Role of lipid microdomains in TLR-mediated signalling

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1

ABSTRACT

Over the last twenty years, evidence has been provided that the plasma membrane is partitioned with microdomains, laterally mobile in the bilayer, providing the necessary microenvironment to specific membrane proteins for signalling pathways to be initiated. We discuss here the importance of such microdomains for Toll-like receptors (TLR) localization and function. First, lipid microdomains favour recruitment and clustering of the TLR machinery partners, i.e. receptors and co-receptors previously identified to be required for ligand recognition and signal transmission. Further, the presence of the so-called Cholesterol Recognition Amino-Acid Consensus (CRAC) sequences in the intracellular juxtamembrane domain of several Toll-like receptors suggests a direct role of cholesterol in the activation process.

KEYWORDS

2

ABBREVIATIONS

ABCA1/G1: ATP-binding cassette, sub-family A/G, member 1; AP-1: activator protein 1; CARC: inverted "CRAC" domain; CD 14/36/55: cluster of differentiation 14/36/55; CRAC: Cholesterol Recognition Amino-Acid Consensus sequence; CXCR4: C-X-C chemokine receptor type 4; DAMP: danger-Associated Molecular Patterns; ECD: extracellular domain; EGF: Epidermal growth factor; EGFR: Epidermal growth factor receptor; EtOH: Ethanol; GM-1: monosialotetrahexosylganglioside; GPCR: G protein–coupled receptors; GPI: glycosyl phosphoinositol; HsP70/90: 70/90 kilodalton heat shock proteins; IFN β/γ: Interferon β/γ;IL-1: Interleukin-1; IRF : Interferon regulatory factor; LPS: lipopolysaccharides; LRR: Leucine-rich Repeats; MAL: MyD88 adapter-like - also known as TIRAP; MβCD: methyl β- cyclodextrin; MD-2: myeloid differentiation protein-2; MPL: Monophosphoryl Lipid A; MyD88 : myeloid differentiation factor 88; NF-κB : nuclear factor kappa B; PAMP: Pathogen-Associated Molecular Patterns; PIP_2 or $PtdIns(4,5)P_2$: Phosphatidylinositol 4,5-bisphosphate; RTK: Receptor tyrosine kinases; SM: sphingomyelin; Src kinase: Sarcoma proto-oncogene tyrosine-protein kinase; TIR: toll-interleukin 1 receptor; TIRAP: TIR domain containing adaptor protein - also known as MAL; TLR: toll-like receptor; TM: transmembrane; TMD: transmembrane domain; TRAM: TRIF-related adaptor molecule; TRIF: TIR domain-containing adaptor inducing IFN-β.

Table of Content

1 <u>INTRODUCTION</u>

It has been shown for years that membrane protein activity is modulated by the lipids that surround them. Some proteins sense directly the membrane physico-chemical properties such as fluidity, polarity, curvature, hydrophobic mismatch, thickness and lateral membrane tension. In other cases, specific interactions between defined protein motifs and given lipids have been evidenced. Since the seminal hypothesis of a fluid mosaic model membrane [1], an overwhelming body of evidence was collected that supports the existence in the membrane of differentiated domains characterized by a specific chemical composition. One example of such microdomains is the so-called membrane rafts that were proposed to be transient domains enriched in sphingolipids, cholesterol and saturated lipids [2,3]. These lipids are laterally mobile in the bilayer, leading to changes in lipid bilayer thickness and allowing recruitment of specific proteins [2,3]. It has been proposed that lipid microdomains might provide specific membrane proteins with the necessary microenvironment for the initiation of signalling pathways [4].

2 <u>TOLL-LIKE RECEPTORS</u>

Toll-like receptors (TLRs) are Type I transmembrane receptors in charge of recognition of conserved patterns characteristic of bacterial, viral or fungal invasions, but distinguishable from host molecules, the so-called Pathogen-Associated Molecular Patterns (PAMPs), and some endogenous intracellular molecules or extracellular matrix components released by activated or necrotic cells designed as Danger-Associated Molecular Patterns (DAMPs) [5,6]. They are major contributors of the innate immune system of mammalian organisms and their activation induces the secretion of pro-inflammatory mediators like cytokines, chemokines, type I interferon (IFN), reactive oxygen species, and other mediators.

The structure of TLRs is characterized by a N-terminal extracellular domain (ECD) made of leucine-rich repeats (LRRs), short motifs that fold into a characteristic solenoid structure giving to the ectodomain a horseshoe-shape, a single transmembrane domain, and a C-terminal intracellular signalling domain that contains a conserved region called the Toll/IL-1 receptor (TIR) domain, characteristic of the Interleukin-1 receptor / toll-like receptor superfamily [7-9].

Human TLR family comprises 10 proteins, each of them being specialized in the recognition of a specific class of PAMPs. The most studied Toll-like receptor, TLR4, recognizes bacterial lipopolysaccharides of the outer membrane of Gram-negative bacteria, which are responsible for most dramatic clinical manifestations of bacterial infections in mammals. TLR1, 2 and 6 recognize bacterial and fungal lipoprotein and peptidoglycan; TLR3, TLR7 and 8 recognize respectively viral double stranded RNA, single stranded DNA and single stranded RNA while bacterial flagella and unmethylated CpG sequences characteristic of bacterial DNA activate TLR5 and TLR9-dependent signalling cascades, respectively. The function of human TLR10 is still unclear but seems to cooperate with the TLR1/2/6 family [8,10].

TLRs can be divided into two subgroups, depending on their cellular localization: plasma membrane TLRs include TLR1, TLR2, TLR4 and TLR5, while other TLRs (TLR3, TLR7, TLR8 and TLR9) localize to and signal from acidified compartments of the endolysosomal pathway.

The signalling cascades induced by Toll-like Receptor ligands can be divided into two main cascades depending on the intracellular adaptor molecules they recruit. The myeloid differentiation primary response protein 88 (MyD88) is the main adaptor of the first signalling cascade leading to

the activation of Nuclear Factor-κB (NF-κB) and Activated Protein-1 (AP-1) transcription factors. The second cascade is dependent on the activation of interferon (IFN)-regulatory factors (IRFs) by TIR domain-containing adaptor protein inducing IFN-β (TRIF). TLR 1/2/6, 7, 8 and 9 activate exclusively the MyD88-dependent pathway (even if growing evidence suggests TLR1/2/6 are also capable to activate the second signalling pathway in some conditions). TLR3 activates exclusively the TRIF-dependent pathway. TLR4 is generally accepted as the only Toll-like Receptor activating both signalling pathways [8,10].

Ligands are recognized by the extracellular domains of TLRs at different binding sites depending on the receptor. The overall shape of the signalling TLR-ligand complexes is similar among all TLRs and consists in a m-shaped TLR ECD dimer, in which the N-termini stretch out to opposite ends and the C-termini interact in the middle. Dimerization of TLR monomers is usually induced by ligand recognition (with the exception of TLR7-8 and 9 existing as an inactive homodimer in non-activated cells, and which will be activated upon ligand binding after cleavage in the endosomes) [6,11]. Formation of dimers upon activation supports the hypothesis that dimerization of the ECDs enforces dimerization of the intracellular signalling TIR domains, hence able to recruit adaptor molecules and trigger downstream signalling pathways [6,11].

3 LIPID REGULATION OF TOLL-LIKE RECEPTOR ACTIVITY

3.1 *TLR signalling is initiated in lipid microdomains*

The existence of membrane lipid microdomains, the so-called lipid rafts, has been proposed in the nineties by Kai Simons [2]. It is generally accepted that these short-lived (~100ms) microdomains are cholesterol and sphingolipids-enriched regulate the biological activity of proteins inserted into these lipid domains by influencing the membrane physico-chemical properties or through direct binding to proteins. Membrane lipid partitioning may also lead to the formation of multicomponent transduction complexes [2] and explain the spatial segregation of certain signalling pathways emanating from the cell surface [3].

3.1.1 *Role of cholesterol*

First, it was shown that cholesterol loading of macrophage plasma or endosomal membranes enhanced the inflammatory activity of TLR4 and 3 agonists [12]. More convincingly, depletion of cholesterol or disruption of lipid rafts by different drugs downregulate the inflammatory signalling. The treatment with the cholesterol depleting agent, methyl β-cyclodextrin (MβCD), causes a decrease of the NF-κB recruitment into nucleus or a decreased activity of the MyD88-dependent pathway [13-18]. Furthermore, depletion of cholesterol using, statins or α-tocopherol derivatives [14,16,19,20] confirmed the importance of cholesterol-rich domains in TLR initiation. Ethanol, on its turn, modulates TLR4 activation mechanism by LPS by mimicking the raft-disrupting agents streptolysin-O or saponin, hence affecting TLR4 partitioning into rafts after LPS stimulation [16,21,22]. Similarly, the down-regulating effect of surfactants or oxidized phospholipids on LPS activation was correlated to a decreased TLR4 translocation into lipid rafts [23,24] while neither LPS binding nor TLR4 surface expression were reduced.

Finally, macrophages from mice lacking the cholesterol efflux pump ATP-binding cassette subfamily A1 (ABCA1) presented an enhanced proinflammatory response to LPS [25]. It was

further demonstrated that this deficiency cause an accumulation of free cholesterol within lipid rafts and localization of TLRs into rafts explaining the enhanced activation of MyD88-dependent signalling pathways by TLR4, 2, 7 and 9 [26]. Modification of lipid rafts by treatment with MβCD or nystatin corrected the abnormal hyper-responsiveness of ABCA1 mutants to LPS [27]. Similar results were obtained with ABCA1 and ABCG1 double mutants, presenting a hyper-responsiveness to TLR2, 3 and 4, but not TLR 7 or 9 agonists which was abolished following treatment with MβCD or filipin [28].

3.1.2 *Role of sphingomyelin*

Recruitment of TLR4/MD2 upon LPS stimulation decreases in mutants for sphingomyelin synthases SMS1 and SMS2 (responsible for the synthesis of sphingomyelin from ceramide and phosphatidylcholine) [29-31]. As a consequence, NF-κB activation by LPS in these mutants was attenuated. Similarly, a partial deficiency in the Serine Palmitoyl-CoA Transferase (SPT) - the key enzyme of the *de novo* biosynthetic pathway of sphingolipids - also resulted in a reduced recruitment of TLR4/MD2 on cell surface and decreased NF-κB activation upon LPS stimulation [32]. It should be noted however that SPT deficiency was linked to an increase of ABCA1 and ABCG1 cell surface expression, increasing the cholesterol efflux, which means that SPT deficiency affects both sphingomyelin synthesis and cholesterol accumulation into plasma membrane. Moreover, macrophages depleted in sphingomyelin presented attenuated LPS responses [30,33,34].

Finally, one should note that LPS stimulation is followed by a rapid production of ceramide (resulting from sphingomyelin hydrolysis by the neutral or acid sphingomyelinase), apparently crucial for LPS activity since inhibition of the sphingomyelinase blocks signalling events [33,35,36].

Similarly to TLR4, TLR3 activation up-regulates genes involved in sphingolipid metabolism and increases sphingomyelin and ceramide lipid contents [37].

3.1.3 *Importance of lipid partitioning for the formation of "activation clusters"*

TLR4 is not detected in lipid raft-enriched fractions isolated from non-activated cells but is recruited in GM-1 ganglioside (a raft associated lipid) and CD14 enriched fractions, after LPS stimulation [14]. This leads to the concept that TLR activation requires the formation of "supramolecular activation clusters" [38,39] within lipid raft microdomains, offering a platform that brings receptor molecules close together, allowing their activation and signal transduction [40,41]. Such assemblies have been characterized by confocal microscopy or fluorescence resonance transfer (FRET) between raft constituents, co-receptors and TLR4 [14,42,42,43], affinity chromatography or peptide-mass fingerprinting experiments [42,44-46]. The role of these raft-associated TLR4 activation cluster proteins has been largely discussed [47] and we will focus here on CD14 and CD36, the two main raft-associated co-receptors of TLR2 and 4.

Apart from the membrane co-receptors, intracellular co-adaptors for TLR signalling cascades are also associated to lipid microdomains: MAL (also known as TIRAP), the co-adaptor of MyD88, and the co-adaptor for TRIF, TRAM (TRIF-related adaptor molecule). Here again, it was suggested that lipid rafts favour the increased density of lipid-associated adaptor molecules at the site of TLR4 clustering, as evidenced by the increased TLR4 clustering and signalling following stabilization of lipid rafts with cholera toxin B (binding GM-1 in lipid raft microdomains)[48].

3.1.3.1 *CD14*

The Cluster of Differentiation CD14 exists in two forms, one expressed on the cell surface thanks to a glycosylphosphatidylinositol anchor (mCD14) and a soluble form found in the serum (sCD14)[49,50]. Both forms were shown to facilitate LPS recognition by the TLR4/MD-2 complex, but most studies have focused on deciphering the role of the membrane form in the LPS machinery. After LPS binding, mCD14 transfers LPS to the TLR4/MD-2 complex which dimerizes and triggers the MyD88-dependent pathway and the resulting secretion of pro-inflammatory cytokines [38,51]. Once the MyD88-pathway cascade has been triggered, the TLR4/MD-2/LPS complex is endocyted and activates the TRIF-dependent production of type I interferons [50,52].

CD14 greatly enhances the detection of LPS by TLR4 but its requirement depends also on the LPS phenotype or structure: although CD14 is required for the detection of smooth LPS (LPS with abundant O-glycosylation, the more common phenotype of LPS), it is not required by rough LPS (LPS lacking the O-antigen), MPL (Monophosphoryl Lipid A) and CRX-527 to activate the MyD88-dependent pathway [53-56].

Thanks to its GPI anchor, mCD14 is localized in the cholesterol- and sphingolipid-rich microdomains of the plasma membrane [14,57] and is essential for the TLR4/MD-2 recruitment into lipid microdomains, a process required for efficient dimer formation [58]. This role was further confirmed by deciphering the anti-inflammatory activity of a cationic lipid (lipofectamine) on the LPS-induced activation of TLR4: this lipid was shown to uncouple LPS binding by preventing CD14-TLR4 interactions [59]. The same hypothesis has been formulated for EtOH effect, altering LPS-related partition of CD14 [16]. It is also believed to be the main mechanism of modulation of immune cascades by oxidized phospholipids [60].

The higher hydrophobicity of rough LPS, MPL and CRX-527, as compared to smooth LPS phenotype, allows a better incorporation of these ligands into biological membranes and enhanced mobility, and may therefore explain the non-requirement of mCD14 for activating the MyD88-dependent pathway [53,54]. Importance of membrane insertion of LPS has been highlighted by several studies using model membranes or giant liposomes, showing a preferential incorporation of LPS into sphingomyelin and cholesterol-rich domains [61-63] and the ability of rough LPS, as compared to smooth form, to induce the formation of lipid microdomains into model membranes [64,65].

However, LPS is unable to activate the TRIF-dependent pathway in macrophages expressing a truncated inactive variant of CD14, irrespectively of the LPS phenotype [53]. Since CD14 controls the LPS-induced endocytosis of the activated TLR4 [66], which is required for the activation of the TRIF-dependent pathway [52], it is generally accepted that CD14 also controls the internalization of the TLR4/MD-2/LPS complex required to induce the TRIF-dependent pathway [50,66]. This role has been confirmed by forcing endocytosis of LPS, using latex beads or cationic liposomes, in the absence of CD14 [66-68].

Clathrin-mediated endocytosis is believed to be the principal endocytic process [52,69,70] but blocking of this specific endocytic pathway using potassium depletion and Dynasore treatment did not inhibit completely the internalization of membrane TLR4 upon LPS stimulation. Interestingly, this specific internalization of membrane TLR4 could be abrogated by depletion of plasma membrane cholesterol with MβCD [69]. Therefore the role of lipid-raft or caveolin-dependent mediated endocytosis has emerged [69,71-73].

Apart from TLR4 signalling, CD14 is also a co-receptor for TLR2, TLR3, TLR7 and TLR9 [74] and its role in the re-location of TLR2 into lipid rafts, formation of the TLR2/ligand complex and subsequent activation is well documented [75-77].

3.1.3.2 *CD36*

CD36, a member of the class B scavenger receptor family, acts as a co-receptor for TLR2 [40,78-81] but also for a non-canonical TLR4/TLR6 heterodimer [82]. CD36 is a membrane protein, found in lipid microdomains, and which is thought to adopt a hairpin-like structure with palmitoylated cytoplasmic C and N terminal tails [83,84]. Similarly to CD14, it has been suggested that the role of CD36 is to recruit TLR1/TLR2 heterodimers into lipid rafts upon stimulation [40]. CD36 is also involved in endocytic uptake of different components including oxidized LDL [85] amyloid β peptide [86], and possibly LPS [87], which are all activators of TLR4 or TLR2-dependent cascades. This suggests that CD36, similarly to CD14, also regulates internalization of TLRs. Indeed, TLR2/6 and CD36 co-localized intracellularly following activation with TLR2 ligands and this localization was prevented by treatment with either filipin or nystatin [40]. Similarly, CD36, TLR4 and TLR6 co-localized in intracellular compartments following treatment with oxLDL and inhibition of dynamin-dependent endocytosis blocked CD36-TLR4-TLR6 signalling induced by oxidized LDL[82].

3.1.3.3 *MAL*

The adaptor protein MAL (TIRAP) contains a N-terminal phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2 or PIP2) binding motif, rich in positively-charged lysine residues [88]. Thanks to this anchor, MAL is pre-localized into raft lipid microdomains enriched with PtdIns(4,5)P_2 [88]. The recruitment of TLRs into rafts would therefore favour the interactions with MAL and MyD88 TIR domains, which is a prerequisite for the induction of the signalling pathway.

13

3.1.3.4 *TRAM*

Similarly to MAL, the adaptor protein TRAM is localized in the plasma membrane. TRAM contains a N-terminal myristoylation site, similar to that found in mammalian Src kinases, which is modified by the addition of a myristoyl group on a specific glycine residue. Myristoylation of TRAM allows its association with the membrane (most probably into lipid microdomains enriched in saturated acyl chain lipids) but is probably not sufficient to confer stable membrane binding. It was suggested that several lysine residues close to the myristoylation site may serve as a polybasic cluster stabilizing membrane binding by electrostatic interactions with phospholipid head groups of the lipid membrane [89]. Mutation of this myristoylation site leads to improper localization of TRAM and affects the TLR4-dependent LPS response [89].

3.2 *Lipid-binding sequences within TLRs*

TLR dimerization process (required for activation of the TLR1 to TLR6) is believed to be dependent on both ectodomains (sufficient to form *in vitro* dimers after ligand recognition) and transmembrane regions [90]. Isolated transmembrane domains have indeed a strong tendency to oligomerize [91,92] in a homotypic manner. Heterotypic interactions of transmembrane domains from different TLR showed a preference for their native dimer partners (i.e. TLR2 transmembrane domain heterodimerize with TLR1, TLR6 or TLR10 TMDs but not with TLR4 or TLR5) providing evidence of specific transmembrane domain interactions [91]. Since truncation of the TLR4 ectodomain leads to constitutive activation after dimerization of the transmembrane and TIR domains, it was proposed that ectodomain exerts an autoinhibitory effect to prevent spontaneous, ligand-independent dimerization [93].

14

Upon activation, TLRs migrate to specific lipid microdomains where they are activated and able to transmit signals [14,40,94]. It is likely that the transmembrane region and/or the regions surrounding this sequence are involved in the localization of TLRs. It was demonstrated indeed that the intracellular localization of TLR9 and TLR7 are governed by their transmembrane domains [95,96] and that the localization of TLR3 depends on the juxtamembrane domain [96].

Specific amino acid sequences have been associated with the localization of proteins within membrane lipid microdomains [97,98]. The presence of a (or several) Cholesterol Recognition Amino-Acid Consensus (CRAC) sequence and its counterparts, the CARC sequence, in or near the transmembrane region is considered as indicative of cholesterol dependency for efficient protein function [99,100].

3.2.1 *CRAC domains*

The sequence analysis of human Toll-like receptors reveals the presence of CRAC (defined as -[LV]-X(1,5)-Y-X(1,5)-[RK]-) and/or CARC sequences (defined as -[RK]-X(1,5)-Y-X(1,5)-[LV]-) in the cytoplasmic domain close to the transmembrane region (see Table 1a).

Although most TLRs (with the exception of TLR5 and TLR9) show CRAC-like (or CARC) sequences located close to their transmembrane domain, only TLR2, TLR4, TLR7 and TLR8 possess both CRAC and CARC sequences. Interestingly, CARC-CRAC-CARC domains into TLR4 are located close to the membrane, before the TIR domain. This strongly suggests that this intracellular region of TLR4 binds specifically cholesterol. This region corresponds to a sequence previously identified as a small hydrophobic segment adjacent to the transmembrane region and the TIR domain with the consensus sequence FYFHLxLxxGC among mammalian species (see Table 1b) which was demonstrated to be required for both activation and dimerization of TLR4 [101].

15

It is likely that the increased bilayer thickness of the lipid bilayer in rafts (due to the high content of saturated acyl chain lipids) favours the insertion of the first CARC motif into lipid bilayer membrane, and its interaction with cholesterol will lead to structural rearrangements required for an efficient activation. The presence of CRAC motifs in TLR4, and the fact that cholesterol is required for activation, suggests the interaction of this region with the membrane (and more specifically the cholesterol) occurs upon activation and is required for efficient dimerization of the receptors.

Toll-like receptors belongs to the family of Type-I transmembrane receptors which includes receptor tyrosine kinases (RTKs) which are activated by ligand-induced dimerization of the receptors [102,103]. The most studied RTK is the Epidermal Growth Factor (EGF) Receptor (EGFR) which dimerizes upon recognition of EGF inducing a dramatic conformational change in both ectodomains and juxtamembrane regions [102,104,105]. Moreover, EGFR has been also found to be inserted into lipid rafts [104,106] and was reported to be regulated by gangliosides [107-110] (a specific type of glycosphingolipid located in lipid rafts), as suggested for TLR2 and TLR4 [111-113] reinforcing the structural and functional similarities between TLRs and EGFR.

The juxtamembrane cytosolic sequence of EGFR contains several basic amino acids which are sequestered in the membrane by interacting with anionic phospholipid head groups in the inactive conformation. Upon activation, several residues of two EGFR molecules (in an active dimeric complex) are pulled out of the plasma membrane and reassemble as antiparallel α-helices with a kinase activity. Based on this mechanism, it is likely that cytosolic juxtamembrane sequences of the TLRs interact with the membrane [6].

3.2.2 *Sphingolipid domains*

Similarly to cholesterol, sphingolipids are major components of lipid microdomains. A signature sequence to be found into the transmembrane domains has been defined recently [97]. The

16

sequence analysis of human Toll-like receptors reveals the presence of sphingolipid binding-like sequences in the transmembrane region of TLR3 and TLR5 (see Table 2). Interestingly, TLR3 which presents a CARC motif in the juxtamembrane segments close to the membrane (possibly inserted into membrane bilayer in raft microdomains) also presents two successive sphingolipid binding-like motifs in its transmembrane segment. Such coexistence of both specific sequences has not been reported earlier and strongly suggests that TLR3 interacts with both sphingolipids and cholesterol. This is in line with the fact that cholesterol accumulation into rafts enhances TLR3 activation (see 3.1.1) and that TLR3 activation upregulates sphingolipid metabolism (see 3.1.2).

4 CONCLUSIONS

Although compelling evidences demonstrate that Toll-like Receptor signalling depends on the lipid membrane composition, little is known about the mechanism by which lipid composition influence TLR activity.

It has been proposed that lipid microdomains favour clustering of the TLR activation complexes i.e. TLR and co-receptors like CD14 or CD36. Upon ligand recognition, all co-receptors migrate into rafts, bringing TLRs in close proximity thereby allowing TLR dimerization and activation.

Since CRAC and CARC domains are located into the juxtamembrane domain of TLR4, it can be suggested that, upon ligand recognition and recruitment into lipid microdomains (with the help of CD14 or another co-receptor), a structural rearrangement occurs allowing the insertion of the first CARC motif of the juxtamembrane segment of TLR4 region into the transmembrane stretch (Figure 1 - upper panel). This rearrangement would bring the TIR domain closer to the membrane in a way it interacts with MAL (associated to the membrane thanks to its PIP_2 anchor) and initiates the signalling cascade [114]. After insertion into the lipid bilayer, the juxtamembrane segment would adopt an alpha-helix secondary structure (as predicted by secondary prediction programs),

17

extending the existing transmembrane helix (Figure 1 - lower panel). This helix would rigidify the juxtamembrane segment, stabilize the TIR domains and favour the formation of TIR/TIR dimers. This is in agreement with previous statements that the cytosolic juxtamembrane domain has rotational flexibility thanks to the presence of several glycine residues able to adopt a wider range of dihedral angles [115].

BEFORE STIMULATION

CARC   CRAC   CARC

SLNITCQMNK**TIIGVSVLSVLVVSVVAVLVY**KFYFHLM**LLAGCIKYGRG**ENIYDAFVIYSSQDEDW

Transmembrane region   **TIR domain**

UPON STIMULATION

CARC   CRAC   CARC

SLNITCQMNK**TIIGVSVLSVLVVSVVAVLVYKFYFHLMLL**AGCIKYGRGENIYDAFVIYSSQDEDW

Transmembrane region   **TIR domain**
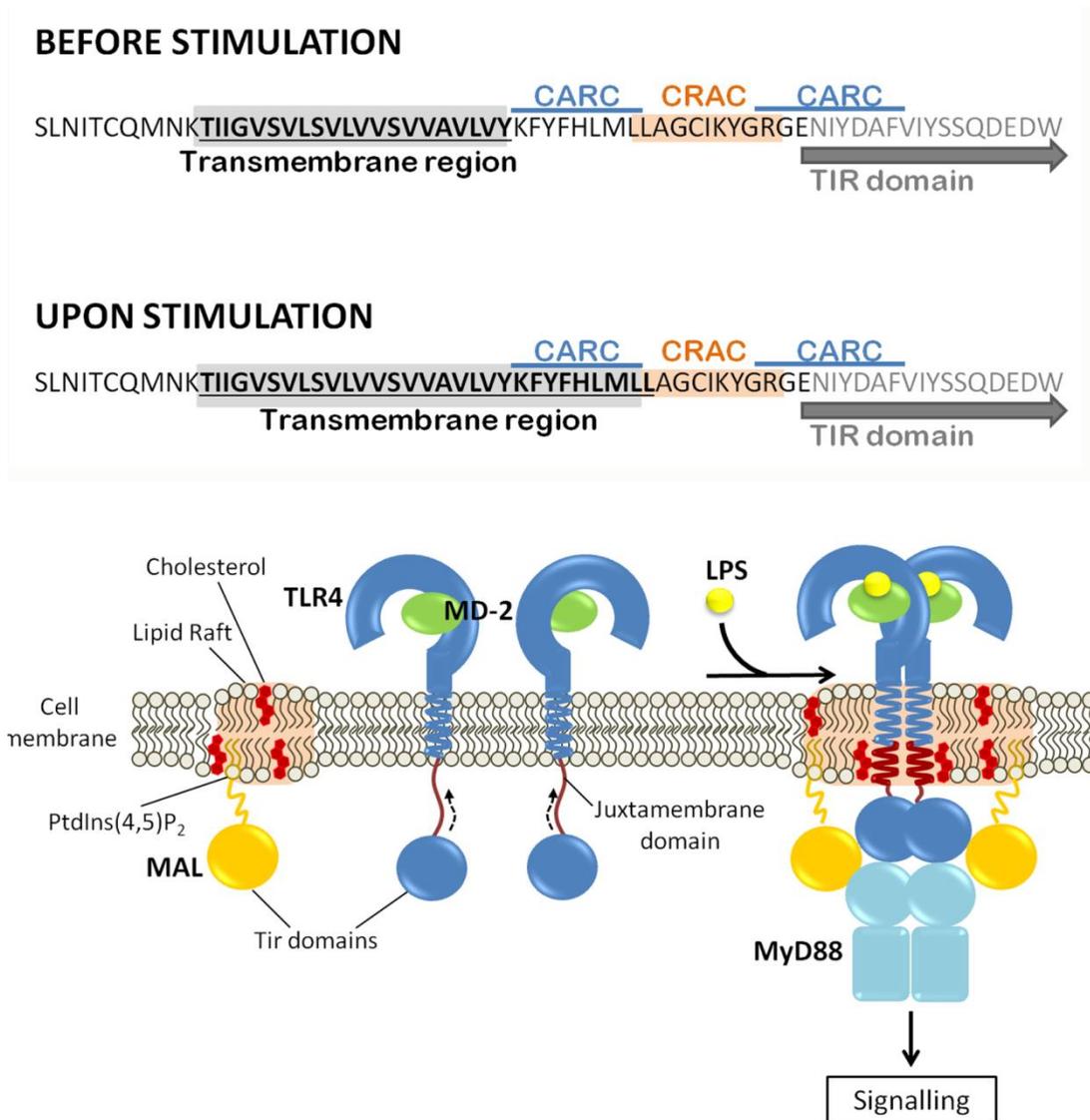
Figure 1: Activation of TLR4 before and after stimulation by bacterial lipopolysaccharides (LPS). Inactive TLR4s exist as monomers in lipid membranes before stimulation and are recruited into lipid microdomains upon stimulation. Upper panel shows the sequence of human TLR4 (622-

687) and respective CARC and CRAC domains. Lower panel is a schematic view of the activation process (shown for the MyD88-dependent pathway only). Due to membrane thickness increase in the raft domains, part of the juxtamembrane domain (in red) containing a CARC sequence adopts a helical structure upon insertion into the lipid bilayer. This leads to a shortening of the cytosolic juxtamembrane region and allows TIR domain to get closer to the membrane, and to interact with membrane-associated co-receptors like MAL, initiating the MyD88-dependent signalling cascades. It may also suggest that a rotation of the long helix made of TLR4 transmembrane domain (in blue) and part of the juxtamembrane domain (red) would significantly modify the orientation of the cytosolic TIR domains with respect to each other.

5 <u>ACKNOWLEDGMENTS</u>

6 REFERENCES

[1] S.J. Singer, G.L. Nicolson, The fluid mosaic model of the structure of cell membranes, Science 175 (1972) 720-731.

[2] K. Simons, E. Ikonen, Functional rafts in cell membranes, Nature 387 (1997) 569-572.

[3] U. Coskun, K. Simons, Membrane rafting: from apical sorting to phase segregation, FEBS Lett. 584 (2010) 1685-1693.

[4] K. Simons, M.J. Gerl, Revitalizing membrane rafts: new tools and insights, Nat. Rev. Mol. Cell Biol. 11 (2010) 688-699.

[5] T. Kawai, S. Akira, Toll-like receptors and their crosstalk with other innate receptors in infection and immunity, Immunity. 34 (2011) 637-650.

[6] N.J. Gay, M.F. Symmons, M. Gangloff, C.E. Bryant, Assembly and localization of Toll-like receptor signalling complexes, Nat. Rev. Immunol. 14 (2014) 546-558.

[7] B.S. Park, D.H. Song, H.M. Kim, B.S. Choi, H. Lee, J.O. Lee, The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex, Nature 458 (2009) 1191-1195.

[8] N.J. Gay, M. Gangloff, Structure and function of Toll receptors and their ligands, Annu. Rev. Biochem. 76 (2007) 141-165.

[9] D.H. Song, J.O. Lee, Sensing of microbial molecular patterns by Toll-like receptors, Immunol. Rev. 250 (2012) 216-229.

[10] O. Takeuchi, S. Akira, Pattern recognition receptors and inflammation, Cell 140 (2010) 805-820.

[11] I. Botos, D.M. Segal, D.R. Davies, The structural biology of Toll-like receptors, Structure. 19 (2011) 447-459.

[12] Y. Sun, M. Ishibashi, T. Seimon, M. Lee, S.M. Sharma, K.A. Fitzgerald, A.O. Samokhin, Y. Wang, S. Sayers, M. Aikawa, W.G. Jerome, M.C. Ostrowski, D. Bromme, P. Libby, I.A. Tabas, C.L. Welch, A.R. Tall, Free cholesterol accumulation in macrophage membranes activates Toll-like receptors and p38 mitogen-activated protein kinase and induces cathepsin K, Circ. Res. 104 (2009) 455-465.

[13] H. Arima, Y. Nishimoto, K. Motoyama, F. Hirayama, K. Uekama, Inhibitory effects of novel hydrophilic cyclodextrin derivatives on nitric oxide production in macrophages stimulated with lipopolysaccharide, Pharm. Res. 18 (2001) 1167-1173.

[14] M. Triantafilou, K. Miyake, D.T. Golenbock, K. Triantafilou, Mediators of innate immune recognition of bacteria concentrate in lipid rafts and facilitate lipopolysaccharide-induced cell activation, J Cell Sci. 115 (2002) 2603-2611.

[15] J. Cuschieri, Implications of lipid raft disintegration: enhanced anti-inflammatory macrophage phenotype, Surgery 136 (2004) 169-175.

[16] Q. Dai, J. Zhang, S.B. Pruett, Ethanol alters cellular activation and CD14 partitioning in lipid rafts, Biochem. Biophys. Res. Commun. 332 (2005) 37-42.

[17] K. Motoyama, H. Arima, Y. Nishimoto, K. Miyake, F. Hirayama, K. Uekama, Involvement of CD14 in the inhibitory effects of dimethyl-alpha-cyclodextrin on lipopolysaccharide signaling in macrophages, FEBS Lett. 579 (2005) 1707-1714.

[18] K. Motoyama, Y. Hashimoto, F. Hirayama, K. Uekama, H. Arima, Inhibitory effects of 2,6-di-O-methyl-alpha-cyclodextrin on poly I:C signaling in macrophages, Eur. J Pharm. Sci. 36 (2009) 285-291.

[19] P. Chansrichavala, U. Chantharaksri, P. Sritara, N. Ngaosuwankul, S.C. Chaiyaroj, Atorvastatin affects TLR4 clustering via lipid raft modulation, Int. Immunopharmacol. 10 (2010) 892-899.

[20] W. Duan, J. Zhou, S. Zhang, K. Zhao, L. Zhao, K. Ogata, T. Sakaue, A. Mori, T. Wei, ESeroS-GS modulates lipopolysaccharide-induced macrophage activation by impairing the assembly of TLR-4 complexes in lipid rafts, Biochim. Biophys. Acta 1813 (2011) 772-783.

[21] A. Dolganiuc, G. Bakis, K. Kodys, P. Mandrekar, G. Szabo, Acute ethanol treatment modulates Toll-like receptor-4 association with lipid rafts, Alcohol Clin. Exp. Res. 30 (2006) 76-85.

[22] S. Fernandez-Lizarbe, M. Pascual, M.S. Gascon, A. Blanco, C. Guerri, Lipid rafts regulate ethanol-induced activation of TLR4 signaling in murine macrophages, Mol. Immunol. 45 (2008) 2007-2016.

[23] W. Abate, A.A. Alghaithy, J. Parton, K.P. Jones, S.K. Jackson, Surfactant lipids regulate LPS-induced interleukin-8 production in A549 lung epithelial cells by inhibiting translocation of TLR4 into lipid raft domains, J Lipid Res. 51 (2010) 334-344.

[24] K.A. Walton, X. Hsieh, N. Gharavi, S. Wang, G. Wang, M. Yeh, A.L. Cole, J.A. Berliner, Receptors involved in the oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine-mediated synthesis of interleukin-8. A role for Toll-like receptor 4 and a glycosylphosphatidylinositol-anchored protein, J Biol. Chem. 278 (2003) 29661-29666.

[25] X. Zhu, J.Y. Lee, J.M. Timmins, J.M. Brown, E. Boudyguina, A. Mulya, A.K. Gebre, M.C. Willingham, E.M. Hiltbold, N. Mishra, N. Maeda, J.S. Parks, Increased cellular free cholesterol in macrophage-specific Abca1 knock-out mice enhances pro-inflammatory response of macrophages, J Biol. Chem. 283 (2008) 22930-22941.

[26] X. Zhu, J.S. Owen, M.D. Wilson, H. Li, G.L. Griffiths, M.J. Thomas, E.M. Hiltbold, M.B. Fessler, J.S. Parks, Macrophage ABCA1 reduces MyD88-dependent Toll-like receptor trafficking to lipid rafts by reduction of lipid raft cholesterol, J Lipid Res. 51 (2010) 3196-3206.

[27] M. Koseki, K. Hirano, D. Masuda, C. Ikegami, M. Tanaka, A. Ota, J.C. Sandoval, Y. Nakagawa-Toyama, S.B. Sato, T. Kobayashi, Y. Shimada, Y. Ohno-Iwashita, F. Matsuura, I. Shimomura, S. Yamashita, Increased lipid rafts and accelerated lipopolysaccharide-induced tumor necrosis factor-alpha secretion in Abca1-deficient macrophages, J Lipid Res. 48 (2007) 299-306.

[28] L. Yvan-Charvet, C. Welch, T.A. Pagler, M. Ranalletta, M. Lamkanfi, S. Han, M. Ishibashi, R. Li, N. Wang, A.R. Tall, Increased inflammatory gene expression in ABC transporter-deficient macrophages: free cholesterol accumulation, increased signaling via toll-like receptors, and neutrophil infiltration of atherosclerotic lesions, Circulation 118 (2008) 1837-1847.

[29] T.K. Hailemariam, C. Huan, J. Liu, Z. Li, C. Roman, M. Kalbfeisch, H.H. Bui, D.A. Peake, M.S. Kuo, G. Cao, R. Wadgaonkar, X.C. Jiang, Sphingomyelin synthase 2 deficiency attenuates NFkappaB activation, Arterioscler. Thromb. Vasc. Biol. 28 (2008) 1519-1526.

[30] S. Gowda, C. Yeang, S. Wadgaonkar, F. Anjum, N. Grinkina, M. Cutaia, X.C. Jiang, R. Wadgaonkar, Sphingomyelin synthase 2 (SMS2) deficiency attenuates LPS-induced lung injury, Am. J Physiol Lung Cell Mol. Physiol 300 (2011) L430-L440.

[31] Z. Li, Y. Fan, J. Liu, Y. Li, C. Huan, H.H. Bui, M.S. Kuo, T.S. Park, G. Cao, X.C. Jiang, Impact of sphingomyelin synthase 1 deficiency on sphingolipid metabolism and atherosclerosis in mice, Arterioscler. Thromb. Vasc. Biol. 32 (2012) 1577-1584.

[32] M. Chakraborty, C. Lou, C. Huan, M.S. Kuo, T.S. Park, G. Cao, X.C. Jiang, Myeloid cell-specific serine palmitoyltransferase subunit 2 haploinsufficiency reduces murine atherosclerosis, J Clin. Invest 123 (2013) 1784-1797.

[33] J. Cuschieri, E. Bulger, J. Billgrin, I. Garcia, R.V. Maier, Acid sphingomyelinase is required for lipid Raft TLR4 complex formation, Surg. Infect. (Larchmt.) 8 (2007) 91-106.

[34] T.K. Hailemariam, C. Huan, J. Liu, Z. Li, C. Roman, M. Kalbfeisch, H.H. Bui, D.A. Peake, M.S. Kuo, G. Cao, R. Wadgaonkar, X.C. Jiang, Sphingomyelin synthase 2 deficiency attenuates NFkappaB activation, Arterioscler. Thromb. Vasc. Biol. 28 (2008) 1519-1526.

[35] M.L. MacKichan, A.L. DeFranco, Role of ceramide in lipopolysaccharide (LPS)-induced signaling. LPS increases ceramide rather than acting as a structural homolog, J Biol. Chem. 274 (1999) 1767-1775.

[36] S. Jozefowski, M. Czerkies, A. Lukasik, A. Bielawska, J. Bielawski, K. Kwiatkowska, A. Sobota, Ceramide and ceramide 1-phosphate are negative regulators of TNF-alpha production induced by lipopolysaccharide, J Immunol. 185 (2010) 6960-6973.

[37] A.W. Borkowski, K. Park, Y. Uchida, R.L. Gallo, Activation of TLR3 in keratinocytes increases expression of genes involved in formation of the epidermis, lipid accumulation, and epidermal organelles, J Invest Dermatol. 133 (2013) 2031-2040.

[38] K. Triantafilou, M. Triantafilou, S. Ladha, A. Mackie, R.L. Dedrick, N. Fernandez, R. Cherry, Fluorescence recovery after photobleaching reveals that LPS rapidly transfers from CD14 to hsp70 and hsp90 on the cell membrane, J Cell Sci. 114 (2001) 2535-2545.

[39] M. Triantafilou, K. Triantafilou, Receptor cluster formation during activation by bacterial products, J Endotoxin. Res. 9 (2003) 331-335.

[40] M. Triantafilou, F.G. Gamper, R.M. Haston, M.A. Mouratis, S. Morath, T. Hartung, K. Triantafilou, Membrane sorting of toll-like receptor (TLR)-2/6 and TLR2/1 heterodimers at the cell surface determines heterotypic associations with CD36 and intracellular targeting, J Biol. Chem. 281 (2006) 31002-31011.

[41] M. Triantafilou, P.M. Lepper, R. Olden, I.S. Dias, K. Triantafilou, Location, location, location: is membrane partitioning everything when it comes to innate immune activation?, Mediators. Inflamm. 2011 (2011) 186093.

[42] A. Pfeiffer, A. Bottcher, E. Orso, M. Kapinsky, P. Nagy, A. Bodnar, I. Spreitzer, G. Liebisch, W. Drobnik, K. Gempel, M. Horn, S. Holmer, T. Hartung, G. Multhoff, G. Schutz, H. Schindler, A.J. Ulmer, H. Heine, F. Stelter, C. Schutt, G. Rothe, J. Szollosi, S. Damjanovich, G. Schmitz, Lipopolysaccharide and ceramide docking to CD14 provokes ligand-specific receptor clustering in rafts, Eur. J. Immunol. 31 (2001) 3153-3164.

[43] M. Triantafilou, K. Triantafilou, Heat-shock protein 70 and heat-shock protein 90 associate with Toll-like receptor 4 in response to bacterial lipopolysaccharide, Biochem. Soc. Trans. 32 (2004) 636-639.

[44] K. Triantafilou, M. Triantafilou, R.L. Dedrick, A CD14-independent LPS receptor cluster, Nat. Immunol. 2 (2001) 338-345.

[45] C.A. Byrd, W. Bornmann, H. Erdjument-Bromage, P. Tempst, N. Pavletich, N. Rosen, C.F. Nathan, A. Ding, Heat shock protein 90 mediates macrophage activation by Taxol and bacterial lipopolysaccharide, Proc. Natl. Acad. Sci. U. S. A 96 (1999) 5645-5650.

[46] H. Heine, V.T. El-Samalouti, C. Notzel, A. Pfeiffer, A. Lentschat, S. Kusumoto, G. Schmitz, L. Hamann, A.J. Ulmer, CD55/decay accelerating factor is part of the lipopolysaccharide-induced receptor complex, Eur. J Immunol. 33 (2003) 1399-1408.

[47] A. Plociennikowska, A. Hromada-Judycka, K. Borzecka, K. Kwiatkowska, Co-operation of TLR4 and raft proteins in LPS-induced pro-inflammatory signaling, Cell Mol. Life Sci. (2014).

[48] P.G. Motshwene, M.C. Moncrieffe, J.G. Grossmann, C. Kao, M. Ayaluru, A.M. Sandercock, C.V. Robinson, E. Latz, N.J. Gay, An oligomeric signaling platform formed by the Toll-like receptor signal transducers MyD88 and IRAK-4, J Biol. Chem. 284 (2009) 25404-25411.

[49] S. Akashi, S. Saitoh, Y. Wakabayashi, T. Kikuchi, N. Takamura, Y. Nagai, Y. Kusumoto, K. Fukase, S. Kusumoto, Y. Adachi, A. Kosugi, K. Miyake, Lipopolysaccharide interaction with cell surface Toll-like receptor 4-MD-2: higher affinity than that with MD-2 or CD14, J Exp. Med. 198 (2003) 1035-1042.

[50] R. Ostuni, I. Zanoni, F. Granucci, Deciphering the complexity of Toll-like receptor signaling, Cell Mol. Life Sci. 67 (2010) 4109-4134.

[51] C.J. Da Silva, K. Soldau, U. Christen, P.S. Tobias, R.J. Ulevitch, Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. transfer from CD14 to TLR4 and MD-2, J Biol. Chem. 276 (2001) 21129-21135.

[52] J.C. Kagan, T. Su, T. Horng, A. Chow, S. Akira, R. Medzhitov, TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta, Nat. Immunol. 9 (2008) 361-368.

[53] Z. Jiang, P. Georgel, X. Du, L. Shamel, S. Sovath, S. Mudd, M. Huber, C. Kalis, S. Keck, C. Galanos, M. Freudenberg, B. Beutler, CD14 is required for MyD88-independent LPS signaling, Nat. Immunol. 6 (2005) 565-570.

[54] M. Huber, C. Kalis, S. Keck, Z. Jiang, P. Georgel, X. Du, L. Shamel, S. Sovath, S. Mudd, B. Beutler, C. Galanos, M.A. Freudenberg, R-form LPS, the master key to the activation ofTLR4/MD-2-positive cells, Eur. J Immunol. 36 (2006) 701-711.

[55] A. Legat, S. Thomas, P. Hermand, M.M. Van, M. Goldman, W.D. De, CD14-independent responses induced by a synthetic lipid A mimetic, Eur. J Immunol. 40 (2010) 797-802.

[56] N. Tanimura, S. Saitoh, U. Ohto, S. Akashi-Takamura, Y. Fujimoto, K. Fukase, T. Shimizu, K. Miyake, The attenuated inflammation of MPL is due to the lack of CD14-dependent tight dimerization of the TLR4/MD2 complex at the plasma membrane, Int. Immunol. 26 (2014) 307-314.

[57] J. Pugin, V.V. Kravchenko, J.D. Lee, L. Kline, R.J. Ulevitch, P.S. Tobias, Cell activation mediated by glycosylphosphatidylinositol-anchored or transmembrane forms of CD14, Infect. Immun. 66 (1998) 1174-1180.

[58] R.W. Finberg, E.A. Kurt-Jones, CD14: chaperone or matchmaker?, Immunity. 24 (2006) 127-129.

[59] M. Leon-Ponte, M.G. Kirchhof, T. Sun, T. Stephens, B. Singh, S. Sandhu, J. Madrenas, Polycationic lipids inhibit the pro-inflammatory response to LPS, Immunol. Lett. 96 (2005) 73-83.

[60] C. Erridge, C.M. Spickett, Oxidised phospholipid regulation of Toll-like receptor signalling, Redox. Rep. 12 (2007) 76-80.

[61] J.M. Alam, M. Yamazaki, Spontaneous insertion of lipopolysaccharide into lipid membranes from aqueous solution, Chem. Phys. Lipids 164 (2011) 166-174.

24

[62] F. Ciesielski, B. Davis, M. Rittig, B.B. Bonev, P. O'Shea, Receptor-independent interaction of bacterial lipopolysaccharide with lipid and lymphocyte membranes; the role of cholesterol, PLoS. One. 7 (2012) e38677.

[63] F. Ciesielski, D.C. Griffin, M. Rittig, I. Moriyon, B.B. Bonev, Interactions of lipopolysaccharide with lipid membranes, raft models - a solid state NMR study, Biochim. Biophys. Acta 1828 (2013) 1731-1742.

[64] J. Kubiak, J. Brewer, S. Hansen, L.A. Bagatolli, Lipid lateral organization on giant unilamellar vesicles containing lipopolysaccharides, Biophys J 100 (2011) 978-986.

[65] K. Nomura, M. Maeda, K. Sugase, S. Kusumoto, Lipopolysaccharide induces raft domain expansion in membrane composed of a phospholipid-cholesterol-sphingomyelin ternary system, Innate. Immun. 17 (2011) 256-268.

[66] I. Zanoni, R. Ostuni, L.R. Marek, S. Barresi, R. Barbalat, G.M. Barton, F. Granucci, J.C. Kagan, CD14 controls the LPS-induced endocytosis of Toll-like receptor 4, Cell 147 (2011) 868-880.

[67] C. Lonez, K. Irvine, M. Pizzuto, B. Schmidt, N. Gay, M. Vandenbranden, J. Ruysschaert, M. Gangloff, C. Bryant, Cationic Lipid Nanocarriers Induce Toll-like Receptor 4 Activation through a Novel Binding Site, Submitted (2014).

[68] S. Watanabe, Y. Kumazawa, J. Inoue, Liposomal lipopolysaccharide initiates TRIF-dependent signaling pathway independent of CD14, PLoS. One. 8 (2013) e60078.

[69] W. Cai, A. Du, K. Feng, X. Zhao, L. Qian, R.S. Ostrom, C. Xu, Adenylyl cyclase 6 activation negatively regulates TLR4 signaling through lipid raft-mediated endocytosis, J Immunol. 191 (2013) 6093-6100.

[70] H. Husebye, M.H. Aune, J. Stenvik, E. Samstad, F. Skjeldal, O. Halaas, N.J. Nilsen, H. Stenmark, E. Latz, E. Lien, T.E. Mollnes, O. Bakke, T. Espevik, The Rab11a GTPase controls Toll-like receptor 4-induced activation of interferon regulatory factor-3 on phagosomes, Immunity. 33 (2010) 583-596.

[71] T. Shuto, K. Kato, Y. Mori, S. Viriyakosol, M. Oba, T. Furuta, T. Okiyoneda, H. Arima, M.A. Suico, H. Kai, Membrane-anchored CD14 is required for LPS-induced TLR4 endocytosis in TLR4/MD-2/CD14 overexpressing CHO cells, Biochem. Biophys. Res. Commun. 338 (2005) 1402-1409.

[72] Y. Zhang, L. Zhang, Y. Li, S. Sun, H. Tan, Different contributions of clathrin- and caveolae-mediated endocytosis of vascular endothelial cadherin to lipopolysaccharide-induced vascular hyperpermeability, PLoS. One. 9 (2014) e106328.

[73] M. Pascual-Lucas, S. Fernandez-Lizarbe, J. Montesinos, C. Guerri, LPS or ethanol triggers clathrin- and rafts/caveolae-dependent endocytosis of TLR4 in cortical astrocytes, J Neurochem. 129 (2014) 448-462.

[74] M. Di Gioia, I. Zanoni, Toll-like receptor co-receptors as master regulators of the immune response, Mol. Immunol. 63 (2015) 143-152.

[75] G. Schmitz, E. Orso, CD14 signalling in lipid rafts: new ligands and co-receptors, Curr. Opin. Lipidol. 13 (2002) 513-521.

[76] H.M. Shamsul, A. Hasebe, M. Iyori, M. Ohtani, K. Kiura, D. Zhang, Y. Totsuka, K. Shibata, The Toll-like receptor 2 (TLR2) ligand FSL-1 is internalized via the clathrin-dependent endocytic pathway triggered by CD14 and CD36 but not by TLR2, Immunology 130 (2010) 262-272.

[77] M. Triantafilou, M. Manukyan, A. Mackie, S. Morath, T. Hartung, H. Heine, K. Triantafilou, Lipoteichoic acid and toll-like receptor 2 internalization and targeting to the Golgi are lipid raft-dependent, J Biol. Chem. 279 (2004) 40882-40889.

[78] K. Hoebe, P. Georgel, S. Rutschmann, X. Du, S. Mudd, K. Crozat, S. Sovath, L. Shamel, T. Hartung, U. Zahringer, B. Beutler, CD36 is a sensor of diacylglycerides, Nature 433 (2005) 523-527.

[79] M.J. Jimenez-Dalmaroni, N. Xiao, A.L. Corper, P. Verdino, G.D. Ainge, D.S. Larsen, G.F. Painter, P.M. Rudd, R.A. Dwek, K. Hoebe, B. Beutler, I.A. Wilson, Soluble CD36 ectodomain binds negatively charged diacylglycerol ligands and acts as a co-receptor for TLR2, PLoS. One. 4 (2009) e7411.

[80] M. Mae, M. Iyori, M. Yasuda, H.M. Shamsul, H. Kataoka, K. Kiura, A. Hasebe, Y. Totsuka, K. Shibata, The diacylated lipopeptide FSL-1 enhances phagocytosis of bacteria by macrophages through a Toll-like receptor 2-mediated signalling pathway, FEMS Immunol. Med. Microbiol. 49 (2007) 398-409.

[81] M. Triantafilou, F.G. Gamper, P.M. Lepper, M.A. Mouratis, C. Schumann, E. Harokopakis, R.E. Schifferle, G. Hajishengallis, K. Triantafilou, Lipopolysaccharides from atherosclerosis-associated bacteria antagonize TLR4, induce formation of TLR2/1/CD36 complexes in lipid rafts and trigger TLR2-induced inflammatory responses in human vascular endothelial cells, Cell Microbiol. 9 (2007) 2030-2039.

[82] C.R. Stewart, L.M. Stuart, K. Wilkinson, J.M. van Gils, J. Deng, A. Halle, K.J. Rayner, L. Boyer, R. Zhong, W.A. Frazier, A. Lacy-Hulbert, K.J. El, D.T. Golenbock, K.J. Moore, CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer, Nat. Immunol. 11 (2010) 155-161.

[83] N. Tao, S.J. Wagner, D.M. Lublin, CD36 is palmitoylated on both N- and C-terminal cytoplasmic tails, J Biol. Chem. 271 (1996) 22315-22320.

[84] D.J. Dorahy, L.F. Lincz, C.J. Meldrum, G.F. Burns, Biochemical isolation of a membrane microdomain from resting platelets highly enriched in the plasma membrane glycoprotein CD36, Biochem. J 319 (Pt 1) (1996) 67-72.

[85] K.J. Moore, M.W. Freeman, Scavenger receptors in atherosclerosis: beyond lipid uptake, Arterioscler. Thromb. Vasc. Biol. 26 (2006) 1702-1711.

[86] R.S. Jones, A.M. Minogue, T.J. Connor, M.A. Lynch, Amyloid-beta-induced astrocytic phagocytosis is mediated by CD36, CD47 and RAGE, J Neuroimmune. Pharmacol. 8 (2013) 301-311.

[87] M. Czerkies, K. Borzecka, M.I. Zdioruk, A. Plociennikowska, A. Sobota, K. Kwiatkowska, An interplay between scavenger receptor A and CD14 during activation of J774 cells by high concentrations of LPS, Immunobiology 218 (2013) 1217-1226.

[88] J.C. Kagan, R. Medzhitov, Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling, Cell 125 (2006) 943-955.

[89] D.C. Rowe, A.F. McGettrick, E. Latz, B.G. Monks, N.J. Gay, M. Yamamoto, S. Akira, L.A. O'Neill, K.A. Fitzgerald, D.T. Golenbock, The myristoylation of TRIF-related adaptor molecule is essential for Toll-like receptor 4 signal transduction, Proc. Natl. Acad. Sci. U. S. A 103 (2006) 6299-6304.

[90] E.M. Reuven, A. Fink, Y. Shai, Regulation of innate immune responses by transmembrane interactions: lessons from the TLR family, Biochim. Biophys. Acta 1838 (2014) 1586-1593.

[91] J.I. Godfroy, III, M. Roostan, Y.S. Moroz, I.V. Korendovych, H. Yin, Isolated Toll-like receptor transmembrane domains are capable of oligomerization, PLoS. One. 7 (2012) e48875.

[92] M. Treeby, J. Vasl, P. Ota, J. Friedrich, R. Jerala, Different functional role of domain boundaries of Toll-like receptor 4, Biochem. Biophys. Res. Commun. 381 (2009) 65-69.

[93] G. Panter, R. Jerala, The ectodomain of the Toll-like receptor 4 prevents constitutive receptor activation, J Biol. Chem. 286 (2011) 23334-23344.

[94] A.F. McGettrick, E.K. Brint, E.M. Palsson-McDermott, D.C. Rowe, D.T. Golenbock, N.J. Gay, K.A. Fitzgerald, L.A. O'Neill, Trif-related adapter molecule is phosphorylated by PKC{epsilon} during Toll-like receptor 4 signaling, Proc. Natl. Acad. Sci. U. S. A 103 (2006) 9196-9201.

[95] G.M. Barton, J.C. Kagan, R. Medzhitov, Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA, Nat. Immunol. 7 (2006) 49-56.

[96] T. Nishiya, E. Kajita, S. Miwa, A.L. DeFranco, TLR3 and TLR7 are targeted to the same intracellular compartments by distinct regulatory elements, J Biol. Chem. 280 (2005) 37107-37117.

[97] F.X. Contreras, A.M. Ernst, P. Haberkant, P. Bjorkholm, E. Lindahl, B. Gonen, C. Tischer, A. Elofsson, H.G. von, C. Thiele, R. Pepperkok, F. Wieland, B. Brugger, Molecular recognition of a single sphingolipid species by a protein's transmembrane domain, Nature 481 (2012) 525-529.

[98] R.F. Epand, A. Thomas, R. Brasseur, S.A. Vishwanathan, E. Hunter, R.M. Epand, Juxtamembrane protein segments that contribute to recruitment of cholesterol into domains, Biochemistry 45 (2006) 6105-6114.

[99] R.M. Epand, Cholesterol and the interaction of proteins with membrane domains, Prog. Lipid Res. 45 (2006) 279-294.

[100] J. Fantini, F.J. Barrantes, How cholesterol interacts with membrane proteins: an exploration of cholesterol-binding sites including CRAC, CARC, and tilted domains, Front Physiol 4 (2013) 31.

[101] T. Nishiya, E. Kajita, S. Miwa, Ligand-independent oligomerization of TLR4 regulated by a short hydrophobic region adjacent to the transmembrane domain, Biochem. Biophys. Res. Commun. 341 (2006) 1128-1134.

[102] N.F. Endres, T. Barros, A.J. Cantor, J. Kuriyan, Emerging concepts in the regulation of the EGF receptor and other receptor tyrosine kinases, Trends Biochem. Sci. 39 (2014) 437-446.

[103] I.N. Maruyama, Mechanisms of activation of receptor tyrosine kinases: monomers or dimers, Cells 3 (2014) 304-330.

[104] N.F. Endres, R. Das, A.W. Smith, A. Arkhipov, E. Kovacs, Y. Huang, J.G. Pelton, Y. Shan, D.E. Shaw, D.E. Wemmer, J.T. Groves, J. Kuriyan, Conformational coupling across the plasma membrane in activation of the EGF receptor, Cell 152 (2013) 543-556.

[105] A. Arkhipov, Y. Shan, R. Das, N.F. Endres, M.P. Eastwood, D.E. Wemmer, J. Kuriyan, D.E. Shaw, Architecture and membrane interactions of the EGF receptor, Cell 152 (2013) 557-569.

[106] A. Balbis, B.I. Posner, Compartmentalization of EGFR in cellular membranes: role of membrane rafts, J Cell Biochem. 109 (2010) 1103-1108.

[107] U. Coskun, M. Grzybek, D. Drechsel, K. Simons, Regulation of human EGF receptor by lipids, Proc. Natl. Acad. Sci. U. S. A 108 (2011) 9044-9048.

[108] E.G. Hofman, M.O. Ruonala, A.N. Bader, D. van den Heuvel, J. Voortman, R.C. Roovers, A.J. Verkleij, H.C. Gerritsen, van Bergen En Henegouwen PM, EGF induces coalescence of different lipid rafts, J Cell Sci. 121 (2008) 2519-2528.

[109] W. Shen, K. Stone, A. Jales, D. Leitenberg, S. Ladisch, Inhibition of TLR activation and up-regulation of IL-1R-associated kinase-M expression by exogenous gangliosides, J Immunol. 180 (2008) 4425-4432.

[110] A.R. Zurita, H.J. Maccioni, J.L. Daniotti, Modulation of epidermal growth factor receptor phosphorylation by endogenously expressed gangliosides, Biochem. J 355 (2001) 465-472.

[111] I. Jou, J.H. Lee, S.Y. Park, H.J. Yoon, E.H. Joe, E.J. Park, Gangliosides trigger inflammatory responses via TLR4 in brain glia, Am. J Pathol. 168 (2006) 1619-1630.

[112] W. Shen, K. Stone, A. Jales, D. Leitenberg, S. Ladisch, Inhibition of TLR activation and up-regulation of IL-1R-associated kinase-M expression by exogenous gangliosides, J Immunol. 180 (2008) 4425-4432.

[113] H.J. Yoon, S.B. Jeon, K. Suk, D.K. Choi, Y.J. Hong, E.J. Park, Contribution of TLR2 to the initiation of ganglioside-triggered inflammatory signaling, Mol Cells 25 (2008) 99-104.

[114] C. Bovijn, P. Ulrichts, A.S. De Smet, D. Catteeuw, R. Beyaert, J. Tavernier, F. Peelman, Identification of interaction sites for dimerization and adapter recruitment in Toll/interleukin-1 receptor (TIR) domain of Toll-like receptor 4, J Biol. Chem. 287 (2012) 4088-4098.

[115] M.R. Nunez, J. Wong, J.F. Westoll, H.J. Brooks, L.A. O'Neill, N.J. Gay, C.E. Bryant, T.P. Monie, A dimer of the Toll-like receptor 4 cytoplasmic domain provides a specific scaffold for the recruitment of signalling adaptor proteins, PLoS. One. 2 (2007) e788.

29

Protein	Ref. Uniprot	Residue Numbers	Transmembrane domain
TLR1	Q15399	546-645	VLEGWPDSYKCDYPESYRGTLLKDFHMSELSCNIT**LLIVTIVATMLVLAVTVTSLC**SYLDLPWYLRMVCQWTQTRRRARNIPLEELQRNLQFHAFISYSG
TLR2	O60603	554-653	VLIDWPANYLCDSPSHVRGQQVQDVRLSVSECHRT**ALVSGMCCALFLLILLTGVLC**HRFHGLWYMKMMWAWLQAKRKPRKAPSRNICYDAFVSYSERDAY ******** ***
TLR3	O15455	670-769	ELSSHYLCNTPPHYHGFPVRLFDTSSCKDSAPFEL**FFMINTSILLIFIFIVLLIHF**EGWRISFYWNVSVHRVLGFKEIDRQTEQFEYAAYIIHAYKDKDW *********
TLR4	O00206	597-696	QRQLLVEVERMECATPSDKQGMPVLSLNITCQMNK**TIIGVSVLSVLVVSVVAVLVY**KFYFHLMLLAGCIKYGRGENIYDAFVIYSSQDEDWVRNELVKNL ********* **********
TLR5	O60602	605-704	PADIYCVYPDSFSGVSLFSLSTEGCDEEEVLKSLK**FSLFIVCTVTLTLFLMTILTV**TKFRGFCFICYKTAQRLVFKDHPQGTEPDMYKYDAYLCFSSKDF
TLR6	Q9Y2C9	551-650	VLEGWPDSYKCDYPESYRGSPLKDFHMSELSCNIT**LLIVTIGATMLVLAVTVTSLC**IYLDLPWYLRMVCQWTQTRRRARNIPLEELQRNLQFHAFISYSE
TLR7	Q9NYK1	805-904	IPYLATDVTCVGPGAHKGQSVISLDLYTCELDLTN**LILFSLSISVSLFLMVMMTAS**HLYFWDVWYIYHFCKAKIKGYQRLISPDCCYDAFIVYDTKDPAV **********
TLR8	Q9NR97	793-892	VKIPRLVDVICASPGDQRGKSIVSLELTTCVSDVT**AVILFFFTFFITTMVMLAALA**HHLFYWDVWFIYNVCLAKVKGYRSLSTSQTFYDAYISYDTKDAS *********
TLR9	Q9NR96	784-883	LPSRVKCGSPGQLQGLSIFAQDLRLCLDEALSWDC**FALSLLAVALGLGVPMLHHLC**GWDLWYCFHLCLAWLPWRGRQSGRDEDALPYDAFVVFDKTQSAV
TLR10	Q9BXR5	543-642	MMVGWSDSYTCEYPLNLRGTRLKDVHLHELSCNTA**LLIVTIVVIMLVLGLAVAFCC**LHFDLPWYLRMLGQCTQTWHRVKTTQEQLKRNVRFHAFISYSE

Table 1a. The regions surrounding transmembrane domains are represented here for each human TLR. CRAC sequence defined as -[LV]-X(1,5)-Y-X(1,5)-[RK]- [100] is annotated by orange highlighted and underlined characters in the sequences while CARC corresponding to the CRAC inverted sequence i.e. -[RK]-X(1,5)-Y-X(1,5)-[LV]- [100], is represented by stars below characters and green highlighted sequence. Predicted transmembrane region is represented in dark blue bold and blue highlighted characters.

TLR4	Ref. Uniprot	Residue Numbers	Transmembrane domain
			FYFHLxLxxGC
Human	O00206	624-705	NITCQMNK**TIIGVSVLSVLVVSVVAVLVY**KFYFHLMLLAGCIKYGRGENIYDAFVIYSSQDEDWVRNELVKNLEEGVPPFQL ********* **********
Mouse	Q9QUK6	622-703	NSTCYMYK**TIISVSVVSVIVVSTVAFLIY**HFYFHLILIAGCKKYSRGESIYDAFVIYSSQNEDWVRNELVKNLEEGVPRFHL **********
Cow	Q6WCD5	625-706	NATCQLSK**TIISVSVVTVLLVSVVGVLVY**KFYFHLMLLAGCKKYGRGESTYDAFVIYSSQDEDWVRNELVKNLEEGVPPFQL ********* **********
Pig	Q68Y56	625-706	NATCQISE**AVISASVLTFLLVSVAGILVY**KFYFHLLLFVGCKKYGRGESTYDAFVIYSSQDEDWVRNELVKNLEEGVPPFHL ********* **********
Gorilla	Q8SPE8	622-703	NITCQMNK**TIIGVSVLSVLVVSVVAVLVY**KFYFHLMLLAGCIKYGRGENVYDAFVIYSSQDEDWVRNELVKNLEEGVPPFQL ********* **********
Horse	Q9MYW3	626-707	NATCQISK**TIVGGSVFSILMVSVIAVLVY**KFYFHLMLLAGCKKYGRGESIYDAFVIYSSQDEDWVRNELVKNLEEGVPPFQL ********* **********
Dog	F1PDB9	625-706	NATCQRSK**TIISVSVFTVLMVSLVAVLAY**KFYFHLMLLAGCKRYNRGESTYDAFVIYSSQDEDWVRNELVKNLEEGVPPFQL ********* **********
Cat	P58727	625-706	NATCQVRK**TIITGSVFTVLLVFLVVVLVY**KFYFHLMLLAGCKKYSRGESTYDAFVIYSSQDEDWVRNELVKNLEEGVPPFQL ********* **********

Table 1b. The regions in TLR4 from different species, surrounding transmembrane domains, are represented here. CRAC sequence defined as -[LV]-X(1,5)-Y-X(1,5)-[RK]- [100] is annotated by orange highlighted and underlined characters in the sequences while CARC corresponding to the CRAC inverted sequence i.e. -[RK]-X(1,5)-Y-X(1,5)-[LV]- [100], is represented by stars below characters and green highlighted sequence. Predicted transmembrane region is represented in dark blue bold and blue highlighted characters.

Protein	Ref. Uniprot	Residue Numbers	Transmembrane domain
TLR1	Q15399	546-645	VLEGWPDSYKCDYPESYRGTLLKDFHMSELSCNIT**LLIVTIVATMLVLAVTVTSLC**SYLDLPWYLRMVCQWTQTRRRARNIPLEELQRNLQFHAFISYSG
TLR2	O60603	554-653	VLIDWPANYLCDSPSHVRGQQVQDVRLSVSECHRT**ALVSGMCCALFLLILLTGVLC**HRFHGLWYMKMMWAWLQAKRKPRKAPSRNICYDAFVSYSERDAY
TLR3	O15455	670-769	ELSSHYLCNTPPHYHGFPVRLFDTSSCKDSAPFEL**FFMIN**<u>**TSILLIFIFIVLLIHF**</u>EGWRISFYWNVSVHRVLGFKEIDRQTEQFEYAAYIIHAYKDKDW
TLR4	O00206	597-696	QRQLLVEVERMECATPSDKQGMPVLSLNITCQMNK**TIIGVSVLSVLVVSVVAVLVY**KFYFHLMLLAGCIKYGRGENIYDAFVIYSSQDEDWVRNELVKNL
TLR5	O60602	605-704	PADIYCVYPDSFSGVSLFSLSTEGCDEEEVLKSLK**FSLFIV**<u>**CTVTLTLFLMTILTV**</u>TKFRGFCFICYKTAQRLVFKDHPQGTEPDMYKYDAYLCFSSKDF
TLR6	Q9Y2C9	551-650	VLEGWPDSYKCDYPESYRGSPLKDFHMSELSCNIT**LLIVTIGATMLVLAVTVTSLC**IYLDLPWYLRMVCQWTQTRRRARNIPLEELQRNLQFHAFISYSE
TLR7	Q9NYK1	805-904	IPYLATDVTCVGPGAHKGQSVISLDLYTCELDLTN**LILFSLSISVSLFLMVMMTAS**HLYFWDVWYIYHFCKAKIKGYQRLISPDCCYDAFIVYDTKDPAV
TLR8	Q9NR97	793-892	VKIPRLVDVICASPGDQRGKSIVSLELTTCVSDVT**AVILFFFTFFITTMVMLAALA**HHLFYWDVWFIYNVCLAKVKGYRSLSTSQTFYDAYISYDTKDAS
TLR9	Q9NR96	784-883	LPSRVKCGSPGQLQGLSIFAQDLRLCLDEALSWDC**FALSLLAVALGLGVPMLHHLC**GWDLWYCFHLCLAWLPWRGRQSGRDEDALPYDAFVVFDKTQSAV
TLR10	Q9BXR5	543-642	MMVGWSDSYTCEYPLNLRGTRLKDVHLHELSCNTA**LLIVTIVVIMLVLGLAVAFCC**LHFDLPWYLRMLGQCTQTWHRVRKTTQEQLKRNVRFHAFISYSE

Table 2. The regions surrounding transmembrane domains are represented here for each human TLR. Sphingo-specific lipid binding region is defined as -[VITL]-X-X-[VITL]-[VITL]-X-X-[VITL]-[YFW]- [97] and is represented by grey highlighted underlined characters. Predicted transmembrane region is represented in dark blue bold and blue highlighted characters.