

TLR2 & Co: a critical analysis of the complex interactions between TLR2 and coreceptors

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ABSTRACT

TLRs play a major role in microbe-host interactions and innate immunity. Of the 10 functional TLRs described in humans, TLR2 is unique in its requirement to form heterodimers with TLR1 or TLR6 for the initiation of signaling and cellular activation. The ligand specificity of TLR2 heterodimers has been studied extensively, using specific bacterial and synthetic lipoproteins to gain insight into the structure-function relationship, the minimal active motifs, and the critical dependence on TLR1 or TLR6 for activation. Different from that for specific well-defined TLR2 agonists, recognition of more complex ligands like intact microbes or molecules from endogenous origin requires TLR2 to interact with additional coreceptors. A breadth of data has been published on ligand-induced interactions of TLR2 with additional pattern recognition receptors such as CD14, scavenger receptors, integrins, and a range of other receptors, all of them important factors in TLR2 function. This review summarizes the roles of TLR2 in vivo and in specific immune cell types and integrates this information with a detailed review of our current understanding of the roles of specific coreceptors and ligands in regulating

TLR2 functions. Understanding how these processes affect intracellular signaling and drive functional immune responses will lead to a better understanding of host-microbe interactions and will aid in the design of new agents to target TLR2 function in health and disease. *J. Leukoc. Biol.* 94: 885–902; 2013.

INTRODUCTION

TLRs play a central role in the innate immune system by recognizing microbe-associated molecular patterns (MAMPs) [1]. TLRs are critical for host defense but are also associated with the pathogenesis of inflammatory and autoimmune diseases via their recognition of endogenous molecules [2, 3]. TLRs can be viewed as critical surveillance receptors for detection of infection, tissue damage, and remodeling [4–6]. Over the past decade, it has become clear that TLRs depend on additional pattern recognition receptors to form functional multireceptor clusters to respond appropriately to microbes and bacterial and endogenous ligands [2, 7, 8]. Many of these pattern recognition receptors (PRR) have their own specific intracellular signaling pathways that intersect with TLR signaling [9]. Engagement of ligands with their corresponding TLR results in the induction of intracellular signaling cascades, including myeloid differentiation primary response gene (MyD88)-dependent phosphorylation of MAPK and the activation of NF- κ B and activating protein-1 (AP-1) signaling [10]. Any additional PRRs can further invoke signal transduction cascades via spleen tyrosine kinase (Syk), PI3K, protein kinase A, and protein kinase C, all of them able to modulate TLR signaling [11–13]. Cellular activation after TLR triggering there-

Abbreviations: β 2-gpl= β 2-glycoprotein I, $\alpha\beta$ =amyloid beta; AP-1=activating protein-1, CLR=C-type lectin, DAMP=damage-associated molecular pattern, DAP12=DNAX-activating protein of 12 kDa, FcR γ =FcR common γ -chain, FRET=fluorescence resonance energy transfer, ICD=intracellular domain, IP=immunoprecipitation, ITAM=immunoreceptor tyrosine-based activation motif, LTA=lipoteichoic acid, LOX-1=lectin-like oxidized LDL receptor-1, LP=lipopeptide, Mal=MyD88-adaptor-like, MALP-1=macrophage-activating lipopeptide-2, MAMP=microbe-associated molecular pattern, MARCO=macrophage receptor with collagenous structure, MyD88=myeloid differentiation primary response gene, PAUF=pancreatic adenocarcinoma up-regulated factor, PGN=peptidoglycan, PIP2=phosphatidylinositol 4,5-bisphosphate, PIP3=phosphatidylinositol-(3,4,5)-trisphosphate, PIP5K=phosphatidylinositol 4-phosphate 5-kinase, PRR=pattern recognition receptor, SAA=serum amyloid A, sCD14=soluble form of CD14, SR=scavenger receptor, Src=proto-oncogene tyrosine-protein kinase Src, SREC-I=scavenger receptor expressed by endothelial cells-I, sTLR2=soluble TLR2, Syk=spleen tyrosine kinase, TDM=6,6'-dimycolate, TIR=Toll-IL-1 receptor, TIRAP=TIR domain containing adaptor protein

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fore depends on the ligand and the cell-specific expression of TLRs and other PRRs, with all of the separate signal cascades integrated in specific lipid membrane microdomains termed lipid rafts [14].

In this review, we focus on TLR2, the most extensively studied receptor in innate immunity. The importance of TLR2 in inflammatory and infectious diseases has been highlighted by studies using TLR2 knockout mice. An overview of such studies, focusing on the variety of roles for TLR2 signaling rather than providing a complete overview of all available data, is shown in **Table 1**. A beneficial role for TLR2 signaling is described in mucosal homeostasis and defense against specific pathogens, whereas TLR2 signaling via other pathogens or after endogenous triggering is correlated with a more severe phenotype in infectious and/or inflammatory disease. In addition, although there is a clear role for TLRs in microbe-host interactions, gut flora studies in TLR2 knockout mice indicate a reciprocal role for TLR2 in host-microbe and microbe-host interactions [15, 16]. However, models based on TLR2 knockouts stress the importance of TLR2, but they do not provide insight into the interactions with actual ligands and/or coreceptors.

TLR2 expression has been found in human cells and tissues in a resting state or after activation [17]. A comprehensive overview of all cells expressing TLR2 and the functional consequence of TLR2 activation is beyond the scope of this review. However, to highlight the differential effects of TLR2 activation, a selection of cells and their effects after ligation by MAMPs or endogenous ligands are presented in **Table 2**, focusing on different outcomes induced by ligands that differ greatly in molecular makeup.

Much is still not known regarding TLR2 function, but it is clear (Table 2) that cellular activation after TLR2 ligation depends on two main factors: first, the cellular expression of additional TLR or PRR coreceptors; and, second, the type of TLR2 ligand. This review provides an update on the current knowledge regarding microbial and endogenous TLR2 agonists. Combined with a comprehensive overview of the existing data on TLR2-coreceptor interactions and the functional consequences thereof, this review aims to identify critical aspects governing TLR2 function from the many different factors that have been highlighted separately in the literature.

TLR2 AND MICROBIAL AGONISTS

TLR2 recognizes microbe membrane constituents such as lipoteichoic acids (LTA), peptidoglycan (PGN), and lipopeptides (LP), which are present in a variety of microbes, predominantly in Gram-positive, but also in Gram-negative bacteria [18]. PGNs have long been used as TLR2 agonists. However, PGN preparations are commonly found to be contaminated with LTAs and LPs and rigorous purification methods established that PGN recognition is more likely to occur via intracellular NOD-like receptors [19, 20]. TLR2 agonistic activity of purified LTA is a matter of controversy [20]. Activity-guided fractionation of *Staphylococcus aureus* LTA preparations showed that LP contamination was the

major determinant of TLR2 activation [21], which was further substantiated by the 100-fold decrease in activity in a comparison of LTA from *S. aureus* with LTA from an LP *S. aureus* mutant [22]. However, both experimental setups still showed residual TLR2 activity, indicating that LTAs are TLR2 ligands. Indeed, preparation of synthetic LTA followed by structure-function analysis revealed a minimal TLR2-active LTA structure comprising an anchor of 2 fatty acids and a short backbone of ≥ 3 glycerophosphate subunits [23, 24]. Bacterial LPs have no shared sequence homology but are characterized by lipid chains attached by ester bonds to a glycerol backbone that is in turn connected to the sulfur atom of the mandatory N-terminal cysteine. Depending on the microbe, an additional lipid chain is connected to the cysteine amino terminus via an amide bond (**Fig. 1**) [25, 26]. Variations in the polypeptide side chain attached to the cysteine were shown to have a minimal affect on TLR2 receptor activation [27]. On the other hand, variations in the peptide part do have a significant effect on TLR2-mediated immunological activity [28–30]. The minimal structure for TLR2 receptor activity is the Cys-Ser/Thr/Gly/Ala lipopeptide containing at least one ester-bound fatty acid acyl group of optimal length (C_{16}) [31–34]. Species-related differences in receptor activity were observed upon varying the length of the O-linked fatty acids in contrast with variations in the N-linked fatty acids, which had no discernible effect [33]. Well-characterized TLR2 ligands are di- and triacylated lipopeptides such as the synthetic lipopeptides Pam₂CSK₄, Pam₃CSK₄ (based on LPs from *Escherichia coli*), and macrophage-activating lipopeptide-2 (MALP-2) and FSL-1 (representing part of the LP from *Mycoplasma fermentans* and *Mycoplasma salivarium*, respectively) [34].

Although TLR2 is able to recognize soluble LPs, in the intact cell wall of microbes these ligands are anchored in the microbial membrane and therefore inaccessible to the receptor [35]. The precise mechanism of TLR activation after microbial encounter is still unknown. However, it is clear that TLRs depend on additional PRRs for microbe tethering, MAMP loading, and subsequent activation (**Table 3**) [8, 35].

TLR2 AND ENDOGENOUS LIGANDS

Inflammation is crucial for host defense against invasive pathogens. Inflammation in response to trauma, i.e., chemically induced, mechanically induced, induced by radiation, or caused by oxygen/nutrient withdrawal, typically occurs in the absence of microbes and therefore has been termed “sterile inflammation”. Similar to the inflammatory response to infection, sterile inflammation is characterized by the release of inflammatory mediators and the recruitment of innate cells. Endogenous molecules that play a role as mediators of inflammation are called damage-associated molecular patterns (DAMPs) [36]. Because MAMPs and DAMPs have a similar functional role in inflammation, PRR recognition of DAMPs is thought to drive the inflammatory response [6]. Research into the role of TLRs as DAMP receptors has been

TABLE 1. Overview of studies using TLR2 knockout mice

Study on	Phenotype	Mechanism of action	Ref.
Mucosal homeostasis	More susceptible to colonic inflammation ^a	Regulating TJ-associated barrier integrity	[73]
	More susceptible to colonic inflammation	Regulation goblet cell differentiation	[182]
	More susceptible to develop colitis-associated colon cancer	Dysregulated proliferation, altered immune response	[183]
	Compared with WT, different in colonic mucosal DNA methylation, immune response, and microbiome patterns	NR	[184]
Obesity	Protected from diet-induced adiposity, insulin resistance, hypercholesterolemia, and hepatic steatosis	Attenuated adipocyte hypertrophy, impaired innate response	[185]
	Leaner type of bacterial composition based on F/B ratio	Altered gut microbiota	[186]
	Protected from diet-induced insulin resistance	Altered gut microbiota	[16]
	Protected from diet-induced insulin resistance	Reduced tissue inflammation, preferential usage of fat as energy	[187]
Sterile inflammation	More resistant to T-cell or drug-induced systemic inflammation	Impaired response to extracellular histones	[188]
	More resistant to develop autoimmune diabetes	Impaired response to apoptotic and SN cells	[189]
	More resistant to atherosclerosis	Impaired response in non-BM cells	[190]
	More resistant to ozone-induced airway disease	Absence of AHR and reduced neutrophilic inflammation	[191]
	More resistant to lung injury after blunt chest trauma	Impaired release of CXCL-1, impaired neutrophilic response	[192]
	More resistant to systemic inflammation and reduced organ damage after I/R injury	Impaired release of proinflammatory cytokine, increased levels of IL-10	[193]
	Protected from oxidative stress induced tissue damage	Impaired inflammatory response	[194]
	More resistant to brain damage after I/R	NR	[195]
Infection	Susceptible to parasite-induced cysticercosis	Th2 vs. Th1 immunity	[196]
	More resistant to disseminated <i>Candida</i> infection	Impaired IL-10 synthesis, diminished amount of Treg	[197]
	More resistant to <i>Yersinia enterocolitica</i> infection	Impaired IL-10 synthesis	[198]
	Susceptible to <i>Staphylococcus aureus</i> infection	Impaired TNF- α synthesis	[199]
	Susceptible to <i>Borrelia burgdorferi</i> infection	Impaired innate response	[200]
	More susceptible to neuroinflammation	Impaired innate cellular and cytokine response	[201]
Neuroinflammation	More susceptible to CNS injury	Impaired innate response and adaptive cell recruitment	[202]
	Increased preservation of synaptic inputs after peripheral nerve injury	Impaired proinflammatory cytokine expression, increased expression of BDNF and GDNF	[203]
	Less susceptible to develop autoimmune diabetes	Impaired immune response to secondary necrotic cells	[189]
Autoimmune disease	Less susceptible to develop systemic lupus erythematosus	Impaired production of autoantibodies	[204]
	Less susceptible to experimental autoimmune encephalomyelitis	Impaired IL-6 response	[205]

AHR=airway hyperresponsiveness; BDNF=brain-derived neurotrophic factor; BM=bone marrow, CXCL-1=circulating chemokine (CXC motif) ligand 1, F/B=ratio of *Firmicutes* to *Bacteroidetes*, GDNF=glial cell-derived neurotrophic factor, I/R=ischemia/reperfusion, NR=not reported, SN=secondary necrotic, TJ=tight-junction, Treg=regulatory T cells, WT=wild-type. ^aTLR2 contribution to intestinal disease pathology is not without controversy. Although there is a clear role for TLR2 in managing DSS-induced acute colitis [73], TLR2 seems to have only a limited impact in a chronic model of intestinal inflammation [206].

TABLE 2. Cellular TLR2 expression and function after TLR2 ligation

Cell type	Ligand	Effect	Coreceptor	Remarks	Ref.
B cells ^{a,b}	Pam ₃ CSK ₄ , Pam ₂ CSK ₄	B-cell proliferation, activation and ASC differentiation	TLR1, TLR6	Similar outcome after TLR2/1 or TLR2/6 ligation	[207]
	HMGB-1	IL-8 release	CD36	Amplified IL-8 release when combined with Pam ₃ CSK ₄	[208]
Dendritic cells	PGN, LTA	TNF- α release	DC-subset dependent TLR1/2/6 expression	PGN, in contrast to LTA induced TNF- α release	[209]
	Various lipopeptides derived from <i>Mycobacterium tuberculosis</i> , Pam ₃ CSK ₄	Inhibition of TLR9 induced IFN- β release	NR	Quantitative differences in effectiveness	[210]
	Various lactobacilli, bifidobacteria, PGN, LTA, zymosan, Pam ₃ CSK ₄ , Pam ₂ CSK ₄	DC maturation and cytokine release	NR	Quantitative and qualitative differences in effectiveness	[71, 211]
	Pam ₃ CSK ₄ , PGN	Cellular activation	NR	Qualitative difference in activation kinetics	[212]
T-cells ^c	Zymosan, Pam ₃ CSK ₄ , Pam ₂ CSK ₄ , FSL-1	Induction of <i>Raldh</i> gene expression	Dectin-1	Quantitative differences in effectiveness	[213]
	Pam ₃ CSK ₄ , FSL-1	Decreased Treg function, Treg to "Th17-like" differentiation	TLR1, TLR6	TLR2/6 ligation did not affect Treg function	[214]
	Pam ₃ CSK ₄ , Pam ₂ CSK ₄ , FSL-1	Decreased Treg function	TLR1, TLR6	Donor-dependent expression of TLR1/6, no difference in TLR2/1 vs. TLR2/6 ligation	[215]
	PSA	Enhanced Treg function	NR	Critically dependent on TLR2 but independent of TLR1 or TLR6 expression	[216]
	HSP60, p277	Enhanced Treg function	NR	Critically dependent on TLR2	[217]
Microglia ^d	A β 42 aggregates	Proinflammatory cytokine release	TLR1, TLR6	TLR1 enhances, TLR6 suppresses cytokine release	[41]
	Oligomeric α -synuclein	Proinflammatory cytokine release	NR	TLR2 activation is ligand conformation sensitive	[42]
	Pam ₃ CSK ₄ , Pam ₂ CSK ₄ , PGN	Cell activation followed by cell death	NR	No difference in effects between ligands	[218]
	Gram-positive bacteria, Pam ₃ CSK ₄	Cell activation	NR	Qualitative difference in cellular activation	[219]
Astrocytes ^d	Pam ₃ CSK ₄ , FSL-1, HKLM	ROS production	TLR1, TLR6	ROS production after TLR2/6 ligation in contrast with TLR2/1	[220]

A β 42=42-aa form of human amyloid β , ASC=antibody-secreting cell, HKLM=heat killed *Listeria monocytogenes*, HSP60=heat shock protein 60, NR=not researched, PSA=polysaccharide A from *Bacteroides fragilis*, p277=HSP60 peptide, Raldh=retinaldehyde dehydrogenase type 2, ROS=reactive oxygen species. ^aMurine B cells are TLR2 responsive; human B-cells are TLR2 responsive after BCR cross-linking. ^bB-cell TLR expression and function are reviewed in ref. [221]. ^cT-cell TLR expression and function are reviewed in ref. [222]. ^dBrain cell TLR expression and function are reviewed in ref. [223].

the subject of much debate [37–39]. Although TLR signaling is often found to be absent when highly purified endogenous molecules are used, they intrinsically contain motifs that promote interaction with MAMPs and subsequently lower the threshold of TLR activation [38]. Data presented in Table 1 signify the importance of TLR2 in sterile inflam-

mation, indicating that the absence of TLR2 is linked with a reduction in inflammatory cytokine levels and an impaired innate response. Endogenous ligands of TLR2 have been reviewed elsewhere [37–39], but a selection of newly discovered TLR2 ligands and ligands in which MAMP contamination is ruled out is discussed here in more detail.

TLR2 pocket: ester-bound lipid interaction

- Chain length \geq C8 (mouse) \geq C12 (human) optimal length = C16
- Minimal structure: one chain of optimal length
- Can tolerate small structural changes

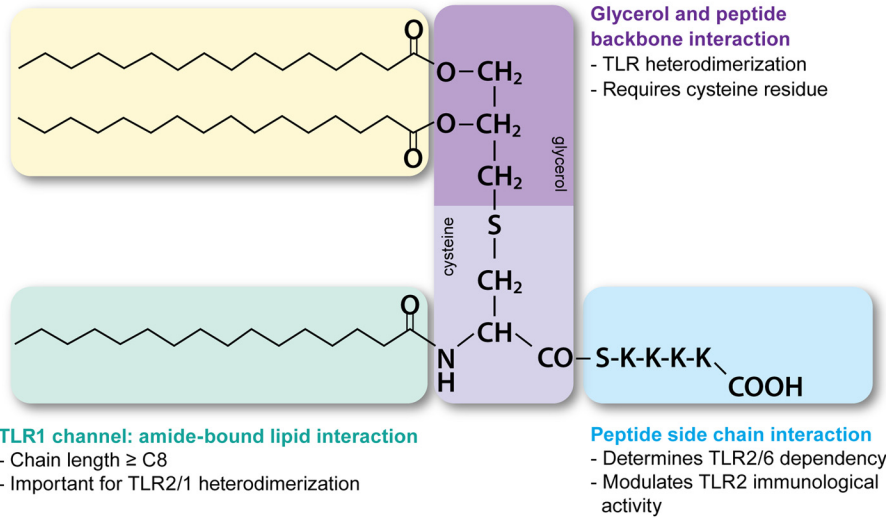


Figure 1. Summary of lipopeptide characteristics involved in TLR2 binding and heterodimerization. Adapted from Kang and Lee [181].

Pancreatic adenocarcinoma up-regulated factor (PAUF)

PAUF was found to associate with TLR2, TLR4, TLR5, and TLR6, but not with TLR3, TLR8, TLR9, or TLR10. Next, using an inhibitor of glycosylation, it was determined that TLR2 and PAUF interacted at the amino acid level. However, PAUF was not able to induce NF- κ B activation in either TLR2 or TLR4 transfectants or THP-1 monocytes. Moreover, depending on the TLR2 coreceptor CXCR4, PAUF signaling negatively regulates NF- κ B activation [40].

Amyloid β ($A\beta$)

Liu et al. [41] established that synthetic peptide aggregates of $A\beta$ 1-42, but not the control $A\beta$ 42-1, specifically associated with TLR2. They further identified the TLR2 DD loop amino acids EKKA (aa 741–744) as being critical for $A\beta$ -induced TLR2 activation. Based on this finding, it was determined that TLR2 activation after $A\beta$ ligation was enhanced by TLR2/1 but reduced by TLR2/6 heterodimers.

 α -Synuclein

In an elegant set of experiments α -synuclein was implicated as an endogenous agonist of TLR2 [42]. Recombinant α -synuclein from cellular as well as bacterial origin activated TLR2. To do so, α -synuclein needs to form oligomers and adapt a β -sheet conformation. In addition, conditioned medium from wild-type mouse primary cortical neurons activated TLR2 in contrast with conditioned medium from α -synuclein-null mice. Cytokine induction, but not uptake, could be inhibited using *Thr2*^{-/-} microglia or a TLR2 blocking antibody, suggesting the involvement of additional PRRs.

Serum amyloid A (SAA)

Full-length SAA expressed by a human cell line, but not SAA deletion mutants, was described to signal via TLR2. TLR2 acti-

vation was sensitive to proteinase K and heat treatment, suggesting that the activity is protein dependant [43].

SNAP-associated protein (Snapin)

Shi et al. [44] showed that human cell-expressed and purified full-length Snapin as well as Snapin (aa 60–136) associated with TLR2 but not TLR4. Snapin-induced TLR2 activation was proteinase K and heat treatment sensitive and could be abrogated using TLR2 antibodies.

 β 2-Glycoprotein I (β 2-gpI)

TLR2 was identified as the receptor for human isolated β 2-gpI. β 2-gpI was found to interact with both TLR2 and TLR4. However, only association to TLR2 was shown using binding studies. Binding of β 2-gpI to cells or TLR2 activation could be inhibited by either TLR2 antibodies or TLR2 knockdown [45].

The above listed endogenous molecules indeed suggest that TLR2 might be activated through other binding sites as suggested for bacterial LPs. However, with regard to the current knowledge on TLR2 activation and the possible microbial contaminants introduced during purification steps or during recombinant production (both microbial and mammalian), additional experiments are needed to rule out MAMP contribution to the observed effects [38]. Experiments showing the affinity of these molecules for various MAMPs and the subsequent affect on the TLR activation threshold are highly valuable for a better understanding of the relation between specific DAMPs, TLR2, and sterile inflammation.

THERAPEUTIC POTENTIAL OF TLR2**TLR2 antagonists**

Data presented in Table 1 suggest that interventions with the aim of suppressing TLR2 activity might be a beneficial ap-

TABLE 3. Overview of TLR2 coreceptors

PRR	Lipid raft	Signal transduction pathway	Endogenous ligand with reported TLR activity	Exogenous ligand with reported TLR activity	Experimental evidence functional association PRR-TLR in response to ligand	
					TLR2	TLR4
TLR1, TLR6	Recruited	MyD88 [170], PI3K [84]	NR	Bacterial LPs[31], microbes	[24, 78]	TLR6 [224]
CXCR4	Recruited	cAMP, PI3K	PAUF [40] ^a	LPS [140], PgFimbriae [143]	[40, 143]	[40, 140]
CD14	Resident	Src kinases [163] ^h	Versican [225], ^a A β ₄₂ [226, 227] ^b	PgLPS [228], PgFimbriae [154], PAM ₃ CSK ₄ [109], LPS [229], LTA [230]	[109, 154, 228, 230]	[229]
SRs						
Class A	Recruited	Internalization motif [231]	Modified LDL [38, 232] ^{b,g}	Microbes [113] ^f		
Class B	Resident	Src kinases [159] ^h	Modified LDL, LDL, HDL, VLDL [38, 232], ^{b,g} F-A β ₄₂ [41, 233], ^a SAA [43, 234] ^a	Microbes [113], ^f LPS [235, 236], LTA, MALP-2 [108]	[80]	
Other	Recruited	Internalization motif [237]	Modified LDL [38, 232] ^{b,g}	Microbes [113], ^f OmpA [122]	LOX-1 [122]	
Dectin-1	Recruited	Src kinases [238]	NR	<i>Mycobacterium abscessus</i> , zymosan [135]	[135]	
Integrins	Recruited	Rho family GTPases [239], Src kinases [240]	ECM, ^d vitronectin [129] ^b	Microbes ^e , PgFimbriae [154], HSV gH/gL [241], Pam ₃ CSK ₄ [131], LPS [242]	β_2 [228], β_3 [129, 241], $\alpha 3$ [131]	β_2 [243]

A β ₄₂=amyloid β peptide 42, ECM=various extracellular matrix proteins reviewed in ref. [244], cAMP=cyclic AMP-dependent protein kinase pathway, F-A β ₄₂=fibrillar amyloid β peptide 42, HSV gH/gL=herpes simplex virus glycoproteins gH/gL, NR=not reported, OmpA=*Enterobacteriaceae* outer membrane protein A, PgFimbriae=fimbriae from *Porphyromonas gingivalis*, PgLPS=LPS from *Porphyromonas gingivalis*, VLDL=very low-density lipoprotein. ^aTLR activity is reported in response to ligand, experimental evidence that ligand binds TLR. ^bTLR activity is reported in response to ligand, no experimental evidence that ligand binds TLR. ^cNo TLR activity reported, ^dECM molecules carrying TLR activity are reviewed in ref. [38]. ^eIntegrins interacting with microbes are reviewed in ref. [245]. ^fSRs interacting with microbes are reviewed in ref. [113]. ^gEndogenous ligands for SRs are reviewed in ref. [232]. Concomitant TLR signaling is reviewed in ref. [38]. ^hCD14 and CD36 are not direct targets of Src kinases.

proach for reducing inflammation after infection or disease-specific sterile inflammation. Indeed, systemic application of the anti-TLR2 antibody T2.5 showed beneficial and specific effects in both a sensitization-dependent and a high-dose TLR2-specific animal model of toxemia [46]. Data obtained with the TLR2 antibody OPN-301 show promise in patients with rheumatoid arthritis because blockade of TLR2 prevents spontaneous release of inflammatory mediators by ex vivo synovial explants [47]. OPN-305, a humanized IgG4 monoclonal anti-TLR2 antibody, significantly reduces infarct size and preserves cardiac function in a pig model of ischemia-reperfusion injury [48]. OPN-305 is currently being evaluated for clinical application in renal transplant patients at high risk of delayed graft function. In addition to TLR2 antibodies, TLR2 antagonists based on oligonucleotides are developed [49]. However, efficacy has yet to be proven.

TLR2 agonists

Synthetic TLR2 ligands have been under investigation with regard to their capacity to stimulate antigen-presenting cells

and function as adjuvants to improve the immunogenicity of otherwise weak immunogens and, in addition, show none of the harmful side effects of other adjuvant formulations [50, 51]. In 1989, it was discovered that coupling of an antigenic determinant to a synthetic lipopeptide induced long-lasting protection without the use of additional adjuvants [52, 53]. LPs are particularly suited because they provide a tool to modulate DC function without being mitogenic for T cells [54]. Coupling antigen peptide sequences (B-cell epitopes as well as T-cell epitopes) directly to synthetic TLR2 ligands creates so-called lipid-based self-adjuvanting synthetic peptides, which, when used as vaccines, provide superior immunogenicity over coadministration of TLR2 ligands and antigens.

Preclinical data on the therapeutic administration of lipopeptides further show promise in the management of allergic diseases [55–57], cancer [58, 59], autoimmune diabetes [60, 61], radiation exposure [62, 63], and respiratory pathogens [64, 65]. However, in contrast to agonists targeting other TLRs, there are currently no clinical data on the use of TLR2 agonists [66, 67].

Probiotics are derived from commensal Gram-positive bacteria, commonly from the species *Lactobacillus* or *Bifidobacterium*, and have been shown to induce immunomodulation (alive, killed, or fragmented) by a variety of cell types and in various diseases [68–70]. A prime receptor candidate for the direct interaction of intact probiotics with cells from the immune system is TLR2. Receptor candidates for fragmented probiotics are TLR2 (cell wall components), TLR9 (bacterial DNA), and NOD-like receptors (PGN fragments) [71, 72]. Oral treatment with either a TLR2-specific synthetic agonist or probiotics has been proven to be beneficial in animal models of inflammatory bowel disease, and clinical results in humans are promising [73–75]. Although much is still not known about the mechanism of action of probiotics and it is even more difficult to delineate the contribution of TLR2 to the observed effects, it is clear, however, that probiotic administration has an effect on intestinal TLR signaling, which contributes to the maintenance of tolerance and immunity [15, 72, 76].

TLR2 HETERODIMERIZATION

Ozinsky et al. [77] were the first to determine that, unlike other TLRs, TLR2 is needed to form heterodimers with TLR1 or TLR6 to be able to initiate cell activation. With the determination of the crystal structures of TLR2/1 and TLR2/6, much insight into the prerequisites for TLR2 heterodimerization and TLR2 ligand binding has been gained. Upon complexing of TLR1/2 with the synthetic triacylated lipopeptide Pam₃CSK₄, it was determined that the 2 glycerol-bound lipid chains of Pam₃CSK₄ docked with the hydrophobic pocket of TLR2 and the third amide-bound lipid chain docked on TLR1, thereby stabilizing the TLR2/1 heterodimer [78]. Although TLR2 can also bind the diacylated lipopeptide Pam₂CSK₄, this does not lead to a stable TLR2/1 heterodimer because of the absence of the amide-bound lipid chain. TLR6 does not contain a lipid-binding groove but instead has an approximately 80% increased exposed hydrophobic surface. Ligand-mediated stabilization of TLR2/6 heterodimerization is therefore achieved by enforcement of the hydrophobic interaction via several strong hydrogen bond interactions between TLR2 and TLR6 and the peptide backbone of the LP [24]. From the above discussion, it becomes clear that only TLR2/1 or TLR2/6 heterodimers are functional, and for these heterodimers to become stable they need to bind a specific ligand. When this fact is taken into account, the question arises: What can be expected to be found on the surface of cells? With use of immunoprecipitation (IP) techniques with transfected “tagged” TLR constructs, TLR2 was associated with TLR1 [79] or TLR6 [77] in cells that normally do not express TLRs. This association was not found to be increased upon ligand binding, which suggests that TLR2 heterodimers preexist on the surface. Later, using sophisticated fluorescence microscopy techniques (fluorescence resonance energy transfer [FRET]) in human primary monocytes, Triantafyllou et al. [80] showed that indeed a percentage of preformed TLR2 heterodimers preexist but, in contrast to the previous finding, the association between the TLR2 heterodimers increased ligand dependently. In addition, it could be argued that TLR2/6 ligand binding reduced the per-

centage of preformed TLR2/1 heterodimers but not vice versa. From these findings, it is tempting to suggest that, in relation to the weak TLR2/1-TLR2/6 heterodimer stability in the absence of a ligand, the percentage of preformed TLR2 heterodimers might reflect temporal associations of TLR2 with TLR1 or TLR6 molecules. A drawback of both of the above-mentioned techniques is that cells are either fixated (FRET) or lysed (IP), reflecting the situation at the surface of the cells at a specific moment, but do not answer the dynamics of heterodimerization. Additional studies by Triantafyllou et al. [81], using a fluorescence microscopy technique on live cells termed fluorescence recovery after photobleaching, established that TLR2 diffused freely over the plasma membrane. In these experiments, only TLR2 was labeled; it therefore remains to be determined how much of TLR2 was in a heterodimeric association. However, it was shown that upon ligand binding, TLR2 lateral diffusion was impaired, suggesting lipid raft translocation. Indeed, with use of lipid raft-disrupting agents [81, 82] or TLR association with lipid raft-restricted GM1 ganglioside [80], it was established that TLR2 heterodimers translocate to lipid rafts, depending on their interactions with specific ligands. It was previously suggested that TLR2 heterodimerization with TLR1 or TLR6 did not induce differences in cellular activation but only functions to broaden the ligand repertoire [83]. However, recent literature reports suggest that intracellular signaling might differ, depending on the type of TLR2 heterodimer. First, the strength of the cellular activation after lipopeptide ligation seems to depend on the intracellular domains (ICDs) of the TLR1 and TLR6 coreceptors [41]. Replacing the ICD of TLR1 with the ICD of TLR6 reduced cytokine release after lipopeptide stimulation [41]. Second, the TLR2 intracellular adaptor MyD88 adapter-like (Mal)/Toll-IL-1 receptor (TIR) domain-containing adaptor protein (TIRAP) directly connects TLR2/6 to PI3K after activation, whereas TLR2/1 depends on a currently unknown adaptor, ultimately leading to differences in phosphatidylinositol-(3,4,5)-trisphosphate (PIP3) generation and macrophage polarization [84]. Third, in apparent contrast to the previous approach, strategies to inhibit Mal/TIRAP and block cellular signaling were effective after TLR4 and TLR2/1 but not TLR2/6 activation [85]. In contrast to the efforts to delineate LPS-TLR4 signal transduction, research addressing TLR2 signal transduction has been confounded by the use of cellular transfectants that endogenously express TLR1 and/or TLR6 (HEK293 cells) [34, 86, 87], the presence of a wide variety of TLR2 ligands (MAMPs and DAMPs), and the finding that ligand dose might overcome TLR2 heterodimer-specific signaling [88]. In addition, Buwitt-Beckmann et al. [34] determined that although activation by lipopeptide is critically dependent on the presence of TLR2, some lipopeptides were found to initiate signal transduction independent of TLR1 and/or TLR6. These findings leave room for the argument that TLR2 might partner with another receptor to induce TLR2 signaling. Moreover, as mentioned in the previous paragraph, TLR2 function but not receptor activity was dependent on the peptide moiety of the lipopeptide, again suggesting that additional TLR2 coreceptors are involved in TLR2 activity.

One last aspect, which is much less known, is the role of TLR10. TLR10 is a member of the TLR1/2/6/10 cluster, and it has been hypothesized to have a function similar to that of TLR1 and TLR6, although little is known regarding the formation of heterodimers with other TLRs and the recognition of specific ligands. Recently, using ligand-induced TLR10 binding assays, binding to triacylated lipopeptides was observed, in contrast with that to diacylated lipopeptides [89]. However, TLR10 homodimers or any other TLR-TLR10 heterodimer were unable to activate NF- κ B [90]. In conclusion, although there are some data to suggest that TLR10 may recognize bacterial products such as lipopeptides, the extent of its role in antibacterial defense is as yet unproven.

Single nucleotide polymorphisms affecting TLR2 heterodimerization

Studies that have investigated the functional consequences of nonsynonymous polymorphisms in the TLR2 gene, mainly comprising the amino acid substitutions R753Q (rs5743708) and P631H (rs5743704), have revealed altered function of the protein for mutant variants with minor frequency. For the P631H polymorphism, defective membrane internalization and consequently a gain of function of the receptor was observed, leading to increased immune activation and immunopathology [91, 92]. Conversely, a loss-of-function effect of the R753Q polymorphism was detected for recognition of its ligand LTA, bacterial and viral components [93–96], which increases the susceptibility to bacterial and viral infections [97]. However, effects of these polymorphisms on pro- and anti-inflammatory cytokine production by peripheral blood mononuclear cells have turned out to be limited [98], which indicates that other mechanistic defects are responsible for the clinical associations of these polymorphisms with human disease. Studies investigating the consequences of the TLR2 nonsynonymous polymorphism on TLR2 heterodimerization are scarce, but, recently, the R753Q polymorphism has been linked with a reduced capacity to form TLR2/6 heterodimers, leading to an impaired response towards LPs and *Mycobacterium* cell wall components [99]. In addition, using published crystallographic models of TLR2 heterodimers, nonsynonymous polymorphisms in TLR1, TLR2, TLR6, and TLR10 can be matched to critical amino acids at the interface of dimer formation. Consequently, al-

though there are no experimental data yet, amino acid alterations at positions L443I, V542A, and T565S in TLR1 might potentially impair TLR2/1 heterodimer formation as has been determined for P315L [100, 101].

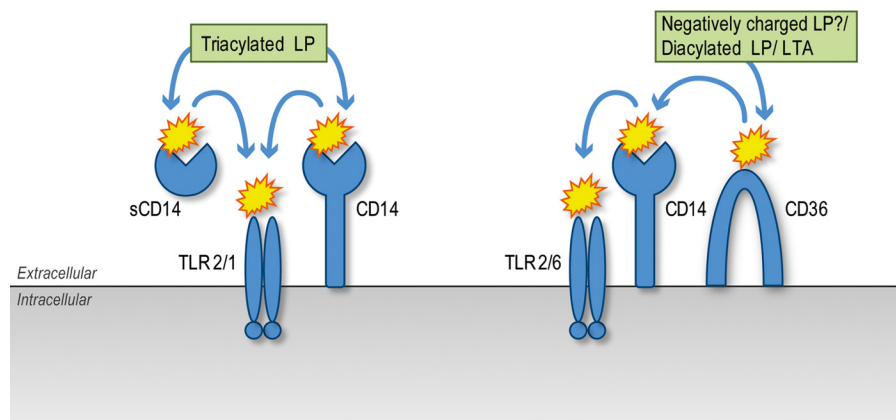
CD14 AS A CORECEPTOR FOR TLR2

Although much of the research being done on CD14 is with regard to its role in endotoxin signaling, CD14 interacts also with TLR2 and TLR4 [102], TLR3 [103], and TLR7 and TLR9 [104]. CD14 as well as the plasma/serum resident soluble form of CD14 (sCD14) directly binds TLR2, and this binding can be prevented with anti-CD14 monoclonal antibodies that were also found to block CD14-mediated LPS loading [105, 106]. With the use of surface plasmon resonance techniques, it was demonstrated that both surface-bound CD14 and sCD14 enhanced TLR2/1 surface recognition of triacylated but not diacylated lipopeptides [107]. An important difference is that sCD14 can replace surface-bound CD14 function in terms of ligand loading, but it cannot do so with regard to TLR2 lipid raft recruitment [80, 107–109]. CD14 was one of the first proteins to be recognized as a PRR, important in the recognition of cell wall structures of Gram-positive as well as Gram-negative microbes [110]. In myeloid cells, CD14 is highly concentrated inside lipid rafts as a glycosylphosphatidylinositol-anchored membrane protein. In the sequence of events, CD14 significantly enhances TLR2 activation by facilitating lipopeptide binding and TLR2 heterodimerization [109]. With use of lipopeptide analogs that differ in the number of acyl chains and peptide sequence, CD14 function seems dependent on triacylated lipopeptides and independent of the peptide moiety [107]. Although CD14 associates with TLR2 upon ligation of both di- and triacylated lipopeptides, the discrepancy in CD14-dependent TLR2/1 vs. TLR2/6 activation can be explained by the additional dependence of TLR2/6 on scavenger receptor (SR) CD36 for ligation of specific diacylated lipopeptides (Fig. 2) [80].

SRs AS CORECEPTORS FOR TLR2

Several classes of SRs are linked to TLR2 function. Class A (macrophage receptor with collagenous structure [MARCO], SR-A),

Figure 2. Role of CD14 and CD36 in ligand loading of TLR2 heterodimers. TLR2/1 activation depends on CD14 or sCD14 delivery of triacylated LPs. Diacylated LPs or LTA loading of TLR2/6 requires initial binding by CD36. The CD36-ligand interaction might be based on the net charge of the ligand. CD14 is hypothesized to act as an intermediate facilitating TLR2/6 loading. Adapted from Jimenez-Dalmaroni et al. [118].



class B (SR-B1, CD36), class F (scavenger receptor expressed by endothelial cells-I [SREC-I]), and class E (lectin-like oxidized LDL receptor-1 [LOX-1]) SRs are associated with TLR2 with regard to uptake of either endogenous ligands, microbial ligands, or intact microbes. SRs belong to the family of PRRs and comprise a structurally diverse family of transmembrane receptors with a phagocytic function that are commonly found on macrophages and DCs [111]. Foremost, they are implicated in the recognition of modified LDL, but evidence about their uptake of microbial ligands or Gram-positive and Gram-negative microbes is accumulating [112, 113]. MARCO is the preferred receptor of the *Mycobacterium tuberculosis* cell wall lipid trehalose 6,6'-dimycolate (TDM) and signaling to TDM-coated beads was shown to be CD14 and either TLR2 or TLR4 dependent [114]. Class A SRs are further reported to bind Gram-positive bacteria by binding their major cell wall lipid LTA [114] and various cell wall ligands from *Neisseria meningitidis* [115], *Streptococcus* species [116], and *E. coli* and *S. aureus* [117]. In most cases, cell signaling was dependent on MyD88, suggesting the involvement of TLRs, although specific TLR activation was not investigated. CD36 was discovered as a sensor for microbial lipopeptides in mice with an *N*-ethyl-*N*-nitrosourea nonsense mutation of CD36. These mice were hyporesponsive to some but not all diacylated lipopeptides (LTA and MALP-2 vs. Pam₂CSK₄). Responses to triacylated lipopeptides (e.g., Pam₃CSK₄) remained intact [108]. With use of the ectodomain of mouse CD36 (mCD36ED), binding to diacylated LP FSL-1 (similar in molecular makeup to MALP-2) and LTA but not to diacylated Pam₂CSK₄ or triacylated Pam₃CSK₄ was subsequently confirmed [118]. Signaling in response to ligand binding was CD14 dependent. Because mCD36ED does not directly interact with TLR2 as was shown by IP, it was hypothesized that the function of CD36 is to capture ligands and transfer them to TLR2 in a CD14-dependent fashion (Fig. 2) [118]. CD36 and other class B SRs have also been found to mediate phagocytosis of various Gram-positive and Gram-negative bacteria, but direct involvement of TLRs has not been investigated [119–121]. The SRs SREC-I and LOX-1 activate macrophages and DCs in a TLR2-dependent way upon binding a major component of the outer membrane part of *Klebsiella pneumoniae* but only LOX-1 was found to colocalize and cooperate with TLR2 [122].

Overall, SRs offer interesting candidates as TLR2 coreceptors, but more research is needed to determine SR ligand specificity and TLR2 coreceptor mechanics. Notably, with regard to the crucial role of CD36 in TLR2 function, CD36 might also offer an explanation for the difference in TLR2 immunological activity of diacyl lipopeptides with a different peptide moiety. Because the peptide part of FSL-1 or MALP-2 has a larger negative charge than the peptide part of Pam₂CSK₄, the discrepancy in diacylated lipopeptide binding by CD36 might be explained by the finding that CD36 binds negatively charged moieties more avidly [123].

INTEGRINS AS CORECEPTORS FOR TLR2

Current literature reports show that integrin activation intersects with TLR signaling; however, only a few studies reported the direct interaction of integrins and TLRs. On the one hand, integrin activation induced changes in membrane phos-

phoinositide composition enhancing TLR activation by attracting specific TLR adaptor molecules to lipid rafts. On the other hand, integrin-mediated phagocytosis is critically dependent on the activation of coreceptors (specifically but not limited to TLRs) [124–126]. Integrins are heterodimeric receptors that mediate cell-cell and cell-extracellular matrix interactions. They typically comprise an α - and a β -subunit. So far 18 α - and 8 β -subunits have been identified in humans, which combine to recognize a multitude of different ligands that play a role in cell adhesion, migration, and tissue differentiation. The function of integrins is dependent on the type of activation. Integrins can be activated to receive extracellular ligands by “inside-out” signaling, mediated by activation of G-protein-coupled membrane receptors. Integrins relay ligand binding activation via an “outside-in” signal, through activation of intracellular proto-oncogene tyrosine-protein kinase Src (Src) and Syk [127, 128]. Integrin subunits β_1 , β_2 , and β_3 are associated with microbe phagocytosis [126]. The integrin subunit β_3 interacts directly with TLR2 on the surface of resting monocytes. Rapid dissociation of the TLR2-integrin β_3 complex was found upon stimulation with a synthetic triacylated lipopeptide. Blocking β_3 function reduced monocyte TNF- α release upon TLR2 stimulation. Knockdown of the β_3 gene (*ITGB3*) in monocytes further substantiated these findings and also showed that this effect was TLR2 specific [129]. The natural ligand of the integrin β_3 is vitronectin, a soluble protein present in blood and extracellular matrix, the function of which is, among others, to opsonize microbes, which facilitates microbe adhesion to host cells [130]. Because of its characteristics, vitronectin was proposed to concentrate TLR ligands and to interact with β_3 , which subsequently tethers the complex to the membrane and by doing so facilitates TLR2 loading [129]. A set of experiments performed by Marre et al. [131] showed that although membrane integrin $\alpha_3\beta_1$ does not directly bind the lipopeptide Pam₃CSK₄, blocking α_3 function reduced the TLR2-mediated IL-6 release in U937 macrophages, which was independent of the presence of serum proteins. For a complete IL-6 response and induction of a type I IFN response after TLR2 activation, integrin α_3 -mediated uptake and subsequent endosomal TLR2 signaling is required. There clearly is a role for integrins as coreceptors for TLR2 and vice versa. The current literature, however, does not provide enough insight into whether these interactions are TLR specific or a more general function of the concerted interaction of integrins and TLRs in cellular activation and phagocytosis. Notably, integrin signaling, in addition to TLR signaling, influences adjuvant activity. Synthetic Pam₂C-based lipopeptides engineered to contain a functional integrin motif (RGDS) were found to efficiently target and activate DCs. Compared with similar Pam₂C-based lipopeptides having no integrin motif, Pam₂CRGDS showed stronger in vivo antitumor activity [132].

DECTIN-1 AS A CORECEPTOR FOR TLR2

Cooperative interaction between Dectin-1 and TLR2 was established in response to zymosan [133] and various *Mycobacterium* species [134]. Dectin-1 and TLR2 physically associate upon binding of either *Mycobacterium abscessus* or zymosan by mouse

Raw264.7 macrophages and human THP-1 monocytes as was shown by IP. Blocking Dectin-1 function reduced *M. abscessus* uptake and reduced TLR2 and Syk-dependent cytokine release by bone marrow-derived macrophages [135].

Dectin-1 is a member of the C-type lectin (CLR) family of pattern recognition receptors. It is a glycosylated type II transmembrane receptor with a CLR-like domain, which mediates ligand binding and a cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM)-like signaling domain. Dectin-1 can predominantly be found on the surface of myeloid cells where it recognizes fungal and plant cell wall components (e.g., β 1,3-linked glucans) as well as undefined mycobacterial ligands [136]. Although the simultaneous use of synthetic TLR ligands and β -glucans has been shown to synergistically enhance cytokine responses (i.e. IL-6, TNF- α , IL-10, and IL-23) and to reduce IL-12 p70 cytokine release, this was not exclusive to TLR2 agonists [137, 138].

CXCR4 AS A CORECEPTOR FOR TLR2

The signaling pathway of CXCR4 intersects with TLR-induced signaling and, depending on the TLR ligand dose, either suppresses [139] or enhances the TLR-induced cell activation [140]. CXCR4 is a 7-transmembrane G-protein-coupled chemokine receptor for stromal-derived factor-1, found on leukocytes, endothelial cells, and epithelial cells. Next to its role in leukocyte trafficking, it has received a lot of attention concerning its function as a coreceptor for HIV [141]. The first suggestion that CXCR4 might also be regarded as a pattern recognition receptor came from a publication by the group of Triantafyllou et al. [142], who found that CXCR4 was a functional part of a CD14-independent LPS receptor cluster. Functional coassociation of CXCR4 and TLR2 inside lipid rafts led to a down-modulation of the subsequent TLR2 response upon binding of fimbriae of *Porphyromonas gingivalis*. Moreover, unlike CXCR4 modulation of TLR4 signaling, suppression of TLR2-induced NF- κ B signaling was functional over a broad dose range [143]. Intriguingly, a recently discovered factor released by pancreatic cancers (PAUF) was found to be associated with TLR2 and CXCR4. This association, analogous to the effects of the fimbriae of *P. gingivalis*, reduced TLR2-mediated NF- κ B activation and promoted metastasis [40].

LIPID RAFTS

A major property of TLR signaling is determined by the association of coreceptors, adaptor proteins, and signaling kinases, all participating in a receptor complex. The simple interaction of lipopeptides with TLR2 is barely sufficient to induce downstream gene activation but is greatly amplified by the presence of lipid raft resident coreceptors [14]. In addition, disrupting the function of lipid rafts has a significant effect on TLR2-induced immune responses [80, 82, 144, 145]. Coreceptor-induced TLR2 translocation can therefore be regarded as a crucial step in TLR2 immunological function.

Lipid rafts are membrane islands composed of highly ordered saturated lipids and cholesterol within the more fluid

membrane bilayer of largely unsaturated lipids. In resting cells rafts are small and highly dispersed but upon activation via clustering of membrane components, they coalesce into larger platforms [146]. Thus, in resting cells they compartmentalize the plasma membrane and therefore segregate specific protein-lipid and protein-protein interactions. Upon activation, coalescence of smaller raft patches leads to accumulation of receptors, providing a scaffold to bring together and concentrate intracellular adaptor molecules (i.e., MyD88, TIR-domain-containing adapter-inducing interferon- β , Src kinases, and Rho GTPases) that are necessary for signaling [14, 147]. Molecules associated with lipid rafts function to read out specific intracellular internalization motifs and sort transmembrane proteins to endosomes [148, 149].

SRs are associated with lipid rafts; however, current data suggest that class A SRs functionally depend on additional coreceptors to facilitate lipid raft recruitment, signaling, and phagocytosis [87, 150]. In contrast, class B SRs have a palmitoylated N- and C-terminal cytoplasmic tail, which targets them to lipid rafts [147, 151]. Class F and E SRs both associate with lipid rafts upon ligand binding [152, 153]. To initiate integrin-dependent phagocytosis, integrins rely on the activity of coreceptors [8, 126]. Inside-out activation of integrins can be achieved by TLR activation from within lipid rafts. For example, induction of the high-affinity state of the β_2 integrin CD11b/CD18 was mediated through CD14-facilitated TLR2 activation by fimbriae of *P. gingivalis* [154, 155]. A similar manner of inducing activation of the $\alpha_3\beta_1$ integrin was found by means of PAM₃CSK₄- or *Borrelia burgdorferi*-induced TLR2 activation [131]. Outside-in activation of β_3 integrins by MAMP-loaded vitronectin leads to TLR loading and translocation to lipid rafts [129], similar to the role of β_2 integrin-facilitated TLR4 activation [124]. Dectin-1 has also been found to translocate into lipid rafts in response to ligand binding because Dectin-1 engagement of either zymosan or curdlan leads to colocalization of Dectin-1 and lipid raft marker cholera toxin B [156]. An overview of the different PRRs interacting with TLR2 is presented in Table 3. In addition, Table 3 shows PRR lipid raft status, PRR signal transduction moieties, and PRR endogenous or exogenous ligands with reported TLR2 activity and, where possible, indicates whether there is experimental evidence for the functional association of PRR-TLR2 in response to TLR2 agonists. A schematic diagram representing the interaction of PRR and TLR2 signal transduction routes is found in Fig. 3.

Pivotal role of CD14 and CD36

CD14 and CD36 are PRRs for a broad range of ligands, which can be of endogenous origin (components of apoptotic cells, amyloid peptides, ceramides, extracellular matrix, and oxidized LDL) and microbial origin (LTA, LPS, and LPs). CD14 as well as CD36 are lipid raft-resident molecules, which both apparently lack cytoplasmic tails with a discernible signaling motif but are associated with cellular activation and/or ligand uptake [157, 158]. Recently, in a very elegant study by Heit et al. [159], CD36 was found to engage Src family kinases. They elucidated that cross-linking of CD36 leads to recruitment of tetraspanins (CD9 and/or CD81), which facilitates subsequent

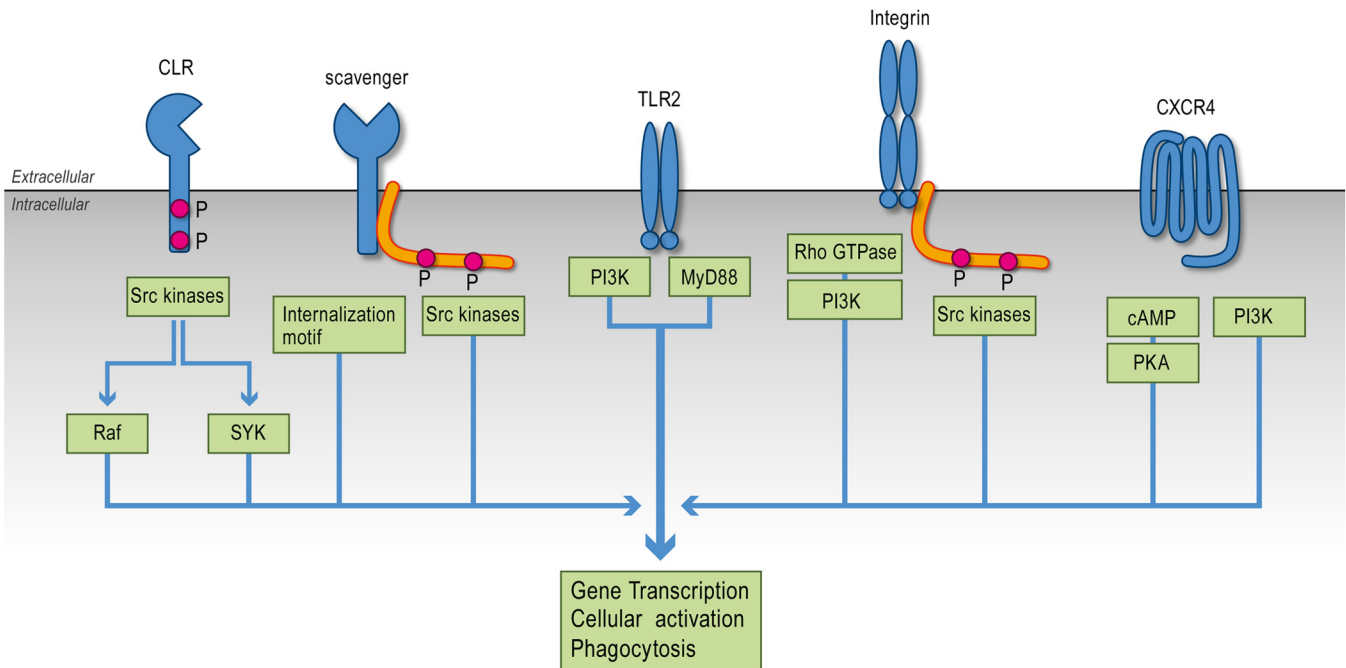


Figure 3. Schematic diagram of the intersection between PRR and TLR2 signaling pathways.

functional interaction with β_1 and β_2 integrins. This signaling complex was found to associate with the intracellular ITAM adapters FcR common γ -chain (FcR γ) and DNAX-activating protein of 12 kDa (DAP12), enabling CD36 ligands to signal via Syk and its downstream effectors. Although specific evidence is lacking, a similar mode of signaling can be hypothesized for CD14, because CD14, tetraspanins, and integrins are functionally associated in response to LPS [160–162]. In addition, Src family kinases are linked to cellular activation after LPS stimulation, independently of TLR4 but exclusively dependent on CD14 [163]. The above results suggest that ligand binding by CD14 and/or CD36 initiates the proximal membrane event, leading to PRR/TLR accumulation, as depicted in **Fig. 4**. On the whole, because it now becomes clear that almost all TLR2 coreceptors engage Src kinase pathways, Src kinase activity after TLR2 activation is more the rule than the exception.

In general, FcR γ - and DAP12-mediated Src kinase signaling inhibits TLR activation because cells deficient in FcR γ and/or DAP12 are hyperresponsive to TLR stimulation [164]. Indeed, DAP12-deficient macrophages secrete increased amounts of proinflammatory cytokines after PGN, Pam₃CSK₄, and zymosan stimulation [165]. CLRs, having an intracellular ITAM, do not require recruitment of signaling adaptors to activate Src kinase pathways [166]. However, Src kinase activation of Raf-1 after the combined stimulation of CLRs and TLR2 has been shown to additionally direct NF- κ B-mediated cytokine expression [167]. Moreover, the differential requirement of Src kinase pathways is thought to underlie neutrophil regulatory responses after MAMP as well as bacterial ligation by TLR2 [168].

The interaction with integrins further facilitates TLR activation by recruitment of phosphatidylinositol 4-phosphate 5-ki-

nase (PIP5K) [169]. PIP5K phosphorylates phosphatidylinositol 4-phosphate, which results in the generation of phosphatidylinositol 4,5-bisphosphate (PIP₂), which mediates Mal/TIRAP recruitment to the plasma membrane. Mal/TIRAP then functions to facilitate MyD88 delivery to activated TLR2 [124]. In addition, Mal/TIRAP recruits PI3K, which phosphorylates PIP₂ to PIP₃, which is necessary to invoke downstream Akt activation [84] (**Fig. 4**).

CONCLUDING REMARKS

It has become clear that, in contrast to TLR2 receptor activity, TLR2 function and downstream signaling indispensably rely on the presence of coreceptors and lipid raft translocation, suggesting that TLR2 interaction with additional PRRs in TLR2 function is more the rule than the exception. Many reports suggest a similar relationship for TLR4 but, in contrast to TLR2, functional evidence for the direct interaction of TLR4 with PRRs (other than CD14) is lacking (**Table 3**). This either suggests a research bias to investigate TLR2 function more in detail, or, more likely, TLR2 has unique attributes that facilitate TLR2-PRR interaction. The central dogma of TLR activation dictates that TLRs must either heterodimerize (TLR2) or homodimerize (all other TLRs) to initiate signal transduction [170]. However, two lines of evidence support a view that TLR2 might be able to initiate signal transduction as a homomer or homodimer. First, as discussed in the paragraph on TLR2 heterodimerization, data on the surface expression of TLR2 are not conclusive on whether TLR2 heterodimers are preformed or reflect temporal associations, leaving room to argue that TLR2 is free to associate with other

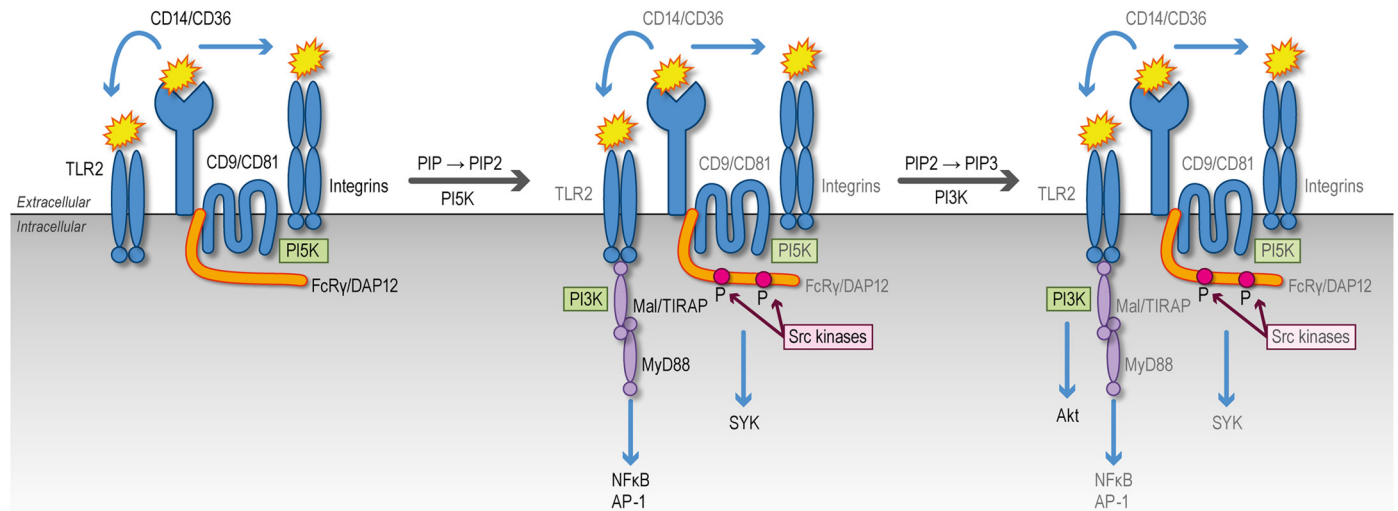


Figure 4. Pivotal role of CD14/CD36 receptor complex in TLR2 activation. Ligand capture and subsequent ligand loading onto TLR2 and additional PRRs by CD14/CD36 initiates the formation of the receptor complex, which leads to the recruitment of the intracellular adaptor FcRγ and/or DAP12 and the enzyme PI5K. PI5K phosphorylates PIP to PIP2 necessary to recruit the TLR2 signal adaptor Mal/TIRAP. Mal/TIRAP subsequently recruits the signal adaptor MyD88, leading to activation of transcription factors AP-1 and NF-κB. Furthermore, Mal/TIRAP recruits the enzyme PI3K. Next, Src kinases phosphorylate the ITAM on FcRγ/DAP12, recruiting and activating Syks. PI3K further phosphorylates PIP2 to PIP3, resulting in the recruitment and activation of Akt.

receptors. Second, some specific LPs can induce TLR2 activation independent of TLR1 and TLR6 [34]. These lines of evidence together with the breadth of data describing the functional association of PRRs and TLR2 suggest that specific PRRs might take on the role of the TLR2 receptor partner in TLR2 activation.

Taking into account the wide variety of current reports on TLR2 coreceptors, the emerging picture shows two main roles of PRRs in TLR2 function:

To act as a transport/tether/capture receptor of microbial ligands or host components, potentially contaminated with microbial ligands, delivering ligands to TLR2 at the cell surface and/or intracellularly.

To function as a signal modifier, assisting in matchmaking between TLR2 signaling adaptor molecules and coreceptor specific signal transduction pathways.

Although the above items describe the essential contributions of PRRs to TLR2 function, analysis of the data did not yield a potential candidate PRR that could replace either TLR1 or TLR6 in TLR2 receptor activation. In addition, whether or not TLR2 has additional ligand binding sites that allow for the interaction with protein or sugar residues remains obscured because of technical restrictions such as detection and removal of MAMP contaminations from TLR2 agonists [38] and the inherent nature of many endogenous proteins to interact with hydrophobic molecules [171]. To obtain more insight into the mechanism of action of TLR2, an overview of the critical aspects governing TLR2 function is listed in **Table 4**.

After the initial description of the crucial involvement of TLR2 in infection and (sterile) inflammatory diseases, it has

become clear that TLR2 activity plays a prominent role in the pathogenesis of a variety of acute and chronic inflammatory diseases. A further clue regarding the importance of controlling TLR2 activity might be derived from the production of a natural TLR2 agonist soluble TLR2 (sTLR2). sTLR2 is the result of postprocessing proteolytic cleavage, is constitutively released by monocytes, and can be detected in plasma, human milk [172], saliva [173], and amniotic fluid [174]. sTLR2 has been found to be protective against HIV-1 infection [175] and to participate in regulating the inflammatory response to microbes or MAMPs [172–174, 176].

From a therapeutic point of view, lipid-based self-adjuvanting synthetic peptides might benefit by endowing vaccines with specific engineered lipopeptides containing motifs that additionally target non-TLR pattern recognition receptors to optimize delivery or fine-tune desired vaccination responses as been shown by Akazawa et al. [132] and van Kooyk et al. [177]. Current available synthetic TLR2 ligands are based on cell wall constituents of (potential) pathogens, and adjuvant research would possibly benefit from elucidating the variations in LP make-up of probiotic strains. With regard to the indispensable role of PRRs in facilitating microbe induced TLR2 function, determination of specific PRRs involved in the recognition of probiotic strains would aid research on the mechanism of action of probiotics. In addition, because microbial manipulation of PRR-TLR crosstalk is used by pathogens to subvert appropriate immune responses, determination of the specific PRRs involved could lead to new therapeutic approaches [178].

Notably, although many lipopeptide analogs have been designed and tested, none of them have shown antagonistic func-

TABLE 4. Critical aspects in the analysis of TLR2 function

Technical aspects:

- Endogenous expression of TLR2 coreceptors. To determine the precise interaction of agonists with the different TLR2 receptor forms, TLR2 transfected cells offer a valuable tool. However, endogenous expression of TLR2 coreceptors TLR1, TLR6, TLR10 should be controlled.
- MAMP contamination. To determine whether or not a molecule is a genuine TLR2 agonist, it is important to consider the following factors:
 - Recombinant microbial expression systems inherently produce MAMP contaminated molecules.
 - Purification and/or modification methods might introduce MAMP contamination.
 - Serum by its very nature contains MAMPs; therefore, it is advisable to use serum-free formulations.
 - With the current technology, it is impossible to separate MAMP contamination from your molecule of interest.

Cell-intrinsic aspects:

- Cellular expression of TLR and/or PRRs. TLR2 function depends on the availability of TLR2 coreceptors; therefore, cellular activity after TLR2 ligation largely depends on its diversity of TLR and PRR expression.
- Cell type and function. In addition to its surface expression of TLRs and PRRs, cell type and function determine cellular responses after TLR2 activation.
- Nonsynonymous polymorphisms. Changes in protein makeup potentially affect TLR2-TLR and TLR2-PRR interactions.
- Cell-extrinsic aspects:
 - Soluble factors that participate in TLR2 agonist-receptor interaction. Serum contains many factors that affect TLR2 function (i.e., sTLR2, sCD14, LPS-binding protein, and vitronectin).
 - Soluble factors that affect cell activation. Factors that condition or activate cells (i.e., cytokines and chemokines) alter cellular expression of TLRs, PRRs, and molecules involved in intracellular signaling.

tion. They either bind and induce a level of activity or they do not bind at all [30–32]. A series of novel synthetic phospholipids that are antagonists of TLR2 have been designed, but functional data on the use of these compounds are mostly lacking [179]. Recently an exoprotein of *S. aureus*, superantigen-like protein 3, was found to function as a TLR2-specific antagonist, potentially offering interesting therapeutic applications [180].

TLR2 is a very interesting receptor highly involved in the field of adjuvant, pathogen, and probiotic research. Although much effort has been put forward to determine TLR2 ligand requirements and TLR2 receptor activity, many questions remain regarding the regulation of the downstream signaling and resulting effect on immunological responses, depending on the large family of reported coreceptors. Increasing the understanding of these mechanisms could offer attractive therapeutic targets and opportunities.

AUTHORSHIP

J.v.B. initiated the review. J.v.B., T.S.P., A.D.K., and A.P.V. have written the review. M.G.N., and J.G. contributed to the scope and setup of the manuscript. M.G.N., J.G., G.F., and L.A.B.J. edited and critically reviewed the manuscript.

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KEY WORDS:

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