence mechanisms that in most cases effectively prevent the development of invasive microbial diseases. These defence mechanisms comprise physical or anatomical (skin, mucosal lining), mechanical (ciliated cells from the respiratory tracts, tight junctions) and biochemical barriers (tears or saliva containing antimicrobial lysozyme) as well as two inducible immune defence systems: the innate and the adaptive immune systems. These two systems are sequentially activated during infection and work cooperatively to eradicate the microbial agent. The innate immune system is the first line of host defence toward microbial infections, while the adaptive immune system is elicited later, about four to seven days post infection and includes a specific and long lasting immunity that is based on the rearrangement and the clonal expansion of a random repertoire of antigen receptors (TCR and BCR) on lymphocytes. In this review, we will focus on the early innate responses and the role of the Toll-Like Receptors (TLRs).

The innate immune system gives protection to a broad variety of pathogens and is based on a limited repertoire of germ-line encoded receptors called pattern recognition receptors (PRRs) because they recognize conserved microbial components known as pattern-associated molecular patterns (PAMPs). The PRRs include among others the members of the Toll-Like Receptor (TLRs) family and the nucleotide-binding oligomerization domain

proteins (NOD-like receptors, NLRs) (5, 46). Here we will primarily discuss the role of TLRs in host protection against bacterial infections.

The Toll-Like Receptors

The Toll receptors are evolutionary conserved and homologous receptors are found in plants, insects, worms (*Caenorhabditis elegans*) and vertebrates. The first member of this family, named Toll was initially identified in the fruit fly, *Drosophila melanogaster* (10). This receptor has been shown to be responsible for the embryogenic dorsoventral development of fruit flies and to play an important role in the protection against fungi in adult flies (100). The Toll-Like Receptors (TLRs) are the mammalian homologues of Toll and totally, thirteen mammalian TLRs have been identified so far; ten human (TLR1-10) and twelve murine (TLR1-9 and 11-13) receptors, of which some are homologues (135).

TLRs are type I transmembrane proteins that are characterized by an extracellular leucin rich domain (LRR) and an intracellular or cytoplasmic domain homologous to the interleukin-1 receptor (IL-1R) and therefore called Toll/IL-1 receptor (TIR) domain (3, 83). The homology between TLRs and IL-1R is restricted to their cytoplasmic domain, while their extracellular domains are remarkably different. Whereas IL-1R has an immunoglobulin (Ig) -like structure, TLRs contain LRR. The LRR domains consist of 19-25 tandem repeats where each repeat has a length of 24-29 amino acids. These domains are responsible for the recognition of PAMPs from bacteria and parasites but also from fungi and viruses (1, 21, 66, 144, 165). At least one ligand for each TLR has been identified so far (Figure 1 and Table 1).

TLR4 is the most extensively studied PPR and it recognizes a variety of ligands (mannan from yeast, host heat shock proteins and fibrinogen and envelope proteins from virus, pneumolysin, a cytotoxin from *Streptococcus pneumoniae*) but is mostly known as the lipolysaccharide (LPS) receptor (74). TLR2 also recognizes a broad range of ligands, such as bacterial lipopeptides, yeast zymosan, parasite and viral proteins and lipoteichoic acid (LTA) from Gram-positive bacteria. The variety of ligands recognized is believed to be due to heterodimer formation of TLR2 with two other TLRs, TLR1 or TLR6, which can discriminate subtle changes in the ligand structure (19, 150, 154). The heterodimer of TLR1/TLR2 has been suggested to recognize triacylated lipoproteins, while TLR2/TLR6 recognizes diacylated lipoproteins (154, 155). TLR5 detects a conserved domain on flagellin monomers, the main structural protein that forms the flagella on Gram-negative bacteria. Flagella are bacterial motor organelles responsible for virulence, chemotaxis, adhesion and invasion of host surfaces (61). TLR9 recognizes nucleic acids such as hypomethylated CpG, motifs, which are common among prokaryotic DNA and absent in eukaryotic genomes (19, 63). Also, TLR9 has been shown to be activated by hemozoin, a heme containing degradation product of haemoglobin generated in erythrocytes infected by malaria parasites (32). TLR3, 7 and 8 recognize nucleic acids like TLR9, but single-stranded and double–stranded RNA rather than DNA (33, 145, 162). The expression of TLRs differ with cell types and cellular localization where some have been found to be expressed primarily extracellularly (TLR1, 2, 4, 5, 6 and 11) and others intracellularly (TLR3, 7, 8 and 9) on numerous

myeloid cells (macrophages, dendritic cells, neutrophils, T and B cells) but also on non-myeloid cells (epithelial cells, fibroblasts).

The Gram-positive TLR ligands

Gram-positive bacteria have a thick multilayer cell wall consisting mainly of peptidoglycan, a polymer of carbohydrates (N-acetylmuramic acid and N-acetylglucosamine) cross-linked through peptide bonds, that surrounds the cytoplasmic membrane (114, 137). The Gram-positive cell wall contains polyalcohols called teichoic acid, some of which are lipid-linked to form lipotechoic acids (Figure 2). Lipoteichoic acids (LTA) are anchored in the cytoplasmic membrane *via* lipid moieties whereas wall teichoic acids (WTA) are covalently bound to the peptidoglycan (Figure 2). Due to the presence of phosphodiester bonds between teichoic acid monomers, teichoic acids give the Gram-positive cell wall an overall negative charge. Many Gram-positive pathogens however, such as *Streptococcus pyogenes* and *Streptococcus pneumoniae* express teichoic acids that are D-alanylated, decreasing the net negative surface charge thereby increasing resistance to cationic antibacterial peptides present in the host (93, 171). Teichoic acids of *Streptococcus pneumoniae* also contain choline residues providing binding sites for a series of choline binding proteins (54, 119). Purified WTA is not inflammatory whereas purified LTA is moderately inflammatory through its diacylated moiety being recognized by TLR2/TLR6 (56, 64, 89, 141). Recent data however, suggest that lipoproteins of

Staphylococcus aureus might be more dominant TLR2 ligands as compared to LTA (58).

The Gram-negative TLR ligands

Gram-negative cell walls are more complex than their Gram-positive counterpart (Figure 2). They consist of a thin peptidoglycan layer adjacent to the cytoplasmic membrane and an outer membrane of lipopolysaccharides (LPS), phospholipids and proteins, which face into the external environment (39, 114).

LPS, the main component of the outer leaflet of the outer membrane, is highly charged and confers an overall negative charge to the Gram-negative cell wall. The chemical structure of the outer membrane LPS is often unique to specific bacterial strains (i.e. sub-species) and is responsible for many of the antigenic properties of these strains. The outer membrane of Gram-negative bacteria also contains channel proteins called porins that allow passive transport of many ions, sugars and amino acids across the outer membrane. The cytoplasmic and the outer membranes are separated by the periplasmic space, which contains the peptidoglycan layer (Figure 2). LPS, also known as endotoxin, is the most studied PAMP of Gram-negative bacteria (Figure 2) (39). This structure protects the bacteria from bile salts, hydrophobic antibiotics and complement activation and is crucial for bacterial survival. LPS is generally composed of an O-linked polysaccharide, which is attached to the lipid A moiety *via* the core polysaccharide (Figure 2). With the exception of *Neisseria meningitidis*,

LPS is crucial for the viability and growth of the bacteria (7, 48, 149). The classical lipid A has a mono or biphosphorylated disaccharide backbone, which has been acylated with fatty acids. Lipid A anchors LPS to the outer membrane *via* its fatty acids. The classical lipid A of *E. coli* is hexa-acylated. Lipid A is the active component of LPS. This component is probably the most potent immunostimulatory molecule of all the PAMPs and is responsible for most of the acute inflammatory response to bacterial LPS observed during toxic shock (161). The level of Lipid A acylation is critical to the immunostimulatory effects of LPS. Thus, penta-acylated LPS from a *WaaN* mutant of *E. coli* is much less potent than wild type (wt) LPS in eliciting a proinflammatory cytokine response from cultured uroepithelial cells (13, 14). Induction of innate immune responses by *E. coli* and purified LPS correlate with organ- and cell-specific expression of TLRs within the human urinary tract (13, 14).

Not all Gram-negative bacteria express similar LPS and changes in the LPS and/or lipid A structure can occur during various environmental conditions, which can result in modulation of the host responses and may thereby confer specific advantages to certain bacterial species under changing environmental host conditions (39, 161). For example, LPS from *Porphyromonas gingivalis*, an oral anaerobic bacterium, is less potent in eliciting an innate immune response than LPS from *E. coli* (16, 17, 134). Also, clinical isolates of the gastric pathogen *Helicobacter pylori* expresses LPS that is penta-acylated and therefore not as immunostimulatory as LPS of many enteric commensals that produce hexa-acylated LPS (159). Furthermore, *Salmonella* is able to down regulate the endotoxicity of its LPS by a lipid A deacetylase (PagL),

which is under the global control of the PhoP/PhoQ regulon (84). However, not only acylation but also other modifications of the lipid A moiety (fatty acid composition, phosphate patterns) are crucial for host recognition (37, 85-87). LPS is recognized by TLR4 but TLR4 is not sufficient for the signalling (110). It also requires accessory proteins. LPS binds first LPS binding protein (LBP), which is an acute phase protein that circulates in the bloodstream and binds to glycosylphosphatidylinositol (GPI) linked co-receptor CD14, which is expressed on the cell surface. LPS is then transferred to a small accessory soluble protein, MD-2 that is also part of the TLR4 receptor complex (116).

The Gram-negative cell wall also contains lipoproteins located either in the cytoplasmic or outer membrane. Particularly, lipoproteins from *Borrelia burgdorferi*, the agent of Lyme disease have been shown to activate inflammatory cells through TLR2 and TLR1 (25, 27, 30).

Other bacterial ligands

Peptidoglycan (PG) is a common component of both Gram-positive and Gram-negative bacteria (Figure 2). TLR2 has been reported to recognize PG however this is controversial. Indeed, it has been suggested that PG purified directly from bacterial cultures may contain contaminations (such as LPS, LTA or covalently bound lipoproteins), which can account for the TLR2-dependent inflammatory responses observed (49, 50, 160). Instead the intracellular NOD-like receptors (NLRs) have been shown to recognize muropeptides derived from the peptidoglycan. NLR-stimulating ligands have also been shown to synergize the proinflammatory effects of TLR ligands and *vice versa* (96). Since, the NLRs are intracellular, muropeptides need to reach them in

the cytosol for activation. It is therefore not surprising that the NLRs have been shown to play a particular role for defence against intracellular bacterial pathogens able to escape from the vacuolar compartment and replicate in the cytosol such as *Shigella* and *Listeria monocytogenes* (51, 95). The primarily extracellular pathogen *Helicobacter pylori* has been proposed to deliver muropeptides to intracellular NLRs *via* its Type IV secretion system encoded by the *cag* pathogenicity island, providing one explanation why *cag* positive *H. pylori* are more proinflammatory than strains lacking this island (167).

Bacterial DNA contains hypomethylated CpG motifs, which are almost non-existent in mammalian genomes. These CpG motifs are immunostimulatory *via* TLR9 recognition (19, 139). Since TLR9 is located intracellularly in the endosome, bacterial DNA must be taken up and transported to the endosome in order for it to interact with this receptor. Simultaneously, with the transport of CpG DNA from the early endosome to the endosome, TLR9 is recruited from the ER to CpG DNA-containing compartment (97). In the endosome, the double stranded DNA is cleaved into smaller single stranded CpG motifs that will be recognized by TLR9. It was also shown that a small proportion of TLR9 is surface accessible on the plasma membrane after exposure to CpG DNA (97).

Flagellin is the main subunit protein of the flagellum (20, 130). Different species of bacteria have different numbers and arrangements of flagella. For instance, *Vibrio cholerae* has only one flagellum while *E. coli* has several flagella expressed all around the bacteria and pointing in all directions. Flagellin monomers are recognized by TLR5 while the flagellum is not (61). The amino acid residues responsible for TLR5 recognition have

been defined and are located in a highly conserved region that is hidden in the flagellum but is accessible in the monomer (9, 148).

Some bacteria-specific ligands have also been described, such as porins or toxins. Porins are proteins that are prevalent in the outer membrane of the Gram-negative bacteria, which act as a pore through which molecules can diffuse (Figure 2). Porins from *Neisseria meningitidis*, *Haemophilus influenzae* type b and *Shigella dysenteriae* have been shown to be immunostimulatory molecules (47, 108, 147). Porins from *N. meningitidis* are recognized by the heterodimer TLR1/TLR2, while the porin of *S. dysenteriae* signals *via* TLR2/TLR6 (131-133). It has been suggested that the low percentage of protein homology (31%) between neisserial and *Shigella* porin accounts for the difference in the TLR recognition specificity (108).

Pneumolysin is a member of the thiol-activated cytolysin expressed by nearly all clinical isolates of *Streptococcus pneumoniae* (121). Pneumolysin has several functional domains responsible for adherence to epithelial cells, cytolysis and complement activation and is therefore an important virulence factor. It has been suggested that TLR4 recognizes pneumolysin (106).

TLR signalling

Upon recognition of their cognate ligands, TLRs dimerize and initiate a signalling cascade that leads to the activation of a proinflammatory response (3). Ligand binding induces two signalling pathways, one is MyD88-dependent and the other is MyD88-independent inducing the production of proinflammatory cytokines and type I interferons (IFNs) (Figure 3) (83). These two distinct responses are mediated *via* the selective usage of adaptor

molecules recruited to the TIR domains of the TLRs after ligand recognition and binding. Four adaptor molecules have been identified so far, MyD88, TIR-associated protein (TIRAP), also called MyD88-adaptor-like (Mal), TIR domain-containing adaptor protein-inducing IFNβ (TRIF) also known as TIR domain-containing molecule 1 (TICAM-1) and TRIF-related adaptor molecules (TRAM), also named TIR domain containing molecule 2 (TICAM-2) (120, 168, 173-175). MyD88 and TIRAP are responsible for the induction of proinflammatory genes and TRIF and TRAM for the induction of the IFNs. One additional adaptor molecule has been found the sterile alpha and HEAT/Armadillo motifs (SARM). Its function in the TLR signalling is not fully understood, even though it was reported that it acts as a negative regulator of TRIF-dependent TLR signalling (29, 76).

MyD88-dependent signalling

All TLRs, except TLR3, signal through MyD88 (2). Upon ligand recognition, MyD88 is recruited and associates with the cytoplasmic domain of the TLRs *via* homophilic interaction between the TIR domains. Then IL-1R-associated kinase 4 (IRAK-4) and IRAK-1 are recruited and activated by phosphorylation. Activated IRAK-4 phosphorylates IRAK-1, which subsequently associates with TNFR-associated factor 6 (TRAF6) (52). TRAF6 activates TGF-β-activating kinase 1 (TAK1). TAK1 phosphorylates IKK-β and MAP kinase kinase 6 (MKK 6) leading to the degradation of IκB and thereby leading to the nuclear translocation of NF-κB which results in the induction of genes involved in inflammatory responses. Activation of the MyD88-dependent pathway results also in the activation of MAPKs such as p38 and JNK, which leads to the

activation of AP-1. In addition to NF-κB and AP-1, the MyD88-dependent pathway can activate a third transcription factor IRF-5. Upon ligand stimulation, IRF-5 can also translocate into the nucleus and bind to IFN-stimulated response elements (ISRE) motifs in the promoter region of cytokines genes. In addition to MyD88, TLR2 and TLR4 needs a second adaptor molecule, TIRAP/Mal in order to signal. It was recently demonstrated that TIRAP/Mal is recruited to the plasma membrane through its phoshatidylinositol 4,5-bisphosphate binding domain, where it then can promote delivery of MyD88 to activated TLR4 (77).

TLR7 and TLR9 activation does not only lead to the induction of proinflammatory cytokines but can also cause the induction of IFN-α in a MyD88-dependent manner. This is specific to plasmacytoid dendritic cells (pDC) that are expressing high levels of TLR7 and TLR9 and are capable of producing high levels of IFN-α. Upon ligand stimulation, a complex consisting of MyD88, IRAK-4, IRAK-1 and TRAF6 is formed at the TIR domain of TLR7 and TLR9 and then the transcription factor IRF-7 is also recruited to this complex. The activation of IRF-7 by phosphorylation leads to its translocation to the nucleus and induction of the IFN response.

MyD88-independent signalling

TLR3 and TLR4 activation triggers the induction of a type 1 IFN response leading to the induction of IFN-α and IFN inducible genes. While TLR3 mediated signalling only requires the adaptor molecule TRIF, TLR4-mediated signalling needs in addition to TRIF another adaptor protein, TRAM. TRAM is considered as a bridging adaptor between TLR4 and TRIF. TRIF interacts

with both receptor-interacting protein 1 (RIP1) and TRAF6 and cooperatively with these two proteins activates NF-κB to induce expression of proinflammatory cytokines (35). Furthermore, TRIF activates also TRAF family members-associated NF-κB activator (TANK) binding kinase 1 (TBK1) *via* TRAF3. In turn, TBK1 phosphorylates directly two transcription factors, IRF-3 and IRF-7 allowing them to translocate in the nucleus and induce IFN-α and IFN inducible genes (136).

In viral infections, the induction of Type 1 interferons has recently been shown to be primarily due to the recognition of double stranded RNA, which is a sign of replicating viruses. The retinoic acid inducible gene I (RIG-I) with its helicase domain has been demonstrated to be an essential regulator for double stranded RNA signalling, that results in the activation of the transcriptional factors NF-κB as well as IRF-3 (151, 176).

TLR signalling regulation and immunomodulators

The TLR signalling needs to be tightly regulated in order to be permissive for the resident microflora, to be restrictive for primary pathogens and to avoid excess inflammation, which can be deleterious for the tissue or organ (109). The first and most basic level of regulation is directly linked to the TLR cellular localization as described above. For instance, the intracellular location of TLR9 allows an increased recognition of endocytosed viral DNA but also prevents recognition of self-DNA (18). Furthermore, in organs like the gut, it was shown that normal primary enterocytes express low levels of TLR2 and TLR4, and the co-receptor MD-2 as well as the membrane-bound CD14 and that, in contrast to macrophages, TLR4 is not localized at the cell surface but

rather at the Golgi apparatus requiring internalization of LPS *via* lipid rafts to activate signalling (70, 71). Expression of membrane bound CD14 is also absent from uroepithelial cells (62).

Beside their cellular localization, TLR signalling can be modulated by the selective usage of the adaptor molecules recruited to the TIR domains of the TLRs after ligand recognition and binding. The intracellular signalling cascade is also negatively regulated at various levels either by protein phosphorylation, degradation, interaction with inhibitory adaptor molecules, or sequestration (40, 109). Some of the main players in this immuno-modulatory regulation are i) Suppressor of cytokine signalling 1 (SOCS-1) ii) Flightless I homologue (Fliih) iii) ST2 iv) Triad3A v) A20 vi) IRAK-M vii) IRAK-1c viii) a short form of MyD88 (MyD88s) and ix) ß-arrestin (26, 28, 31, 65, 91, 170, 180). MyD88s inhibits IL-1 and LPS-induced NF-κB activation. Indeed MyD88s acts as a dominant negative form of MyD88 and replaces formation of MyD88 homodimers by MyD88s-MyD88 heterodimers. These heterodimers still recruit IRAK1 but inhibit phosphorylation of IRAK-1 *via* IRAK-4 and thereby inhibit downstream signaling (26). ST2L is a type I trans-membrane receptor composed of three extra-cellular Ig-like domains and an intracellular TIR domain that was shown to sequester MyD88 and Mal, but not TRIF or IRAK, which in turn negatively regulates IL-1R and TLR4-mediated signaling. Another molecule, Fliih has been shown to act as negative regulator by interacting with MyD88. SOCS-1 mediates Mal degradation and thereby negatively regulates TLR signaling (107, 118). Triad3A is a molecule that promotes the ubiquitination and degradation of TLR4 and 9 via binding the cytoplasmic domain of these two TLRs (31). IRAK-M is a negative regulator

that blocks IRAK-4 activation (91). Similarly, Toll-interacting protein (Tollip) interacts with IRAK-1 and suppresses autophosphorylation of IRAK-1 (180). It was suggested that Tollip regulates the intensity of the response to IL-1ß and LPS (38). IRAK-1c is a spliced variant of IRAK-1 that is non-functional because it cannot be phosphorylated by IRAK-4 but it maintains its ability to bind to MyD88 and TRAF-6. It therefore acts as a dominant negative form of IRAK-1. While ß –arrestin prevents oligomarization of TRAF6, which in turn inhibits the autoubiquitination of TRAF6; A20 removes the ubiquitin from TRAF6. However, both molecules inactivate the TLR signalling (170) (65). In addition, TLR signalling can be down-regulated by anti-inflammatory cytokines. For instance, it has been shown that transforming growth factor-ß, TGF-ß induces MyD88 degradation by the proteasome and suppresses the expression of TLR4 (117).

TLR and Experimental infection models

While *in vitro* studies have highlighted the role of TLR for the recognition of specific bacterial ligands, *in vivo* studies were necessary to elucidate the role of individual TLRs in the recognition of the whole bacterium that can carry several ligands simultaneously. This task was facilitated by the creation of knock out animals in the different components of the TLR signalling pathway (2, 67, 68, 74, 82, 150, 179). The model of choice was the murine model and TLR-deficient mice were profoundly used (4). In several instances, a deficiency in a single TLR has no significant effect on mice susceptibility to a pathogen even though it expresses the ligand for the missing TLR (6, 15, 24,

66). These findings are usually explained by the redundancy in the system with several TLRs recognizing ligands on a given pathogen.

TLR1/2 and TLR2/6

TLR2 has been regarded as being the primary Gram-positive TLR and indeed, TLR2 has been proven critical for host protection in murine models of bacterial infection such *Staphylococcus aureus* (53, 153) and *Listeria monocytogenes* (144, 158). In a meningitis model of *Streptococcus pneumoniae*, TLR2 played a role as well, but in a pneumonia model, TLR2-deficient mice were only marginally affected (6, 41, 42, 90, 92, 166). TLR2 has also been shown to be important for Gram-negative infections such as due to *Legionella pneumophila* (12, 59) and *Samonella* (23, 163). Using a calf model of gastroenteritis, it was recently demonstrated that *Salmonella* curli promote the inflammatory response in the bowel, and that the likely receptor for this class of bacterial amyloids was TLR2 (163). Also, in mice curliated *E. coli* were shown to mediate a more pronounced host response than non-curliated mutant bacteria as evidenced by a more significant blood pressure drop upon infection with curliated as compared to non-curliated *E. coli* (23).

Not much information is available for the role of TLR1 and TLR6 *in vivo* (150). These two TLRs seem to be redundant or have a minor role in pneumococcal infections (6).

TLR4

TLR4-deficient mice have been shown to be highly susceptible to many Gram-negative bacteria among others to *Salmonella spp*, *Haemophilus influenzae* and *Klebsiella pneumoniae* (169, 172). Several inbred mice such as C3H/Hej or C57BL10/ScN have been shown to naturally harbour mutations in TLR4 ORF, which render them highly susceptible to Gram-negative infections.

Using a mouse model of experimental urinary tract infection (UTI), it was shown that TLR4 is required for the immune response, including neutrophil recruitment in order to clear uropathogenic *E. coli* (UPEC) from the mucosa (57, 138, 152). It was further demonstrated that in addition to LPS, the presence as well as the type of fimbriae expressed on the bacteria was necessary to trigger a TLR4-dependent response and neutrophil recruitment in the bladder. Indeed, LPS alone was not sufficient to trigger the immune response, as evidenced by the lack of response to infections by non-fimbriated *E.coli* (45, 138). Interestingly, it is worth noticing that these fimbriae are also crucial for the initial attachment of the bacteria to the epithelial cell surfaces, which highlights the importance of co-receptors involved in the activation of a fully functional TLR signalling (81, 115). This might explain how a limited number of the TLRs can recognize such a broad number of bacteria. For some Gram-negative infections such as acute lung infections due to *Pseudomonas aeruginosa*, the role of TLR4 in host protection is not clear yet (43, 124, 125, 178). TLR4 has not only been suggested to play a role in host protection against Gram-negative bacteria but also against Gram-positive bacteria and one study showed that TLR4-deficient mice might be more

susceptible to Gram positive colonization by *Streptococcus pneumoniae* (106). However, these data have not been confirmed by others (6, 24, 166).

TLR5

TLR5-deficient mice are unable to mount a response to purified flagellin, but are not more susceptible to *Salmonella* given intraperitoneally (ip) or to *Pseudomonas aeruginosa* given intranasally (in), possibly due to the activation of other TLRs (44). MUC1 (in humans) and Muc1 (in mice) are membrane bound mucins that interact with flagellin. Muc1-deficient mice were more capable of clearing *Pseudomonas aeruginosa* from the airways and had a more pronounced proinflammatory response as compared to wt mice, suggesting that Muc1 has an immunosuppressive effect in *Pseudomonas* infections of the airways by interfering with flagellin interaction with TLR5 (103).

TLR7

TLR7 recognizes viral ssRNA and so far, no bacterial ligands have been found. It is, however, possible that this receptor could have an indirect effect on susceptibility to bacterial infections. The artificial TLR7 ligand R-848 has been reported to induce increased endothelial adhesiveness, resulting in a transient depletion of local peripheral blood leukocytes in mice (55). Leucopenia is a common feature of many viral infections and may lead to increased susceptibility to secondary bacterial infections (101, 104, 164). Gunzer *et al.* postulated that TLR7-induced leucopenia could contribute to increased susceptibility to secondary bacterial infections (55).

TLR9

Little is known about the contribution of TLR9 in protection against bacterial infections. Based on their CG dinucleotide content in DNA, different bacteria are more or less prone to induce TLR9 (36). Both *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* have high CG dinucleotide contents, while *Streptococcus pneumoniae* and *Staphylococcus aureus* have low. TLR9 has been shown to cooperate with TLR2 in protection against *M. tuberculosis* in mice (15). Compared to wt, TLR2 or TLR9-deficient mice were only slightly more susceptible to infection with *M. tuberculosis*. The TLR9-deficiency was associated with impaired responses of IL-12p40 and IFNγ *in vivo* and *in vitro*. In comparison to TLR2 or TLR9 single knock out animals, TLR2/9 double knockout mice (DKO) were more susceptible to mycobacterial infection. Higher bacterial numbers were observed in the lungs and spleen of these animals, and there were histopathological signs of severe inflammation in the lungs. Cytokine responses were also more attenuated in TLR2/9 DKO mice than in the single knock out animals. Our group has recently shown that TLR9 protects against infection with an invasive strain of *S. pneumoniae* in mice (6, 8). In contrast to mice lacking MyD88, TLR9-deficient mice were able to control bacterial proliferation in the upper respiratory tract and could mount an inflammatory immune response in the lungs (6, 8). However, TLR9 was crucial to clear the infection in the lungs at the very early stage of infection, *i.e.* before 8 h post infection. *In vitro*, bone marrow-derived macrophages from TLR9-deficient mice could respond to most TLR ligands (LPS, lipidA, Pam3Cys4) as well as whole bacteria, but not to CpG DNA. They were also impaired in their ability to take up and kill pneumococci. Also, resident alveolar

macrophages isolated from TLR9-deficient mice were defective in bacterial uptake, suggesting that the increased susceptibility to pneumococcal infection was due to a deficient clearance of bacteria in the lower respiratory tract early in infection. Whether or not this defect is due to a defective response to CpG containing bacterial DNA or to an inherited phagocytosis defect of $TLR9^{-/-}$ macrophages remains to be elucidated (6, 8).

In addition, TLR9 activation has been shown to participate in the pathology of *P. aeruginosa* keratitis in mice and be important for killing of the bacteria (75). TLR9 is also reported to be essential for the immunomodulatory effects of *Proprionebacterium acnes* (78).

TLR11

Very recently, murine TLR11 was demonstrated to protect against uropathogenic *E. coli* (UPEC) (179). It was shown that knock out mice had a 10 000 fold higher bacterial burden in the kidney as compared to wt mice, but there was no difference in the bladder. It was suggested that while TLR11 plays a crucial role in the protection of the kidney from ascending UPEC, it plays only a minor role in the bladder where UPEC is also recognized by TLR2 and TLR4 (see section above). The *E. coli* ligand involved in the activation of TLR11 has not yet been identified. Interestingly, the human TLR11 is probably non-functional due to a stop codon in its open reading frame (ORF) and it has been speculated that the absence of a functional TLR11 signalling toward UPEC would be the reason why humans are specifically susceptible to urinary tract infections (179). However, this speculation remains questionable.

Recently, *Toxoplasma gondii* profilin, a small actin-binding protein involved in polymerization of actin was shown to activate murine dendritic cells via TLR11 (123). Hence, TLR11 like TLR5 might be the only TLRs known to use microbial protein sequences as ligands.

Immune evasion

Many bacterial pathogens have evolved strategies to dampen host inflammatory responses such as by altering LPS as already discussed above (72, 73). *Salmonella enteritidis* serotype Typhi causes systemic disease unlike *Salmonella enteritidis* serotype Typhimurium that causes local inflammatory disease in the intestine. Serotype Typhi unlike serotype Typhimurium expresses a capsule known as the Vi-antigen. It has been suggested that the Vi-antigen has an immunosuppressive effect evading innate immune recognition of serotype Typhi in the intestinal mucosa promoting systemic spread of the bacteria (126). The important human pathogens *Campylobacter jejuni, Helicobacter pylori,* and *Bartonella bacilliformis* produce subclasses of flagellins that do not act as an agonist for TLR5 (9). Enteric pathogens such as *Shigella spp*, *Salmonella spp*, and *Yersinia spp* owe a major part of their virulence to a specialized secretion system (Type III), allowing the delivery of effector proteins into host cells, with different perturbing effects on host cell functions. Recent data show that many effectors have potent anti-inflammatory effects. For example, YopJ of *Yersinia* has recently been shown to be an acetyl transferase that modifies critical amino acid residues on MAPK6 and IKKβ thereby preventing their phosphorylation, and ability to activate the MAPK and NF-κB pathways (113). Other Type III secretion

effectors target protein degradation. Thus, OspG of *Shigella* has been shown to be a kinase that owes its anti-inflammatory effects to binding to a subset of ubiquitinated E2 (ubiquitine-conjugating) enzymes, including those involved in the degradation of I-κB, thereby preventing translocation of NF κB to the nucleus (88). It may seem strange that *Shigella* causing dysentery, the prototype of an acute inflammatory infection of the bowel, needs an anti-inflammatory strategy in its weapon arsenal, but recent data suggest that this pathogen make use of pro-inflammatory as well as anti-inflammatory approaches during different stages in the infectious cycle.

Polymorphisms, immunodeficiency and susceptibility to infections

In contrast to mice, little is known about the role of the human TLRs in resistance to bacterial infection. Studies on genetic polymorphisms in humans showed that single nucleotide polymorphisms (SNP) could result in an altered susceptibility to infectious or inflammatory disease (142). The Arg677Trp polymorphism of TLR2 was found to be associated with leprosy in a Korean population and with susceptibility to tuberculosis in a Tunisian population (22, 79, 80, 105). Another TLR2 SNP, Arg753Gln, was suggested to protect from the development of late stage of Lyme disease (LD) caused by *Borrelia burgdorferi* via a reduced TLR2/TLR1 signalling. In this study, 155 patients with diagnosed LD were compared with healthy controls to investigate the role of heterozygocity of this specific SNP and it showed that the Arg753Gln SNP was present at lower frequency in patients suffering from LD especially in late stage LD patients as compared with matched healthy controls (140). Two SNPs have been described for TLR4, Asp299Gly and Thr399Ile that account for

hypo-responsiveness to inhaled LPS as well as for increased susceptibility to septic shock during infections with Gram-negative bacteria (69). For instance, homozygotes of the TLR4 (Asp299Gly) polymorphism were significantly more frequent among patients with osteomyelitis, a bone infection mostly caused by *Staphylococcus aureus* but also by Gram-negative bacteria (112). A stop codon polymorphism (TLR5 392STOP) in the ligand recognition domain of TLR5 is associated with susceptibility to pneumonia caused by *Legionella pneumophila*, a flagellated bacterium (60). Recently, SNP haplotype for the IRAK-1 gene, Leu522Ser found in Caucasians, was suggested to be associated with a more severe clinical course and outcome in sepsis as well as an increased mortality (11). Primary immunodeficiency diseases, which represent a group of primarily single-gene disorders of the immune system, are known to predispose patients to invasive disease. Indeed, patients with IL-1 receptor-associated kinase 4-deficiency (IRAK-4) as well as NF-kB essential modulator-deficiency (NEMO) were more prone to develop severe pyogenic bacterial infections (34, 94, 122, 123). Both IRAK-4 and NEMO are involved in the Toll-IL-1R signalling pathway leading to NF-κB activation upon microbe-TLR recognition. Both these defects impaired the TLR signalling. However, a recent study on Caucasian patients with invasive pneumococcal disease and controls for the known Arg579His, Pro631His and Arg753trp polymorphisms in TLR2 and the Asp299Gly polymorphism in TLR4 observed no association between TLR2 and TLR4 polymorphisms and invasive pneumococcal infections (111).

TLR and the intestinal microflora

Colonization of the intestine is initiated soon after birth. Throughout life the intestinal microflora needs to be tightly regulated, and it occurs *via* mechanisms that are poorly understood. It is believed that intestinal bowel disease (IBD) is the consequence of a dysregulation between the microflora and the host innate immune system. In mice, the small intestinal epithelium develops tolerance to LPS soon after birth due to the exposure of exogenous endotoxin. Interestingly, this tolerance development was considerably delayed in mice delivered through Caesarean sections as compared to vaginal delivery (102). Hence, responsiveness of the normal intestinal epithelium is actively repressed, by repeated exposure to PAMPS provided by the microflora as well as by food intake. A disturbance of anti-inflammatory and immunosuppressive mechanisms in the gut is believed to lead to colitis driven by a hyper responsiveness to the commensal flora. TLR signalling may have a dual role in gut homeostasis since it was shown to be required for commensal-dependant colitis in IL10-deficient mice but not in mice deficient in IL2 (127-129). The mechanisms by which the intestinal microflora suppresses inflammatory signalling may be dependant on the non-invasive nature of these organisms, as it was recently reported that activation of TLR9 located in the apical surface domain resulted in tolerance to TLR activation, whereas activation of TLR9 in the basolateral region, only reachable by invasive bacteria caused immune activation (98, 99).

Concluding remarks

Even though a wealth of data are available on TLR signalling in response to bacterial ligands, we need to know much more about the integrated responses that occur when intact bacteria are infecting the host. In the years to come we will learn more about different mechanisms by which primary bacterial pathogens can modulate or suppress innate immune responses by interfering at different levels with TLR signalling, and how opportunistic pathogens may take advantage of for example host responses to viral infections to gain access to deep tissue from local sites. The negative feed back loops controlling the extent and duration of innate immune responses to microbes is probably crucial for controlling the commensal flora without inducing inflammatory disease and need to be examined in much more detail. In the human setting, we will obtain a much deeper understanding of inflammatory bowel disease, its relationship to the intestinal microflora, how PAMPs are recognized by the intestinal mucosa, and how tolerance is developed. In the nasopharynx we are still far from understanding why as many as 60% of all preschool children may carry a potentially devastating pathogen, *Streptococcus pneumoniae* in this locality with only a small number of children coming down with invasive pneumococcal disease. Are those children carrying particular haplotypes affecting TLR signalling or are they being infected by more virulent pneumococcal strains or are these children temporally affected in their innate immune responses by other infections? Since most bacterial infections in humans, at least in the developed world, are caused by opportunistic rather than primary pathogens we need to shift our

attention much more to the former group of organisms, and study how host innate immune functions normally confine these opportunists to local sites.

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Table 1: TLRs and bacterial ligands

TLR	Microbial Ligands	Species	Ref.
TLR1/TLR2	Triacylated lipopeptides	Bacteria and mycobacteria	(30, 146, 155)
TLR2	Lipoteichoic acids (LTA)	Gram-positive bacteria i.e *Staphylococcus aureus*, *Streptococcus pneumoniae etc*	(56, 89, 141)
	Atypical Lipopolysaccharides (LPS)	Gram-negative bacteria i.e *Phorphyromonas gingivalis*	(17, 134)
	Porins	Gram-negative bacteria i.e *Neisseria* sp, *Shigella* sp, *Haemophilus influenzae*	(108, 131, 132)
	Peptidoglycan (PG)	Gram-positive and negative bacteria	(143, 177)
	Lipoarabinomannan	Mycobacteria	(157)
TLR2/TLR6	Diacylated lipopeptides	Mycoplasma	(25, 146)
	Lipoteichoic acids (LTA)	Group B streptococci	(64)
TLR4	Lipopolysaccharides (LPS)	Gram-negative bacteria	(156)
TLR5	Flagellin	Flagellated Gram-positive and negative bacteria	(61)
TLR9	CpG	Gram-positive and negative bacteria including mycobacteria	(19)

TLR11	?	Uropathogenic *E. coli*	(179)

Figure Legends

Figure 1: Schematic representation of the Toll-IL-1R superfamily.

IL-1 receptor (IL-1R) and the Toll-Like Receptors (TLRs) share a common signalling pathway *via* recruitment of an adaptor molecule to their homologous cytoplasmic domain called TIR (Toll/IL-1 receptor domain). The extra cellular domain of the IL-1R has an immunoglobulin (Ig)-like structure while TLRs have leucine rich repeat motifs (LRR). The extra-cellular domains of TLRs, the LRRs, are responsible for the recognition of PAMPs.

Figure 2. Schematic pictures of Gram positive and Gram negative cell wall in relation to TLR recognition.

In the Gram-positive and Gram-negative bacterial cell walls, the inner membrane (IM) or cytoplasmic membrane is composed of a double layer of phospholipids and lipoproteins (LP). A thick layer of peptidoglycan (PG) covers the IM of Gram-positive bacteria while a thinner layer is found in the periplasmic space (PS) in Gram-negative bacteria. In Gram-positive bacteria, lipotechoic acids are attached via its lipid-moiety anchored to the cytoplasmic membrane. In Gram-negative bacteria, an additional membrane, the outer membrane (OM) mainly composed by lipopolysaccharide (LPS), phospholipids, proteins (i.e. porins) and lipoproteins (LP) covers the PS.

Figure 3: TLR signalling

Ligand binding to their cognate TLRs induces two signalling pathways, the MyD88-dependent and the MyD88-independent pathways. Four adaptor proteins (MyD88, TIRAP/Mal, TRIF and TRAM) selectively activate these two

signalling pathways leading either to the production of proinflammatory cytokines or type I interferons (IFNs).

Fig. 1

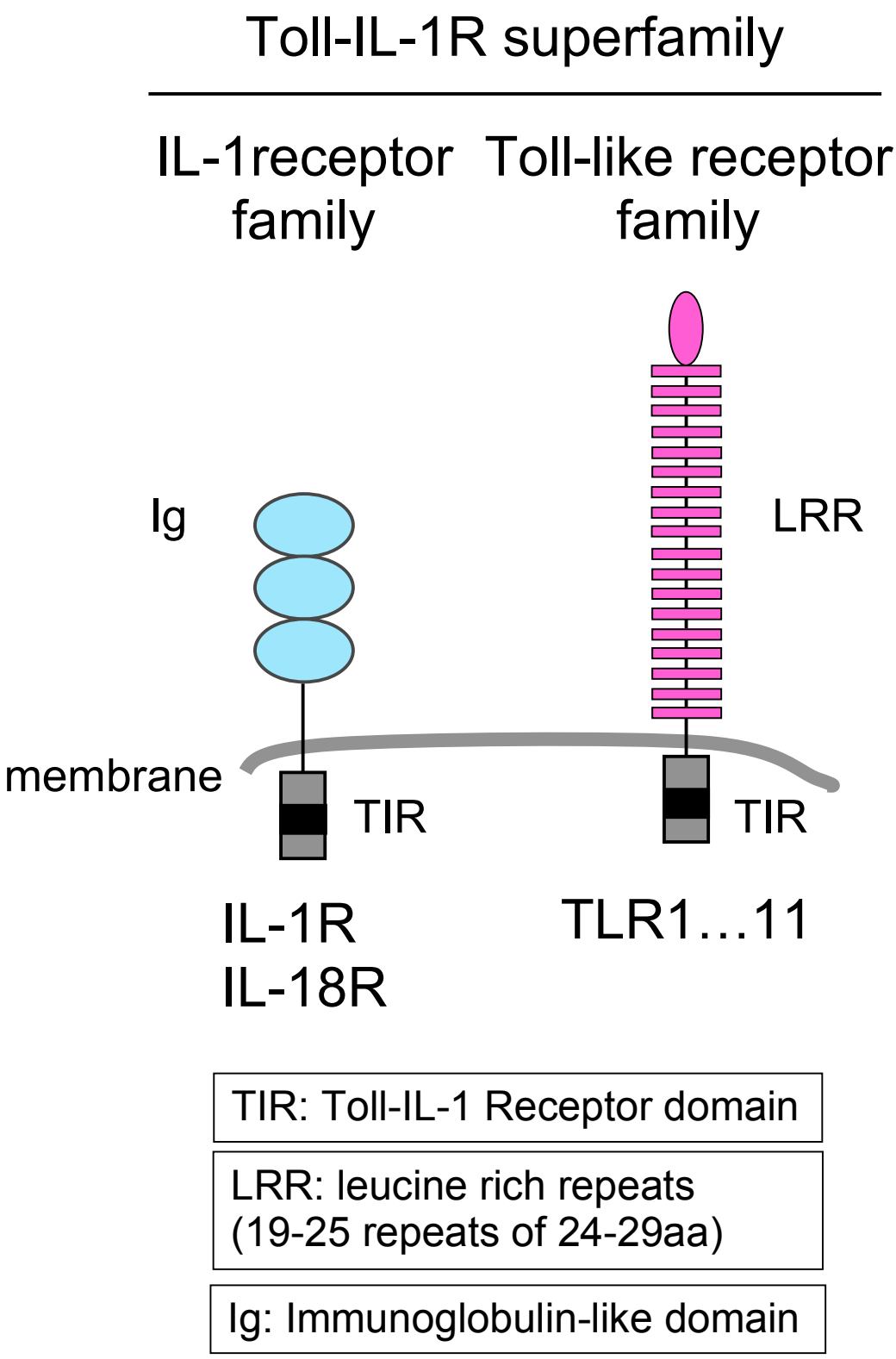
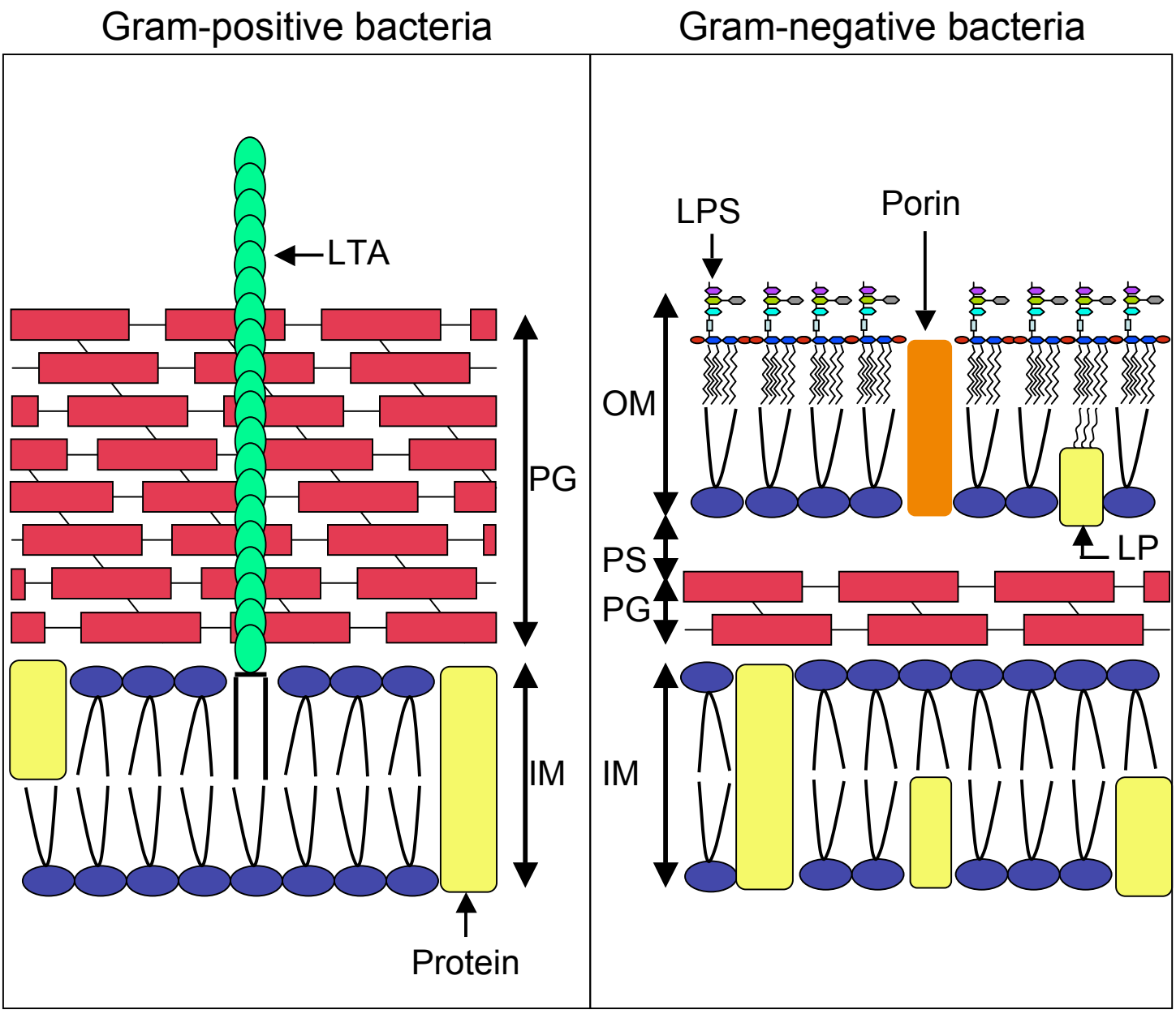
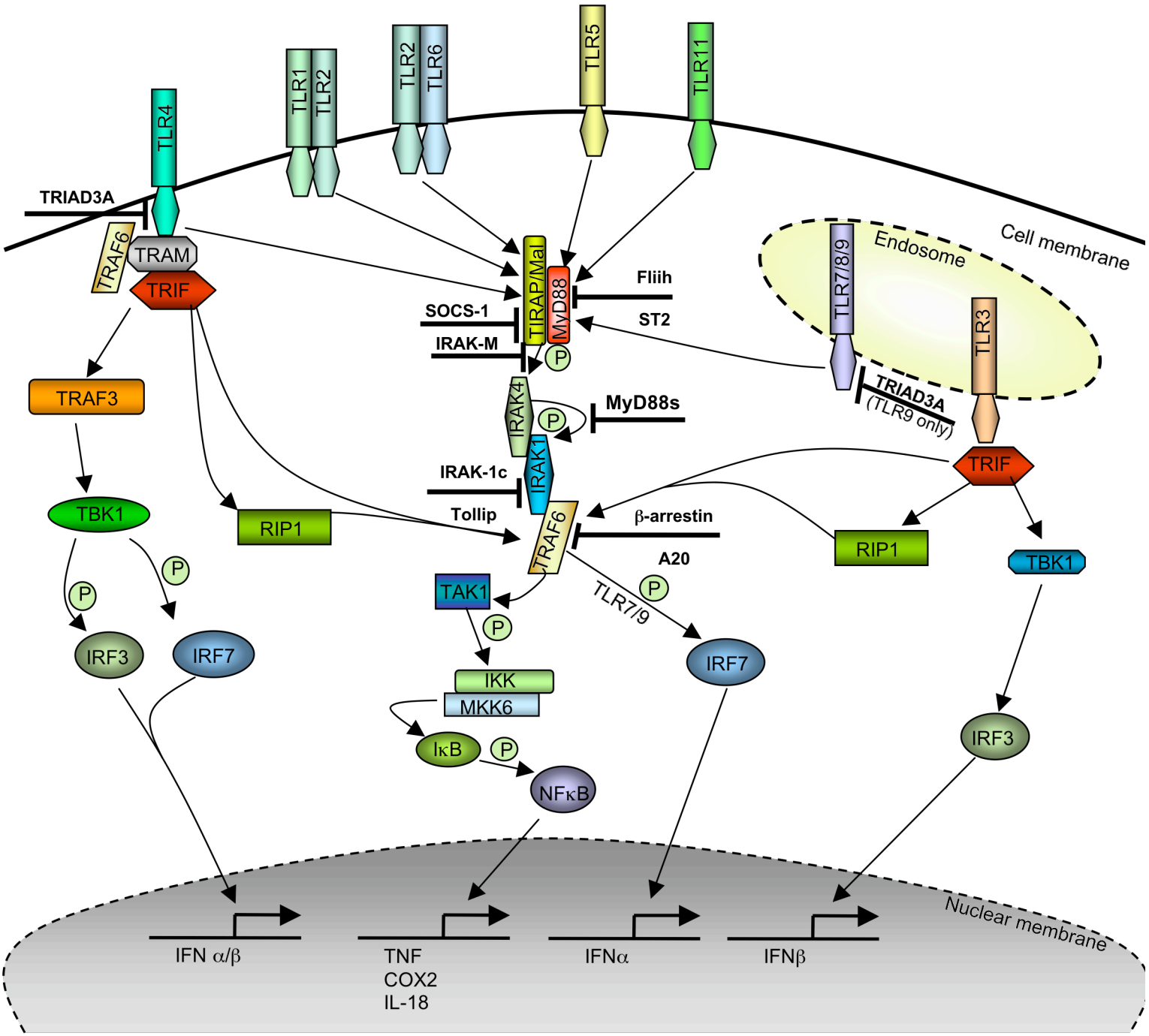

Toll-IL-1R superfamily
IL-1receptor family
Toll-like receptor family
Ig
LRR
membrane
TIR
TIR
IL-1R
IL-18R
TLR1…11
TIR: Toll-IL-1 Receptor domain
LRR: leucine rich repeats (19-25 repeats of 24-29aa)
Ig: Immunoglobulin-like domain

Fig. 2



IM: inner membrane
PS: periplasmic space
OM: outer membrane
PG: peptidoglycan
LTA: lipoteichoic acid
LPS: lipopolysaccharide
LP: lipoprotein

Fig. 3


TLR4
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TLR2
TLR6
TLR5
TLR11
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TRAF6
TRAM
TRIF
TRAF3
TBK1
RIP1
P
IRF3
IRF7
TIRAP/Mal
MyD88
Fliih
ST2
SOCS-1
IRAK-M
IRAK4
MyD88s
IRAK1
IRAK-1c
Tollip
TRAF6
β-arrestin
A20
TAK1
TLR7/9
IKK
MKK6
IκB
NFκB
Endosome
Cell membrane
TLR7/8/9
TLR3
TRIAD3A
(TLR9 only)
TRIF
RIP1
TBK1
IRF3
IRF7
Nuclear membrane
IFN α/β
TNF
COX2
IL-18
IFNα
IFNβ