Toll-like receptors: tools, assays, and implications for in vitro pyrogen tests
Kikkert, R.

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Chapter 1

General introduction

Robert Kikkert
General introduction

Discovery of TLRs – early history

Toll-like receptors (TLRs) are named after Christiane Nusslein-Volhard’s legendary exclamation “toll!” after she observed the effect of a particular gene product on the establishment of dorso-ventral patterning during embryonic development of *Drosophila* flies, which was published in 1985. About a decade later it was discovered that in adult *Drosophila* flies, toll played an essential role in the defence against fungal infection. The discovery of a human homologue of the *Drosophila* toll protein led to the identification of several human mammalian TLRs. When positional cloning of the gene responsible for LPS hyporesponsiveness in C3H/HeJ and C57BL/10ScCr mice as well as the generation of TLR4 knockout mice unequivocally demonstrated that TLR4 is essential for LPS signalling, several research groups started to search for ligands for one of the newly discovered TLRs.

TLRs are a family of homologous type I transmembrane proteins characterized by an extracellular leucin-rich repeat (LRR) domain involved in pathogen-associated molecular pattern (PAMP) recognition, and a cytoplasmic domain that is responsible for signal transduction. The cytoplasmic domain of TLRs is homogolous to the cytoplasmic domain of the IL-1 receptor and is referred to, therefore, as the Toll/IL-1 R homology (TIR) domain.

**TLR signalling**

It has become clear that after TLR triggering by ligands, downstream signalling is only initiated after dimerization with neighbouring TLRs. Dimers are the result of either heterodimerization (e.g. TLR2 with either TLR1 or TLR6) or homodimerization (e.g. TLR4-TLR4). Recently, heterodimerization of particular TLR combinations (TLR7, TLR8, and TLR9) was also demonstrated to inhibit TLR7 and/or TLR8 signalling. TLRs are localized in different compartments. TLR1, TLR2, TLR4, TLR5, and TLR6 are exclusively expressed on the cell surface, whereas TLR3, TLR7, TLR8 and TLR9 are located in the endosomal compartment, although it has been suggested that a small fraction of TLR3 and TLR8 is also located at the cell surface. Following recognition of a certain PAMP and consecutive dimerization of TLRs, intracellular adaptor molecules are recruited to the scaffold formed by the dimerized TIR domains. These adaptor molecules include myeloid differentiation primary response gene 88 (MyD88), MyD88 adaptor-like (MAL)/TIR domain-containing adaptor molecule (TIRAP), TIR domain-containing adaptor inducing interferon-beta (TRIF), or TRIF-related adaptor molecule (TRAM). Recruitment of these adaptor molecules towards the plasma membrane activates a
General introduction

signalling cascade including IL-1-Receptor-Associated Kinase (IRAK) and Tumor Necrosis Factor Receptor-Associated Factor (TRAF) \(^6\). After activation of TRAF6 two different pathways may be activated. One pathway comprises a route that activates Nuclear Factor (NF)-\(\kappa\)B, p38, and c-Jun N-terminal kinase (JNK), whereas the other signalling route culminates in the activation of Interferon Response Factors (IRF1, IRF3, and IRF5)\(^{22,23}\). Both signalling routes eventually lead to the induction of cytokine release, the upregulation of MHC class II as well as B7 co-stimulatory molecules.\(^{22-25}\) Recently, the previously described molecule SARM (sterile \(\alpha\)- and HEAT/armadillo motif-containing protein)\(^{26}\) was found to be a TIR-domain-containing adaptor that negatively regulated NF\(\kappa\)B and IRF activation by interaction with TRIF.\(^{27}\)

An overview of adaptor molecules in TLR signalling is provided in figure 1. At present, numerous adaptors, regulatory proteins, phosphatases, and kinases are being investigated for their involvement in one of the intricate signalling cascades downstream TLR activation.\(^{23,28}\) To illustrate this, in 2008 Luke O’Neill (Trinity College, Dublin, Ireland), who has become the godfather of TLR adaptor biology, published 5 reviews only to keep track of recent developments in TLR adaptor molecules and new signalling cascades.

Specificity of TLRs

Early studies using TLR-transfected cell lines suggested that TLR2 was involved in LPS signalling.\(^{29-31}\) However, TLR2 knock-out mice did not respond to LPS\(^{32}\) and the work by Beutler’s research group clearly demonstrated that in fact TLR4 was the long-sought LPS receptor.\(^6\) Reverse genetic approaches with knockout of TLR mice demonstrated that TLR2 signals the presence of different microbial components of Gram-positive bacteria, such as peptidoglycan,\(^{29-33}\) and lipoproteins.\(^{34-36}\) TLR2 is the most promiscuous TLR as numerous ligands such as constituents of yeast (zymosan),\(^{42}\) schistosomes (phosphatidylserine from Schistosoma mansoni),\(^{41}\) or even viruses (human cytomegalovirus)\(^{43}\) have been identified (see table 1). Both TLR1 and TLR6 were shown to engage in heterodimer formation with TLR2.\(^{9,44}\) TLR1 and TLR2 are involved in recognition of bacterial lipopeptides containing triacetylated cysteine residues, whereas TLR6 and TLR2 recognize mycobacterial lipopeptides with diacetylated cysteines,\(^{9,44}\) which illustrates the specificity of TLR signalling. TLR3 recognizes double-stranded RNA,\(^{45}\) TLR5 signals flagellin from flagellated bacteria,\(^{46}\) and TLR9 detects unmethylated CpG motifs from bacterial DNA.\(^{47}\) TLR7, and in humans also TLR8 recognize small antiviral compounds called imidazoquinolines.\(^{48}\) TLR7 was also claimed to signal the presence of single-stranded RNA viruses such as vesicular stomatitis virus and influenza virus.\(^{49}\)
Figure 1. Overview of transcription factor activation through TIR-domain-containing adaptors for the TLR/IL1R superfamily (adapted from ref. 23). Each adaptor is differentially used by receptor complexes to positively regulate transcription-factor activation. The exception is SARM (sterile α- and armadillo-motif-containing protein), which inhibits TRIF (Toll/IL-1R (TIR)-domain-containing adaptor protein inducing interferon-β (IFN-β))-mediated transcription-factor activation. IL-1R, interleukin-1 receptor; IRF, IFN regulatory factor; mDC, myeloid dendritic cell; MAL, MyD88 (myeloid differentiation primary-response gene 88) adaptor-like protein; NF-κB, nuclear factor-κB; TLR, Toll-like receptor; TRAM, TRIF-related adaptor molecule.
TLR10, which only exists in humans and is lost from the mouse genome, is an orphan receptor as its ligand has not yet been identified. TLR11 is expressed only in mice because of several stop codons in the putative open reading frame of human TLR11. Unlike other TLRs, TLR11 is almost exclusively expressed in the urogenital system and is most likely involved in the recognition of particular proteins from uropathogenic bacteria. In another study, small profilin-like proteins from protozoan parasites have also been identified as ligands for TLR11. The relation between both findings has still to be elucidated. In mice, a TLR closely resembling TLR3 and TLR9 was discovered and baptized TLR13. However, its function is unknown. An overview of TLRs with their respective ligands is presented in table 1.

An important and still underestimated issue when identifying PAMPs as ligands for TLRs is contamination. Early reports claiming that TLR2 was the receptor for LPS were caused by the fact that commercially available LPS was, and still is, frequently contaminated with bacterial lipoproteins or peptidoglycan, which both stimulate TLR2. Repurification by phenol extraction appeared to be a suitable method of removing trace amounts of contaminating peptidoglycan or lipopeptides in commercially available LPS.

TLRs recognize a plethora of ligands. Given the broad range of ligands that are recognized by particular TLRs, Bruce Beutler, one of the leading researchers in TLR biology, has stipulated that the specificity of TLRs is rather low, or alternatively phrased, TLRs possess a large ligand space. Thus, the specificity (or lack thereof) is determined by the TLR itself, the TLR dimerization partner, and the adaptor molecules which are recruited.

**Endogenous ligands for TLRs**

In addition to microbial LPS, also particles/proteins derived from respiratory syncytial virus and from vesicular stomatitis virus were demonstrated to signal through TLR4. However, TLR4 was also reported to signal endogenous ligands such as fibrinogen and fragmented products of hyaluronic acids. In mice, the endogenous protein \( \beta \)-defensin 2 was also identified as an endogenous ligand for TLR4. In men, human heat shock proteins (HSPs) released from injured cells have been reported to activate dendritic cells through TLR2 and TLR4. Tamm-Horsfall glycoprotein (THP) is a protein with unknown function that is exclusively expressed in Henle’s loop of the kidney. Upon damage to the kidney, such as during interstitial nephritis or urinary tract infection, THP leaks out of kidney cells and serves as a ligand for TLR4. Heme, composed of an atom of iron linked to four groups of porphyrin, which together constitute the basis of haemoglobin, is released from cells after cell damage/hemolysis and likewise stimulates TLR4. Although
### Table 1: Toll-like receptors and their respective ligands.

<table>
<thead>
<tr>
<th>TLR human/mouse</th>
<th>Ligand</th>
<th>Endogenous ligand</th>
<th>Reference</th>
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<tbody>
<tr>
<td>TLR1 (hm)</td>
<td>dimerizes with TLR2 (see TLR2)</td>
<td></td>
<td></td>
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<tr>
<td>TLR2 (hm)</td>
<td>peptidoglycan (PGN)</td>
<td></td>
<td>29-33</td>
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<td></td>
<td>lipoproteins</td>
<td></td>
<td>34-36</td>
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<tr>
<td></td>
<td>lipoteichoic acid from Gram+ bacteria</td>
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<td>33</td>
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<tr>
<td></td>
<td>liparabinomannan (LAM) from <em>Mycobacterium tuberculosis</em></td>
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<td>37,38</td>
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<tr>
<td></td>
<td>lipoprotein (19kDa) from <em>Mycobacterium tuberculosis</em></td>
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<tr>
<td></td>
<td>glycophasphatidylinositol anchors from <em>Trypanosoma cruzi</em></td>
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<td></td>
<td>porins from <em>Neisseria meningitides</em></td>
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<td>phosphatidylserine from <em>Schistosoma mansoni</em></td>
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<tr>
<td></td>
<td>zymosan</td>
<td></td>
<td>42</td>
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<tr>
<td></td>
<td>heat shock proteins (HSP60, HSP70, Gp96)</td>
<td>+</td>
<td>54-56</td>
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<td></td>
<td>high mobility group box 1 (HMGB-1)</td>
<td>+</td>
<td>57,58</td>
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<tr>
<td></td>
<td>HMGB1-nucleosome complexes</td>
<td>+</td>
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<tr>
<td></td>
<td>oxygen radicals</td>
<td>+</td>
<td>60,61</td>
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<tr>
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<td>LPS from <em>Porphyromonas gingivalis</em></td>
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<td>OsPA:outer surface protein A from <em>Borrelia burgdorferi</em></td>
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<td>TP47L: lipopeptide from <em>Treponema pallidum</em></td>
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<tr>
<td></td>
<td>MALP-2 (mycoplasmal macrophage-activating lipopeptide-2)</td>
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<td>36</td>
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<td></td>
<td>Secreted factor of group B streptococcus</td>
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<td>64</td>
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<td></td>
<td>heat-killed <em>Listeria monocytogenes</em></td>
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<td>soluble factors released from <em>Neisseria meningitidis</em></td>
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<td>outer membrane protein A from <em>Klebsiella pneumoniae</em></td>
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<td></td>
<td>human cytomegalovirus</td>
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<tr>
<td></td>
<td>respiratory syncytial virus</td>
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<td>68</td>
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<td></td>
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<td></td>
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<tr>
<td>TLR3 (hm)</td>
<td>Poly I:C / double-stranded RNA</td>
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<td>45</td>
</tr>
<tr>
<td></td>
<td>mouse cytomegalovirus</td>
<td></td>
<td>52</td>
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<tr>
<td>TLR4 (hm)</td>
<td>LPS</td>
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<td>6,69</td>
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<td></td>
<td>respiratory syncytial virus</td>
<td></td>
<td>70</td>
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<tr>
<td></td>
<td>vesicular stomatitis virus</td>
<td></td>
<td>71</td>
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<tr>
<td></td>
<td>glucuronoxylomannan of <em>Cryptococcus neoformans</em></td>
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<td>72</td>
</tr>
<tr>
<td></td>
<td>poly M: <em>Pseudomonas aeruginosa</em> mannuric acid polymers</td>
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<tr>
<td></td>
<td>heat shock proteins (HSP60, HSP70)</td>
<td>+</td>
<td>54-56</td>
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<td></td>
<td>HMGB-1</td>
<td>+</td>
<td>57,58</td>
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<tr>
<td></td>
<td>fibrinogen</td>
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<td></td>
<td>hyularonic acid</td>
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<tr>
<td></td>
<td>B-defensin 2</td>
<td>+</td>
<td>76</td>
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<tr>
<td></td>
<td>THP (Tamm-Horsefall Protein)</td>
<td>+</td>
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<tr>
<td></td>
<td>heme</td>
<td>+</td>
<td>78</td>
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<tr>
<td>TLR5 (hm)</td>
<td>flagellin</td>
<td></td>
<td>46</td>
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<tr>
<td>TLR6 (hm)</td>
<td>TLR6 dimerizes with TLR2 (see TLR2)</td>
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<tr>
<td>TLR7 (hm)</td>
<td>imidazooquinolines (imuqimod, R-848)</td>
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<td>48,79</td>
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<td></td>
<td>small antiviral compounds (loxoribine, bropirimine)</td>
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<td>80</td>
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<td></td>
<td>vesicular stomatitis virus</td>
<td></td>
<td>49</td>
</tr>
<tr>
<td>TLR8 (m)</td>
<td>single stranded</td>
<td></td>
<td>48,79</td>
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<td>single stranded RNA from group B cossackieviruses</td>
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<td>81</td>
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<tr>
<td>TLR9 (hm)</td>
<td>CpG DNA</td>
<td>+</td>
<td>47,82</td>
</tr>
<tr>
<td></td>
<td>DNA-containing immune complexes</td>
<td>+</td>
<td>82</td>
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<td>double-stranded DNA</td>
<td>+</td>
<td>83-85</td>
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<td>mouse cytomegalovirus</td>
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<td>52</td>
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<tr>
<td>TLR10 (h)</td>
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<tr>
<td>TLR11 (m)</td>
<td>HKUEC (heat-killed uropathogenic <em>E. coli</em>)</td>
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<td>50</td>
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<tr>
<td></td>
<td>profilin-like molecule (<em>T. gondii</em>)</td>
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<td>51</td>
</tr>
<tr>
<td>TLR12 (m)</td>
<td>unknown</td>
<td></td>
<td>86</td>
</tr>
<tr>
<td>TLR13 (h)</td>
<td>unknown</td>
<td></td>
<td>52</td>
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</table>
endotoxin contamination in these molecules, in particular in HSP preparations, might account for some of the obtained results (which provokes the debate whether or not these substances are genuine TLR-ligands), these reports strongly suggest that TLR4 can recognize both host-derived molecules as well as microbial components. At least the latter two studies which showed that damaged cells release substances, THP and heme respectively, that activate TLR4, were meticulous in demonstrating that the observed effects were not caused by endotoxin contamination.

TLR9 recognizes unmethylated CpG motifs that are present in bacterial and viral DNA, but also in mammalian DNA promoter elements. Using MRL-lpr mice, a well studied murine model of systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), it was demonstrated that both chromatin (=mammalian DNA)-containing immune complexes and CpG DNA were capable of stimulating rheumatoid factor-positive B-cells in a MyD88-dependent manner, suggesting that TLR9 was involved. As TLR9 activation is dependent on endosome maturation/acidification, the finding that inhibitors of the endosome acidification pathway (such as bafilomycin A or chloroquine) also specifically inhibited the stimulatory effect on B-cells, supported the hypothesis that TLR9 was involved. Moreover, endosome acidification inhibitors such as chloroquine have proven successful in the treatment of SLE and RA. Although not yet commonly accepted, recent reports by different groups demonstrated that the recognition by TLR9 of double-stranded DNA was not limited to CpG-motif containing DNA. Despite the fact that synthetic 2'-phosphorothioate (PS)-modified DNA only activated TLR9 when CpG-containing DNA stretches were present, natural DNA with a 2' phosphodiester backbone stimulated TLR9 independent of CpG motifs. These latter findings, next to the report by Leadbetter, make it even more plausible that TLR9 is a receptor for nucleus-derived, mammalian DNA.

Self/Non-self discrimination, the Danger Model, and TLRs

During the last five years data have emerged which strongly suggest that at least TLR2, TLR4, and TLR9 may be involved in the recognition of not only extracellular components of microbial origin, but also of endogenous ligands that are liberated after cell injury. Undoubtedly, more examples of particular TLRs recognizing endogenous “self” material will be reported in near future. The discovery that TLRs are also involved in the recognition of endogenous substances has contributed significantly to a change in the idea that the immune system functions by making a distinction between self and non-self. The original concept of the self/non-self paradigm was that lymphocytes are activated by recognition of foreign material, such as bacteria and viruses. A first modification of this self-nonself hypothesis arose in 1969, when it was discovered that the immune response to foreign
substances only occurred in the presence of T-cell help. Yet another modification of the theory resulted from the finding that this T-cell help could only exist when T-helper cells were co-stimulated by professional antigen-presenting cells (APC). Charles Janeway hypothesized that normally, APC are quiescent and do not provide the co-stimulatory signal for T-helper cells, unless activated by microbes via evolutionary conserved pattern recognition receptors (PRR) of the innate immune system. The discovery of TLR4 as the receptor for infectious and non-self LPS very nicely fitted into this model. In 1994, opposed to the self/non-self hypothesis, Polly Matzinger introduced the conjectural Danger Model hypothesis. This model added a wider scope to the activation of APC and thus to the initiation of the immune response as it proposed that danger/alarm signals from injured cells stimulate APCs. According to this hypothesis danger/alarm signals comprise pathogens (microbes, viruses), toxins, mechanical damage, etc. The danger/alarm signal should not be sent by healthy cells or cells undergoing normal apoptosis, but includes any substance made by, or modified by distressed, damaged, or injured cells. According to this theory, it is not so much the “foreignness” of a particular pathogen that determines whether or not an immune response is triggered. Although TLRs as receptors for foreign pathogenic substances nicely suit the self/non-self hypothesis, they also fit in the Danger Model. Multiple endogenous ligands that activate the immune system via TLRs in a manner which cannot be understood by the classical paradigm of self/non-self recognition, have been discovered (see table 1). Beutler suggested that these responses to damaged or injured cells might have resulted in mutational changes creating so-called neo-ligands for TLRs. This is a tempting speculation, and indeed modified proteins in particular autoimmune diseases have been observed (e.g. citrullinated proteins in rheumatoid arthritis) which may lead to the formation of such neo-ligands. Matzinger hypothesized that the decision of cells whether or not to respond to particular signals is taken in the tissues/organs themselves. For example, under normal healthy physiological conditions THP is present only in the kidney and heme is present only in complex with its porphyrin groups. Upon damage of tissue, THP leaks out of cells and heme is released in its free form. Consequently, these substances become exposed to other cells that normally do not encounter these substances. Combining both the Danger Model and the idea of neo-ligand formation is the recent finding that TLR2 was demonstrated to be essential in the detection of oxidants, confirming similar earlier results obtained by others. Again, the observed responses may either result from the action of neo-ligands (oxidised metabolites) or from abnormal exposure to oxidants of cells/tissues in the peritoneal cavity. Similarly, HMGB1 and nucleosomes are normally contained within the cell nucleus. However, late apoptotic cells release HMGB1-bound nucleosomes which stimulate cytokine production in vitro in a TLR2-dependent manner.
To summarize, there is compelling evidence that TLRs are activated by both exogenous “foreign” and endogenous danger signals upon which APCs are stimulated to mount an appropriate immune response. Because of this new concept, Seong and Matzinger rebaptized PAMPs (pathogen-associated molecular patterns) into DAMPs (damage-associated molecular patterns).\textsuperscript{103,104} The discovery of TLRs as sentinel receptors not only for foreign pathogens (microbes/viruses), but also for danger signals that result from physiological processes which have gone astray, will significantly contribute to new concepts of (innate) immunity.

**Scope of the thesis**

The aim of this thesis was to investigate the role of TLRs in the activation of pro- and anti-inflammatory cytokine production in cellular assays such as in whole blood (WB) and mononuclear cell (MNC) assays. Activation of TLRs in these assays results in cytokine production such as TNF, IL-1(β), IL-6, IL-8, IL-10, and IL-12,\textsuperscript{105,106} which can be measured by ELISA.\textsuperscript{107} At the time we started our investigations, common approaches to study the role of TLRs in the immune response were the use of TLR-deficient mice, and the over-expression of recombinant TLRs in mammalian cells. Alternative tools, such as the use in WB or MNC assays of established neutralizing monoclonal antibodies against TLR, were not available. Therefore, we set out to express the extracellular domain of one of the most versatile TLRs in terms of ligand recognition, TLR2, in recombinant form, with the aim of immunizing mice and developing (neutralizing) monoclonal antibodies against it. As the generation of high affinity antibodies requires proper folding of the antigen, we started out with an expression system using the methyloptropic yeast *Pichia pastoris*. After several unsuccessful attempts in *P. pastoris* we expressed the extracellular domain of TLR2 in multiple bacterial hosts for protein expression, and finally, also in a human protein expression system. The results of our TLR2 expression efforts are addressed in chapter 2.

To obtain monoclonal antibodies against TLR2 and TLR4 we immunized mice with mammalian cells overexpressing TLR2 or TLR4. Prior to immunization, mice were made tolerant to mammalian cells without TLR. To screen hybridomas for anti-TLR immunoglobulin production, a FACS-screening assay was developed which allowed the discrimination of binding of supernatants to “empty” mammalian cells and cells expressing TLR. These tolerization, immunization, fusion and screening experiments are addressed in chapter 3.
Mammalian cells that exclusively express TLR2 or TLR4, and which are activated upon stimulation with TLR2 or TLR4 ligands, are a powerful tool for determining whether or not particular ligands are specific for TLR2 and/or TLR4. Periodontitis, a chronic inflammatory disease initiated by bacteria in dental plaque\(^{108}\) is characterized by a skewed Th2 response.\(^{109-111}\) Because TLRs, and in particular TLR2 and TLR4, have been shown to determine the orientation of the immune response in terms of Th orientation (Th0, Th1, or Th2),\(^{62,112-115}\) we set out to determine whether sonic extracts of a range of Gram-negative, black pigmented oral bacteria associated with periodontitis, stimulated TLR2 and/or TLR4. In addition, we attempted to correlate pathogenic virulence of different isotype strains of \textit{Porphyromonas gingivalis} and \textit{Actinobacillus actinomycetemcomitans} with cytokine production in cellular assays (\textit{chapter 4}).

As cigarette smoking was demonstrated to aggravate periodontal disease,\(^{116}\) we investigated whether this was related to a more pronounced Th2 bias in smokers as compared to non-smokers. To measure this, cytokine production (IL-1\(\beta\), IL-6, IL-8, IL-10, and IL-12) in whole blood of periodontitis patients (smokers and non-smokers) was compared after stimulation with ligands specific for TLR2 and TLR4 (\textit{chapter 5}).

Pyrogens (fever-inducing substances)\(^{117}\) in parenteral pharmaceuticals have been detected using the rabbit pyrogen test\(^{118}\) since the end of World War II and using the Limulus amebocyte lysate test (LAL)\(^ {119}\) since the late 1960s. Because of ethical, financial, and technical objections to the rabbit pyrogen test\(^ {120-124}\) and because of limitations of the LAL-test\(^ {125-127}\) new in vitro tests using human monocytes or human monocytic cell lines have been developed.\(^ {128-130}\) The basis of this so-called monocyte activation test (MAT) is that pyrogenic contaminants, via interaction with TLRs expressed on monocytes, induce cytokine production. A number of different cells with different cytokine read-outs have been established.\(^ {130}\) The aim of our study was to compare in more detail cytokine production in diluted WB, MNC, and in TLR2/4-transfected HEK cells following stimulation with TLR-ligands, to determine which in vitro test is suitable for the detection of pyrogenic contamination (\textit{chapter 6}).

During these studies on the MAT we observed aberrant and increased cytokine production whenever substances that had been dialysed, were tested. Therefore, we investigated the powerful co-stimulatory effects of substances derived from cellulose dialysis membranes on TLR ligand-induced whole blood cytokine production. As (1\(\rightarrow\)3)-\(\beta\)-D-glucans had been reported to leach from cellulose filters or cellulose dialysis membranes,\(^ {131-135}\) we tested several (1\(\rightarrow\)3)-\(\beta\)-D-glucans of different sources, on TLR ligand-induced cytokine production (\textit{chapter 7}).
The extracellular adaptor molecule MD-2 is claimed to be essential for TLR4 signalling.\textsuperscript{136,137} For that reason cDNAs for both TLR4 and MD-2 are normally co-transfected,\textsuperscript{136,138} or alternatively, recombinant soluble MD-2 is supplemented\textsuperscript{139,140} in experiments with TLR4-transfected cells. In our assays using a TLR4-transfected cell line, we noticed complete unresponsiveness toward LPS stimulation, which could be overcome by supplementation with human serum. As serum was well known to enhance the effects of LPS on monocyte cytokine production,\textsuperscript{141,142} we set out to purify the serum protein(s) involved in the potentiation of TLR4 activation by LPS, and to determine whether or not MD-2 was involved (chapter 8).

Activation of WB with ligands for Toll-like receptors (TLRs) expressed on monocytes and/or neutrophils leads to approximately 10-15 fold higher levels of IL-8 compared to IL-6.\textsuperscript{143} However, in T cell-activated WB, abundant IL-8 is produced, whereas IL-6 production was only minimal. Because regulation of IL-6 and IL-8 production is very similar,\textsuperscript{144-146} we investigated which factor(s) in T cell-stimulated WB selectively induced the production of IL-8, and not IL-6 (chapter 9).

In chapter 10 the thesis is summarized and its findings are discussed in a broader perspective.
References

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Chapter 1


