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Review

Regulation of innate immune responses by transmembrane interactions: Lessons from the TLR family☆

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ABSTRACT

The mammalian innate immune response is responsible for the early stages of defense against invading pathogens. One of the major receptor families facilitating innate immune activation is the Toll-like receptor (TLR) family. These receptors are type 1 membrane proteins spanning the membrane with a single transmembrane domain (TMD). All TLRs form homo- and hetero-dimers within membranes and new data suggest that the single transmembrane domain of some of these receptors is involved in their dimerization and function. Newly identified TLR dimers are continuously reported but only little is known about the importance of the TMDs for their dimer assembly and signaling regulation. Uncontrolled or untimely activation of TLRs is related to a large number of pathologies ranging from cystic fibrosis to sepsis and cancer. In this review we will focus on the contribution of the TMDs of innate immune receptors – specifically TLR2–to their regulation and function. In addition, we will address the current issues remaining to be solved regarding the mechanistic insights of this regulation. This article is part of a Special Issue entitled: Membrane Structure and Function: Relevance in the Cell's Physiology, Pathology and Therapy.

Contents

1. Background

1.1. Membrane proteins and their transmembrane domains (TMDs)

Membrane proteins represent about 20–30% of the genome in a variety of different organisms. These proteins cross the cell membrane and are critical for the ability of cells to "sense" their environment, maintain their homeostasis levels of nutrients and ions, detect signals sent from other cells or organs, respond to any infiltration of foreign objects and communicate with other cells. Consequently, membrane protein defects account for diseases ranging from cystic fibrosis to cancer and possibly other pathologies as well [1–5]. The process of protein assembly is considered crucial for most proteins with respect to their function and in particular to membrane proteins. To date, it is clear that the transmembrane domain is involved in the assembly process [6]. This concept has been shown in a wide range of biological systems. For example, it has been shown that functional bacteriorhodopsin [7,8] and lactose permease [9] can be obtained from separate

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transmembrane segments. Other types of membrane proteins, such as immunological related proteins, function only as hetero-oligomers, such as the T-cell receptor [10–12] and MHC class II [13,14].

1.2. Sequences mediating transmembrane domain interactions within the membrane

Although much is known regarding the extra- and intra-cellular portions of membrane proteins, our knowledge of the factors that control protein–protein interactions and recognition of the membrane-embedded domains is still limited. Studies in recent years using computational methods and other cutting edge techniques revealed that TMDs have a role in protein assembly and function [15–18]. Furthermore, in several cases it was shown that TMDs also have further roles in the activation and regulation of their corresponding membrane proteins. However, despite the increase in knowledge regarding the assembly of TMDs, the precise functions and motifs driving TMD assembly are still largely unknown. In general terms, as proteins are inserted into the membrane and their secondary structure is created, tertiary interactions are created between TMD helical fragments. This process is largely attributed to the maximization of Van der Waals contacts through matching knobs-into-holes type interactions. Several motifs mediating a noncovalent association of native TMDs were recognized [19–21], the most common is the GxxxG motif originally discovered in the TMD of Glycophorin A [22–24]. This motif has a significant influence on the ability of membrane proteins to self-assemble [25]. Studies have shown that disrupting this motif by point mutations of the Glycines or enlarging the distance between them lowers the ability of the proteins to form proper dimers [26]. Other transmembrane motifs have been found [27–31]: each related to a certain type of TMD and has its own significance for the assembly and functional processes. These include: (i) the Ser-Thr rich domain, (ii) a helix association domain which was described to be significant in the HCV replication complex, (iii) the polar-xx-polar motif where polar include Ser, Thr, Asn, Asp, Glu, and Gln, (iv) Gly zipper, and (v) aromatic-xx-aromatic motif [31–34]. Additionally, leucine zippers and polar residues expressed in TMDs were shown to be significant for protein function [35,36]. Nevertheless, there are probably other, yet unknown, motifs which control interactions between TMDs.

1.3. The importance of TM–TM interactions for the function of a protein

Receptor assembly has been shown to mediate activity in a variety of signal transduction cascades. One of the most investigated examples is the family of ErbB growth factor receptors (reviewed in [37,38]). This family of receptor tyrosine kinases consists of four members that through their combinatorial association are able to recognize a wide array of ligands [39,40]. Although specificity of binding is mainly driven by recognition through the extracellular regions, it was also postulated that the TM domains of these proteins are able to self-associate, thus influencing biological activity [41–43]. Indeed, in an influential work by Mendrola et al., it was shown that the TMDs of these receptors can self-associate where the association was the strongest for ErbB4 and the weakest for ErbB3 [19]. Further analysis of these interactions revealed that this interaction is mediated by several GxxxG motifs and that their role is in both hetero- and homo-dimerization. While it was established that the GxxxG in the C terminus TMD of ErbB1 and ErbB2 was involved in homo-dimerization, Gerber et al. showed that another GxxxG motif located at the N′ terminus of the TMD is involved in hetero-assembly between ErbB1 and ErbB2 [44]. Thus, it is proposed that these regions allow for fine tuning of these receptors in response to different ligands.

Integrins are a family of membrane receptors that mediate cell adhesion and their TMDs were shown to be directly involved in activation of these molecules [45–47]. A seminal study by Li et al. showed that their TMDs are capable of forming oligomers in SDS PAGE [45] and it was proposed that these interactions are based on the GxxxG motifs present within these regions. Indeed, mutational analysis revealed that the interaction between the TMDs of the integrins αIIB and β3 is GxxxG dependent, and is sensitive to other residues as well. Thus, it was proposed that the GxxxG combined with other flanking residues allow for subtle conformational changes within this region that ultimately affect stability [45]. In a different study, Yin et al. showed that an exogenous peptide that corresponds to the TMD of the integrin αIIb is able to activate these integrins in vitro [48]. The model proposed for integrin activity suggests that in their resting state, the heterodimer TMDs are packed closely together, while in their activated state the heterodimer TMDs dissociated and there is a greater tendency towards homodimer formation. These examples highlight the important role of the TMD in regulating as well as executing the precise outcome in various receptor families.

In addition to the importance of the direct stable association of TMDs, many TMDs enter dynamic associations which are essential for protein function. In the past several years studies have shown that in order for receptors to properly initiate signal transduction, TMD movements are pivotal [49,50]. These movements frequently transmit an extracellular domain ligand-binding event across the bilayer to intracellular domains, thus activating a variety of signaling cascades. Generally, four main potential movements have been proposed to occur in order for a signal to pass through the membrane via a TMD (reviewed in [51,52]). (1) Transmembrane helices can move in the membrane plane to stabilize transient interactions (translational motion). (2) Individual transmembrane helices can move in a piston motion through the lipid bilayer (perpendicular to the membrane). (3) Pivot movement in which transverse helix movements result in tilting of individual helices or in changes in the tilt angle (along an axis parallel to the membrane). (4) Rotation of the helices along an axis perpendicular to the membrane. All of these motions result in a reorientation of intra- and extracellular domains. Consequently, inaccurate localization of the TMD to a less permissive site within the membrane may interfere with these dynamic movements and lead to impairment of receptor activation. In addition to their contribution to signal transduction transmembrane domain segments were shown to directly associate with specific membrane phospholipids. For example, G coupled protein receptors (GPCRs) display a clear preference for PE [53,54]. This specific localization is apparently favorable to protein functionality as described in the case of rhodopsin [53,54].

2. Toll-like receptor (TLR) structure, function and regulation

The Toll protein family was first discovered as a developmental related receptor in **Drosophila** [55]. Since then, homologs of these receptors have been identified in most eukaryotes with the main difference being in the number of proteins in the family [56]. In mammals these receptors play a pivotal role in the innate immune system both as activators of cascades leading to immediate responses, and as linkers for the recruitment and activation of the adaptive immune system (Fig. 1) [57–60]. Furthermore, in recent years, studies proposed that in mammals, TLRs play a significant role in development as well [61–63].

As humans have a small number of innate immune receptors available to respond to an unlimited number of microbial molecules, these receptors must have extensive flexibility [64]. The current dogma for TLR-ligand recognition, that was laid by the late Charles Janeway almost two decades ago, is that they recognize common patterns and are thus referred to as pattern recognition receptors [65,66]. To date, 10 TLRs have been reported in humans and 13 in mice. They are involved in the recognition of multiple groups of microbial molecules that usually are not found in humans, as well as several endogenous ligands termed DAMPs (damage-associated molecular pattern molecules) [67]. The spectrum of TLR ligands is unusually broad for a single family of proteins; they can form stable complexes with molecules ranging from hydrophilic nucleic acids to hydrophobic lipids, which vary in size from small-molecule drugs to large

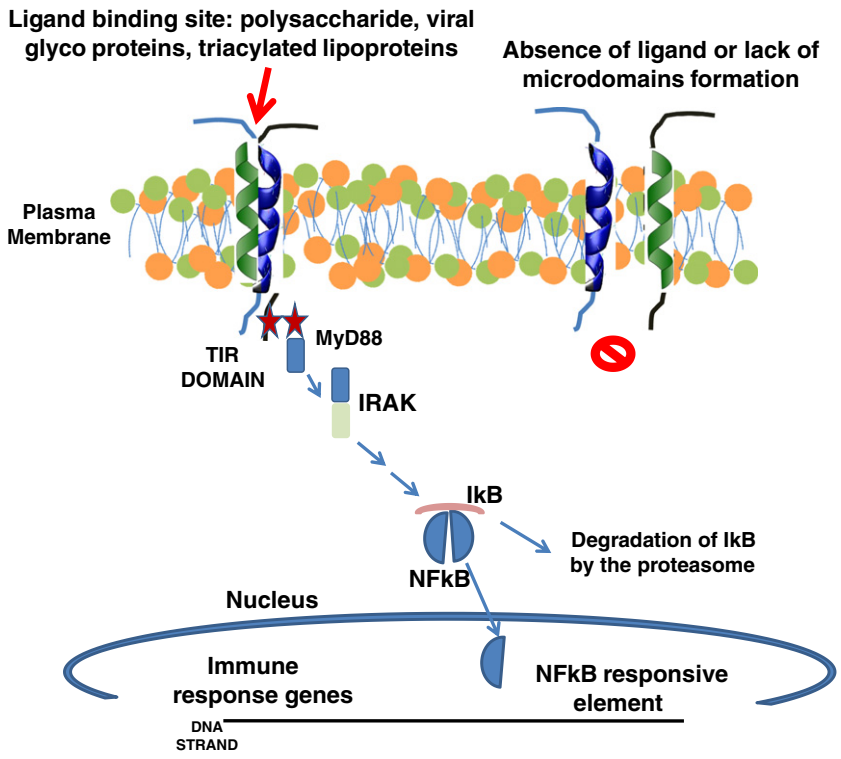

Fig. 1. A brief description of TLR activation and signaling. Upon ligand binding and the formation of a sustainable dimer, TLRs interact with an adaptor protein (eg. MyD88) through their TIR domain (marked as a star). This leads to the binding of IRAK and additional MAP kinases resulting in the translocation of NFkB into the nucleus (together with additional transcription factors). These transcription factors initiate the expression of innate immune response genes such as IL-6, IL-8 TNFα, and other cytokines and chemokines. If membrane microdomains are depleted, proper dimerization is inhibited and the signaling cascade is aborted [57,58].

macromolecules. For example, TLR2 forms heterodimeric complexes with either TLR1 or TLR6 that can bind to lipid conjugates as well as lipopeptides or lipoproteins from bacterial membranes [68]. Uncontrolled activation of TLRs is involved in many infectious and non-infectious diseases and may have fatal outcomes. Thus, TLRs are important targets for a number of immune regulating therapeutics [69]. Recent studies have shown that TLRs, in particular TLR2 and TLR4, are activated in tissues affected by chronic inflammatory diseases, suggesting that they play a role in these pathogenic processes [70].

Due to their involvement in such essential processes, TLRs activity is highly regulated. This regulation is multilayered and involves systemic regulation, signaling regulation and protein–protein interactions, all serving to keep TLR activity well balanced [71–74]. In general, these examples signify the delicate and fine tuning needed for a specific and well balanced response. Notably, another mode of regulation of TLR activity is achieved through specific TLR homo- or hetero-dimers. While some TLRs such as TLR3 and TLR5 are active as homodimers, others such as TLR2 and TLR1/6 work as heterodimers. Recently, TLR4, that was known to be active as a homodimer, was shown to form functional heterodimers with TLR6. These heterodimers are capable of recognizing altered self-molecules such as oxidized low density lipoproteins LDL and aβ amyloid peptides [75]. In addition, a recent study described the interaction between TLR4 and TLR2 upon recognition of the HIV-1 gp120 protein [76]. These intriguing findings raise the possibility that other yet unidentified functional pairs of heterodimers exist, thus creating additional layers of TLR regulation. These findings also indicate that substances which can modulate TLR dimer assembly might be of major importance for regulating TLR activity.

TLRs form homotypic or heterotypic multimers at the plasma membrane, without bound ligands [77,78]. According to current models, these multimers are weakly bound to each other and their TIR domains (Toll/interleukin 1 receptor homology domains; the intracellular sites that the signaling molecules associate with) are relatively far apart. Upon ligand binding, the association between the receptors becomes stronger, bringing their TIR domains closer together, serving as a platform for the initiation of downstream signaling (Fig. 1) [79]. Several studies further shed light on the specificity of the different heterodimers towards their ligands [79,80]. A number of amino acids within the interface between these proteins form hydrophobic and ionic interactions that are important for the stability of the receptor–ligand complex. In addition, recent mutagenesis assays revealed critical amino acids in the cytoplasmic TIR domain that are important for the TLR signaling [81–83]. Despite extensive work focusing on the extracellular and cytoplasmic domains of the different TLRs, only very few studies have focused on the transmembrane region of these proteins. Consequently, this region has been left as a 'black box' waiting to be explored.

3. TLRs activation and signaling

TLRs are reported to be activated within lipid microdomains [78,84,85]. Upon their activation TLRs actively translocate to specific lipid locations within the lipid bilayer [84]. This phenomena represents perhaps a more general theme as it has been shown for other receptors as well [86]. Although the precise mechanism driving TLR accumulation at these lipid microdomains is currently unknown, it is highly reasonable that the TMD sequence and/or the sequences in its closest vicinity, which may also interact with the lipid bilayer, are crucial for these localization events. One example of such a localization signal is the proposed Cholesterol Recognition Amino-Acid Consensus (CRAC) sequence [87–90]. This sequence has been implicated to strongly correlate with the appearance of proteins within distinct membrane microdomains. One classical example is the gp41 of HIV-1 which expresses an LWYIK motif immediately before the TMD. It was described to

depend on cholesterol for its embedment into the membrane as well as to redistribute cholesterol [91]. Furthermore it was shown that mutations in these motifs lead to impairment of the parental proteins' function [89,92,93]. An additional possibility of membrane related regulation of TLR activity rises from the findings of the effect of gangliosides (a specific type of glycospingolipid) on regulation of immune responses. Although to date no studies reported the direct regulation of TLRs by gangliosides, other receptors which function in similar manners (e.g. Insulin receptor and Epidermal Growth Factor receptor) were reported to be regulated by gangliosides by controlling the passage of the extracellular signal through the membrane [94–97].

Since uncontrolled TLR signaling may result in devastating outcomes such as autoimmunity, TLR activation is negatively regulated at the receptor, adaptor, transcription and post-transcriptional levels. For example; the TIR domain of TLR8, a TLR member important in gut homeostasis, intestinal inflammation and colitis-associated tumorogenesis, is required for attenuation of the recruitment of the MyDsome (the complex of Myd88-IRAK4-IRAK2 DD — the platform of the TLR signaling cascade) to the receptor [98]. In all TLR pathways, sophisticated modes of regulation were described to allow for the tight management of the immune response.

4. TLRs in pathology

TLRs are implicated in a number of pathologies. For example, their impact on cancer progression has been widely described [99–101]. They affect the tumor microenvironment in various means as well as affecting the growth patterns of malignant cells. Recent studies showed that in inflammatory bowel disease TLR signaling may on the one hand reduce the severity, while at other time points, may worsen the outcome [102–104]. Emerging studies have indicated the involvement of TLR activation in Alzheimer disease [105–107]. Activation of brain residential microglia as well as infiltrating macrophages induces an immune response in the brain that under certain conditions leads to neuronal cell death. An additional pathology associated with an unregulated immune response is rheumatoid arthritis (RA) [108–111]. In RA the exact mechanism of disease progression is currently not fully known. Nevertheless, it is referred to as an autoimmune disease and it is tightly related to uncontrolled inflammation in the joints. As TLRs are key participants in the initiation of an immune response they are currently under investigation for their participation in RA.

5. The TMD of TLR2 regulates its assembly and function

As such large systemic regulatory networks are being revealed, biochemical and molecular biology approaches are indispensable for understanding the more subtle regulation of these receptors at the protein level. Such examples include regulation by phosphorylation, heterodimerization, recruitment into lipid microdomains and altered trafficking [78,83,112]. Hetero-dimerization with different partners was shown to be extremely important in the case of TLR2 and TLR1/6. Specific interactions of these molecules resulted in recognition of different microbial components. Although TLR4 is known to homodimerize for recognition of LPS [113], a recent article by Stewart et al. has shown a functional heterodimer of TLR4 and TLR6 that is able to recognize altered self-ligands [75]. These ligands are added to the growing list of endogenous ligands that are recognized by TLRs and to the complex layers of regulation for these proteins.

Recent studies provide evidence that the TMDs of TLR2 and 6 are important for the activation and regulation of these proteins and to the nature of the TLR's assembly. The TMDs of TLR2 and TLR6 interact one with each other and have a preference for heterodimers rather than homodimers [114]. It was demonstrated that the TMD of TLR2 also contributes to the specificity of the interactions generated in the presence of a certain ligand. For example, when expressed in the ToxR system, the TLR2 TMD does not interact with the TMDs of TLR4 and TLR5 but only with TLR1/6 and TLR10 [114] (Fig. 2). However, in the presence of a non-canonical activator such as ethanol, TLR2 and TLR4 physically associate and induce an inflammatory response in neuronal cells [115]. Therefore, in response to a specific ligand it is possible that specific conformational changes will occur, which lead to the generation of a specific functional dimer. Recent studies indicate that, at least in part, the TMD participates in this regulatory state [116]. Therefore it was hypothesized that interrupting with TMD associations may lead to impaired responses. In a recent study by Fink et al., synthetic peptides derived from the TMDs of TLR2/6 were added to compete with the native interactions [116]. Indeed, the peptides showed strong association with the TMDs of the native proteins and they further showed high potent inhibition of the in vitro activation of TLR2/6 in a ligand specific manner. This was demonstrated by ELISA of two major cytokines secreted by activated macrophages; IL-6 and TNFα. The inhibition was only achieved upon activation with LTA/PamCysK but not LPS. Through mutational analysis the significance of the exact TMD sequence to drive this interaction was demonstrated; these studies insinuate that the TM domains are important for the hetero-assembly of this complex and that this assembly is a target for interference. A current mode of action for these peptides is shown in Fig. 3. This hypothesis is based on the findings suggesting that the peptides prefer hetero-rather than homo-assembly, as well the fact that they preferentially bind to their reciprocal receptors (TLR2 TMD to TLR6p and TLR6 TMD to TLR2p). In addition, the indications that these TMDs are capable of interacting in the context of a biological membrane within the ToxR reporter system further emphasize this concept.

Several other findings contribute to the notion that these regions functionally participate in TLR activity. A recent study showed that a single nucleotide polymorphism in the TMD of hTLR1 (I602S) correlates with the modified immune response to tri-acylated lipopeptides, insinuating that this region might be involved in the regulation or activation of the TLR1/2 complex [117]. In addition, this SNP was also shown to be associated with Crohn's disease, a pathology that leads to constant chronic inflammation in the ileum [118]. More evidence for the role of the TMDs in TLR activation can be found in a study showing that the hydrophobic domain surrounding the TMD of TLR4 is able to affect the dimerization state of this protein [119]. The corresponding region in TLR2/6 is included in the TLR synthetic peptides previously described, strengthening the idea that the peptides are involved in the dimerization of these receptors. Other studies have shown that the TMD of TLR9 is involved in the trafficking of this receptor [120]. Interestingly, TLR2 contains a CRAC homologous domain [87] within its TM domain, suggesting that the TMD of TLR2 might be involved in recruitment in lipid microdomains (Reuven EM and Shai Y, unpublished data). These assumptions are supported by the studies showing that TLRs are differentially targeted into specific microdomains upon activation [78]. Taken together; all the studies mentioned above strongly support the concept that these previously uncharacterized TM regions of TLR6 and TLR2 are involved in the activation or regulation of the proteins.

6. TLR2 regulation by TLR TMD peptides *in vivo*

TLR regulation is highly critical for the maintenance of body homeostasis. This can be reflected by the fact that over-activation of TLR4 by LPS injection can cause septic shock and death, which is in part attributed to high TNF levels present in the blood stream. Currently, the only available drug to treat severe bacterial sepsis shows only a modest improvement in patient outcome (6% absolute increase in survival) and general use is limited due to severe and lethal side effects [121,122]. As death due to sepsis from bacterial infections is becoming a major health concern, a very recent study [116] provides initial evidence that inhibiting TLR2 in vivo by TMD peptides is a promising way to combat sepsis. For example, when sepsis was induced in mice by pure LTA injection, as well as, by heat killed gram positive bacteria, all the untreated mice died whereas 62.5% and 40%, respectively, survival was found when

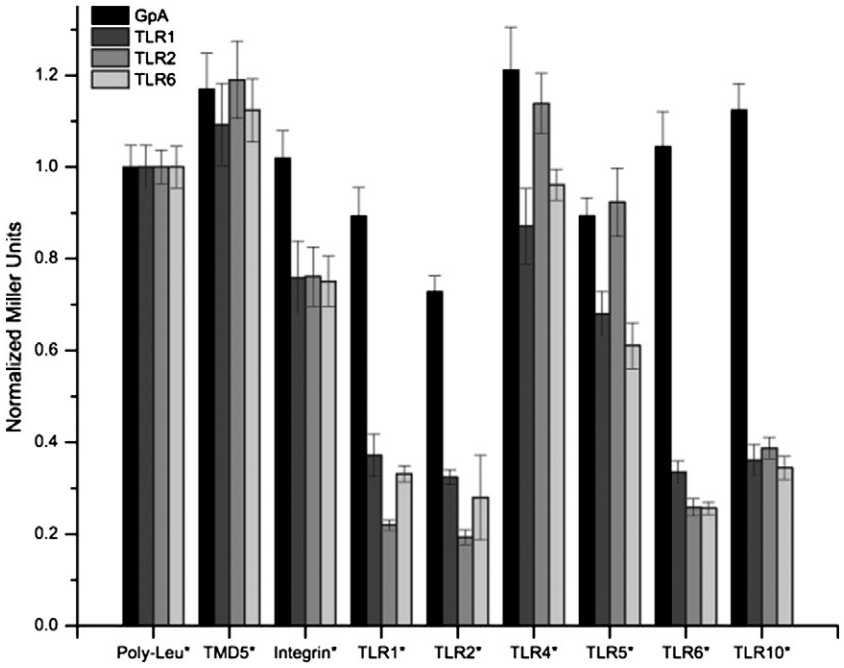

Fig. 2. Cell-surface Toll-like receptor heterotypic interactions. A dominant-negative ToxR assay was used to study heterotypic interactions of the cell-surface TLRs. Two TLR TMDs were encoded in the FHK12 *E. coli* reporter strain, one with a functional ToxR domain (dominant phenotype) and one with a nonfunctional ToxR* domain (negative phenotype). Interaction between the two different TMDs leads to a reduction in signal from that seen for homotypic interactions. The TMDs for GpA, TLR1, TLR2, and TLR6 were used with the functional ToxR domain while TMDs for poly-Leucine, TMD5, integrin allb, TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 were used with the nonfunctional ToxR* domain. Interactions were most prominent for the TLRs known to have heterotypic interactions: TLR2–TLR1 and TLR2–TLR6. TLR10 also showed strong interactions with TLR2. Moderate interaction was seen with other TMDs that could be attributed to non-specific interactions from similar TMD motifs as completely unrelated receptors showed similar levels of knockdown. Each dominant phenotype was done in 3 technical replicates with each negative phenotype and 6 measurements made for each replicate. Error bars depict the standard error of the mean.
Figure taken with permission from Ref. [114].

the mice were treated with the TMD of TLR2 [116]. Furthermore, the peptide also showed some protective effect when injected one hour earlier to the LTA challenge. These results strengthen the idea that TLR2 TMD peptides can target and inhibit TLR2 activation, and can be useful as a platform for further therapeutic research. Interestingly, this peptide did not seem to affect TLR4 driven septic shock. This result is clearly in line with in vitro results and strengthens the idea that the inhibition exerted by these peptides is specific for TLR2 [116]. Combined with other studies showing that TLR2 down regulation was effective in controlling polymicrobial sepsis [123], these findings should lead to additional opportunities to investigate TLR participation in other pathologies where unregulated activation of TLRs in implicated.

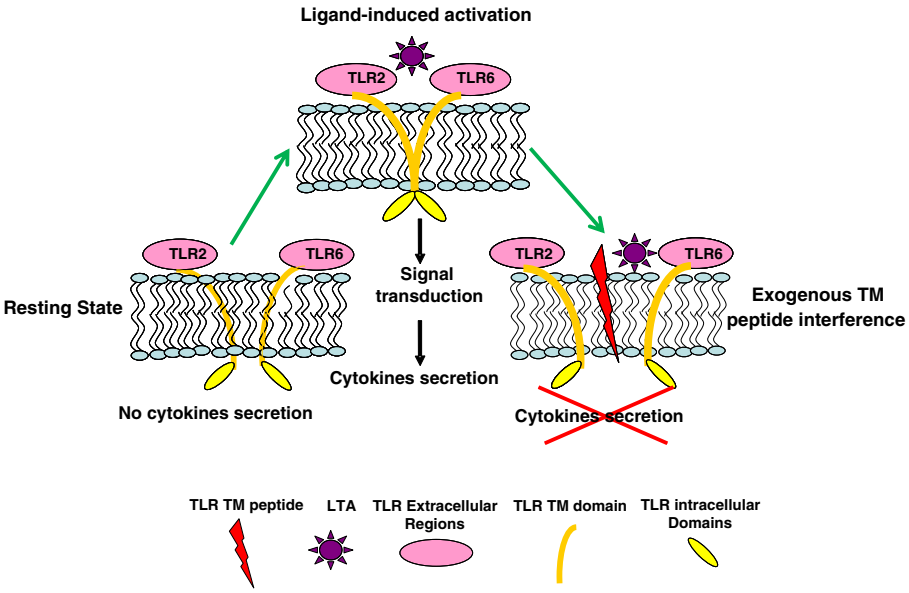

Fig. 3. A proposed mode of action of TMD derived peptides inhibiting TLR activation. TLR molecules latterly diffuse through the membrane and generate temporal non-activating interactions. Upon ligand binding, structural changes occur, including within the TMD of the receptor, leading to exposure of phosphorylation sites and eventually inducing cytokine secretion. TMD peptides interact with their reciprocal TMD of the intact protein and impair proper dimer formation resulting in inhibition of the signal generated and cytokines secreted.

7. Remaining questions regarding the mechanisms of TMD regulation

Although our understanding of the importance of TMDs to their parental protein function has advanced greatly, still major issues are awaiting further investigation. One of the interesting questions is to decipher the precise driving forces of TLR TMDs' interactions, as well as, the exact dynamic motion occurring while the various TLR combinations are generated. It is highly probable that in any given TLR dimer (homo/hetro-dimer) diverse forces induce TM–TM stabilization, as well as, different motions that allow distinct interactions. Additionally, the mutual effects on the TMD motions induced by specific lipid compositions within specific microdomains necessary for TLR signaling, will shed much light on the complete means of regulation of this important family of receptors.

Particularly in the case of the TLR2 TMD another intriguing question is what is the significance of the TMD embedded Cys residues. Using the approach of peptide interference with mutated TMDs, it was found that the cysteines within the TMDs might play a significant role in the activation and regulation of these receptors (unpublished data). While some Cys residues such as Cys606 on TLR6 did not seem to affect peptide activity or in vitro dimerization, Cys609 on TLR2 seemed to enhance peptide activity. Finally, the two adjacent cysteine residues on TLR2 (Cys595 and Cys596) seem to negatively affect the peptide's activity and TM dimerization, most probably due to conformational change resulting in a β sheet structure. It is hypothesized that the cysteines in this region are sensitive to the redox potential of the membrane and that these cysteines might form a molecular switch that prevents prolonged activation under oxidative conditions. In support of this idea, TLR2 has been recently shown to be sensitive to the oxidized lipid product ω-(2-carboxyethyl) pyrrole (CEP), thus relating TLR2 activation to oxidative burst in macrophages [124]. It can be speculated that an oxidative environment can trigger activation through Cys609 on TLR2 while the double Cys on TLR2 can prevent hyper-activation under a highly oxidized environment, as can be seen in highly active phagocytosis. Although at this stage these assumptions are highly speculative, another recent study from our lab might provide some indirect evidence for these results. In this work Ashkenazi et al. have provided evidence for the involvement of Cys residues within the HIV fusion protein GP41 in the fusion process [125]. These residues were neglected so far and considered to have no effect on protein activity. Nevertheless, careful examination of these residues under reduced or oxidized peptides provided evidence for their involvement in the fusion process, mainly in the hemifusion stage [125]. Since these residues were routinely mutated to alanines within inhibitory peptides derived from this region, the actual role of these residues was overlooked. Macrophages transfected with specific plasmids with and without these cysteines or in different combinations will help to provide some more information about these intriguing findings. Supplementary biophysical analysis will help to elucidate the involvement of these residues on TM–TM interactions.

Of note, the therapeutic potential of TMD derived synthetic peptides targeting the TMDs of membrane receptors is very promising. Future studies are ongoing to evaluate the complete range of pathologies such peptides may affect and the means to improve their targeting and specificity in-vivo. They are expected to open new strategies to fight various diseases.

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