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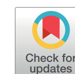
Review

Multiscale modeling of innate immune receptors: Endotoxin recognition and regulation by host defense peptides



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ABSTRACT

The innate immune system provides a first line of defense against foreign microorganisms, and is typified by the Toll-like receptor (TLR) family. TLR4 is of particular interest, since over-stimulation of its pathway by excess lipopolysaccharide (LPS) molecules from the outer membranes of Gram-negative bacteria can result in sepsis, which causes millions of deaths each year. In this review, we outline our use of molecular simulation approaches to gain a better understanding of the determinants of LPS recognition, towards the search for novel immunotherapeutics. We first describe how atomic-resolution simulations have enabled us to elucidate the regulatory conformational changes in TLR4 associated with different LPS analogues, and hence a means to rationalize experimental structure-activity data. Furthermore, multiscale modelling strategies have provided a detailed description of the thermodynamics and intermediate structures associated with the entire TLR4 relay – which consists of a number of transient receptor/coreceptor complexes – allowing us trace the pathway of LPS transfer from bacterial membranes to the terminal receptor complex at the plasma membrane surface. Finally, we describe our efforts to leverage these computational models, in order to elucidate previously undisclosed anti-inflammatory mechanisms of endogenous host-defense peptides found in wounds. Collectively, this work represents a promising avenue for the development of novel anti-septic treatments, inspired by nature's innate defense strategies.

1. Innate immunity and toll-like receptors (TLRs)

The mammalian body is constantly exposed to foreign microorganisms, which all need to be controlled in order to avoid invasive infections and sepsis. The innate immune system represents a first line of defense, providing a rapid response to potential pathogens. The Toll-like receptors (TLRs), of which ten functional members exist within the human genome, are prominent members of this host defense system. Their dysregulation has been linked to numerous disease states, making them important potential therapeutic targets [1]. TLRs recognize evolutionarily conserved molecular motifs referred to as pathogen-associated molecular patterns (PAMPs), a group encompassing a broad spectrum of biomolecules [2,3]. For example, at the cell surface TLR2 can form heterodimeric complexes with either TLR1 or TLR6 to recognize lipopeptides and lipoproteins, whilst homodimeric TLR5 can be activated by bacterial flagellin. Intracellularly, TLR3, TLR7, TLR8, and TLR9 bind nucleic acids derived of various microbial origins. TLR4 recognizes a complex glycolipid from Gram-negative bacterial species termed lipopolysaccharide (LPS) [4], and is of particular biomedical interest given its over-exposure to LPS during serious infections to LPS can lead to sepsis [1].

The N-terminal ectodomain of each TLR is responsible for ligand recognition, and is connected to a cytoplasmic Toll/Interleukin-1 receptor (TIR) domain via a single transmembrane helix. The ectodomain is highly conserved across the TLR family, and is composed of rigid leucine rich repeats (LRRs) that yield curved, solenoidal structures. The typical LRR is ~20-30 residues in length and contains the LxxLxLxxN motif [2,5,6]. The resultant solenoids are composed of parallel β-strands, stabilized on the convex side of the structure by leucines forming the hydrophobic core and asparagines forming a continuous

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backbone hydrogen-bonding network. In typical LRR proteins, the “x” residues form variable structures on the concave, solvent-exposed surface, and often play roles in binding of ligands. In contrast, in TLRs, the convex regions are involved in ligand recognition, and many of the LRR units deviate from the canonical pattern, with unusual structural elements [3,7,8]. Productive PAMP binding by the TLR ectodomain leads to receptor dimerization, resulting in a characteristic “m-shaped” complex with both C-termini at the center. This results in juxtaposition of the two TIR domains, which are then proposed to serve as a scaffold for recruitment of adaptor proteins, leading to downstream activation of transcription factors such as NF-κB and induction of inflammatory cytokines [2,7,9,10].

2. The TLR4 pathway and Sepsis

TLR4 is the archetypal innate immune receptor, and is of particular biomedical interest as a target for anti-inflammatory drugs for the treatment of numerous diseases [1]. Its PAMP, LPS, is a complex glycolipid derived from the Gram-negative bacterial outer membrane, in which it serves a protective role and restricts antibiotic influx. The main bioactive component of LPS is lipid A, which contains multiple acyl tails and a phosphorylated, β(1→6)-linked diglucosamine headgroup, and anchors the molecule to the external leaflet of the membrane. TLR4 does not function in isolation, likely because the hydrophobic lipid A component of LPS strongly disfavors exit from membranous or aggregate phases, thereby hindering exposure for ligand recognition. LPS is first complexed by LPS binding protein (LBP) in serum [11], which serves to increase the effective sensitivity of the host response, though it may not be absolutely essential for stimulation of cells in vivo [12–14]. LPS is next transferred to a glycosyl-phosphatidylinositol (GPI) anchored “adaptor protein”, cluster of differentiation 14 (CD14) [15], which is also composed of LRRs but has no intrinsic signaling capacity [16–18]. CD14 transfers LPS to TLR4 as part of a complex [19–21] with the co-receptor myeloid differentiation factor 2 (MD-2) [22,23], which adopts an immunoblogulin-like fold containing a hydrophobic interior specialized for binding lipid acyl tails.

Tiny amounts of LPS are required to stimulate TLR4, making it uniquely sensitive to potential invading pathogens [24]. Conversely, over-stimulation of TLR4 can be highly deleterious, since systemic inflammation can lead to sepsis and septic shock. It has been estimated that around 6 million deaths result from sepsis worldwide [25], and it remains a primary killer in intensive care units, worsened by the ever-growing antimicrobial resistance crisis. Subtle variations in the chemical structure of lipid A can have a major impact upon the response [24]; hexa-acylated lipid A from *E. coli* is a potent TLR4 agonist, but alterations in the nature of the acylation pattern results in antagonism, as exemplified by its biosynthetic intermediate lipid IVa or the non-toxic lipid A from *R. sphaeroides* [26]. Nevertheless, while numerous studies have supported the concept of TLR4-targeted anti-septic therapy based on lipid A analogues [27–29], none have yet been clinically approved.

3. A computational approach to immune function & inhibition

A better molecular-level understanding of the determinants of LPS transfer and recognition within the TLR4 pathway would facilitate the search for novel immunotherapeutics. To this end, molecular dynamics (MD) simulations provide a classical physics-based framework to generate realistic “movies” of biomolecules in unparalleled spatial and temporal resolution [30,31]. In recent years, simulations have provided a means to extend our knowledge from experimentally derived static “snapshots” of innate immune receptors [16,17,20,21,32] to the dynamic regime, as reported by our group [33–43,78] and others [44–48]. As described below, such an approach has enabled us to elucidate the regulatory conformational changes associated with binding of LPS ligands, and hence molecular rationalization of diverse structure-activity

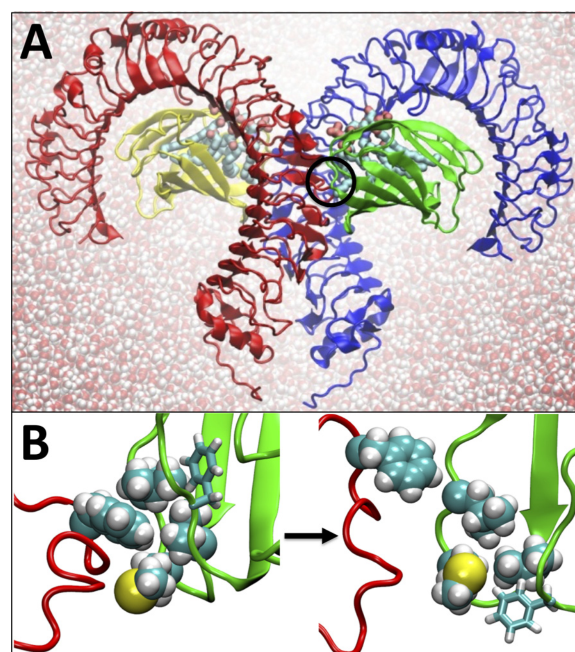

Fig. 1. Atomic-resolution simulations of the $(TLR4/MD\text{-}2)_2$ complex. (A) Snapshot of the receptor complex bound to *E. coli* lipid A agonist. Protein is shown in cartoons format including the two large, solenoidal TLR4 chains and the two small, lipid-bound MD-2 chains. Lipid is shown in CPK wireframe format, and bulk water molecules are shown as van der Waal’s spheres. The region of one the gating loop regions containing F126 is highlighted by a black circle. **(B)** Conformational changes associated with ligand-induced gating. The active state of the complex is maintained in the presence of agonist (left), but in the absence of agonist disassembly occurs as a result of reorientation of F126 (right). Protein chains are colored as in (A), whilst F126 (wireframe) and surrounding hydrophobic residues (van der Waal’s spheres) are shown in CPK format.

data [33–38]. Additionally, coarse-grained (CG) modelling [49,50], in which sets of atoms are grouped together into larger particles to simplify the system of interest, yields orders-of-magnitude increases in the accessible scales that may be simulated, thereby enabling us to study *in silico* the membrane assembly processes of higher-order immune complexes [39,40]. Furthermore, we have begun to leverage this “multiscale” description of the TLR4 pathway and its components, towards the discovery of previously undisclosed antibacterial and antiseptic mechanisms of host-defense peptides (HDPs) that occur naturally during wound healing [41,34–43].

4. Atomic-resolution simulations unravel the mechanisms of TLR4 signaling

To explore the molecular mechanisms by which TLR4 may distinguish between agonistic versus antagonistic ligands, we performed atomic-resolution simulations in the presence of a range of lipid A analogues [33]. In particular, we initiated simulations from the crystallographically observed “active” state of the complex [21], composed of a $(TLR4/MD\text{-}2)_2$ “dimer of dimers” (Fig. 1A). The simulations revealed that the β-cup of MD-2 (like that of related lipid-binding proteins [34]) undergoes “clamshell-like” motions as a result of adaptation to the size and shape of the bound ligand. These motions are coupled to conformational changes in a loop containing a key phenylalanine residue, F126. Consistent with the X-ray structure [21], the sidechain of F126 remained pointing into the MD-2 hydrophobic cavity to interact with the lipid acyl tails during simulations in the presence of agonistic, hexa-acylated *E. coli* lipid A (Fig. 1B, left). In contrast, when bound to under-acylated lipid A analogues, or in the ligand-free state, the F126

loop was consistently observed to reorient, thereby disrupting a hydrophobic cluster at the dimeric interface formed with the carboxy-terminal LRRs (modules ˜15-17) (Fig. 1B, right) which is expected to deactivate the complex. Thus, F126 of MD-2 is proposed to act as a molecular switch in determining TLR4 activation; this is consistent with an F126A mutant which can bind lipids but interferes with signaling [20,21,32], and with NMR measurements of F126 loop dynamics during ligand binding [51].

This work has since been extended, to rationalize the thermodynamic basis for the capacity of the F126 loop to distinguish between different lipid A analogues [35], and also to explore possible larger-scale dynamics in the receptor complex associated with ligand binding [37,38]. In particular, the global collective motions of the active complex were analyzed to investigate how ligand induced conformational changes might be transmitted to the intracellular TIR domains. In the agonistic lipid A bound state, only subtle rotational motions of the ectodomains were observed with respect to one another, suggesting the TIR domains would remain closely apposed. In contrast, in the absence of ligand [37], or in the presence of non-activating free fatty acids [38], significant relative fluctuations of the C-termini were observed, leading to the prediction that TIR domains would become separated and hence block downstream signaling, analogously to the crystallographic measurements made for TLR8 upon ligand unbinding [52]. Combined with single-molecule imaging experiments, our data collectively suggest a two-step model for TLR4 activation, in which agonistic LPS binding triggers conformational changes that favor juxtaposition of TIR domains, forming a nucleating platform for MyDDosome assembly [37].

5. Multiscale simulations trace the pathway to LPS recognition

With the knowledge that TLR4 functions efficiently only as part of a complex relay of receptors and co-receptors, we embarked upon a multiscale modelling study with the aim of tracing the passage of a single LPS molecule from its native bacterial envelope environment to the terminal receptor complex at the plasma membrane surface [40]. This was rooted in the use of the ubiquitous Martini CG forcefield [53,54], which maps approximately four heavy atoms into a single particle, thereby simplifying the description of the system. CG parameters were carefully derived for LPS, GPI-anchored CD14, MD-2, and TLR4 embedded within a mammalian membrane model via a transmembrane helix. Importantly, the dynamics of each component protein in CG resolution were calibrated against their atomistic counterparts, to ensure accurate reproduction of the membrane-association and ligand binding behavior known to be mechanistically important [33,39]. By utilizing “enhanced sampling” simulation methodologies to estimate the equilibria between ligand-bound and free states [55], we were able to rigorously calculate the binding energy of LPS to each receptor and coreceptor in both CG [40] and atomistic detail [35,42]. Based on these calculations, LPS molecules traversing the receptor cascade were confirmed to fall into a “thermodynamic funnel”, such that the large energetic penalty for extraction of LPS from the bacterial membrane or from lipid aggregates is overcome by the favorable affinity gradient presented by CD14 and the terminal receptor complex (Fig. 2).

Multiscale modelling of the TLR4 relay also enabled elucidation of the previously undisclosed structure of the CD14 ligand bound state [16,17,56,57], and confirmed that its hydrophobic cavity is highly malleable [58,59], helping to explain its promiscuity for recognizing multiple PAMPs. Extended simulations revealed that the GPI-anchored CD14 ectodomain spontaneously adopts tilted orientations such that its binding pocket may “dock” with that of TLR4-bound MD-2, with the carboxy-terminal LRRs 13–15 of TLR4 primarily responsible for the physical interaction with CD14 [40], consistent with single-molecule studies [60]. The exchange of LPS between the two cavities was consequently shown to proceed via a contiguous “hydrophobic bridge” which also encompasses the key F126 residue in MD-2 [40]. Collectively, these observations have provided high-resolution insights into the key determinants governing PAMP recognition, and a structural basis for potential design of novel immunomodulatory molecules.

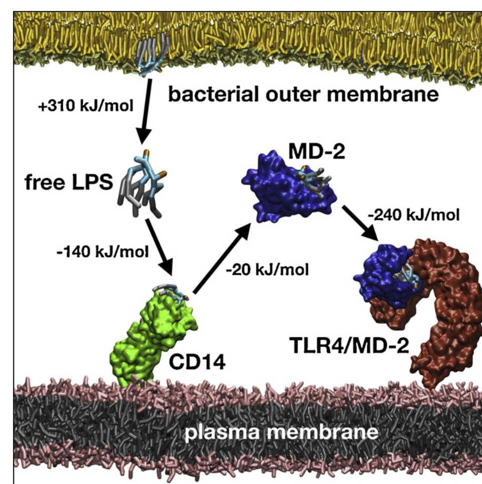

Fig. 2. The thermodynamics of LPS transfer in the TLR4 “funnel”. Biased simulation approaches were used to estimate the free energies of transfer of an LPS molecule between the various states depicted in the figure; transfer to TLR4/MD-2 is overall a favorable process. Proteins are shown in molecular surface format, and lipids are shown in wireframe. Each system and the associated free energies between them are labeled inset.

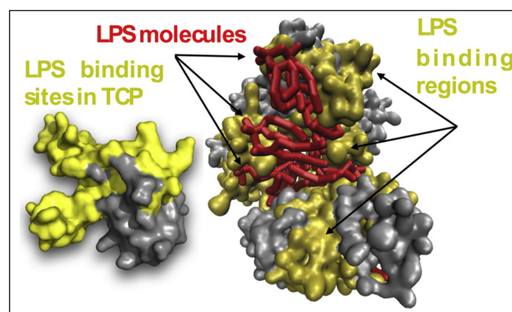

Fig. 3. TCP96 aggregation as a means of LPS scavenging. Simulations were used to study the spontaneous process of aggregation of multiple TCP96 and LPS molecules, to explain their capacity to form amorphous, amyloid-like aggregates in wounds. The LPS binding regions are indicated on the surface of a single TCP96 on the left, whilst the resultant aggregates with LPS are shown on the right. TCP96 is in molecular surface format, whilst LPS molecules are shown in thick wireframe format.

6. Anti-inflammatory scavenging of LPS by endogenous peptides

Microbial infection in wounds triggers multiple arms of the innate immune system, including the deployment of a large family of HDPs, which are diverse in structure but typically amphipathic and cationic [61]. Many such HDPs have been the subject of simulation studies [62], particularly in the context of elucidating mechanisms of bacterial membrane disruption ([63,64];). HDPs have also been shown to exhibit immunomodulatory activities [65,66]; many have a strong affinity for LPS, and this is thought to enable endotoxin “scavenging” and clearance, thereby dampening TLR4-based signaling [67]. In recent years, a group of HDPs associated with the clotting cascade have received special interest due to their multifunctionality. Thus, TLR4 stimulation by LPS triggers the upregulation of tissue factors and formation of thrombin, leading to coagulation and fibrin formation [68], but proteolysis of thrombin by human neutrophil elastase also results in the formation of multiple thrombin-derived C-terminal peptides (TCPs) of around 2 kDa in wounds, as exemplified by FYT21 (NH_2-FYTHVF-RLKKWIQKVIDQFGE-COOH) and HVF18

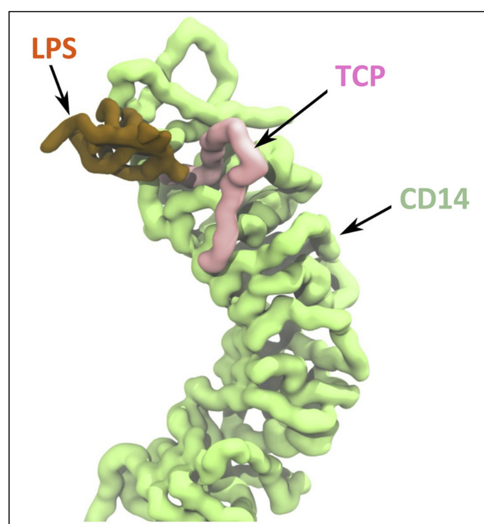

Fig. 4. Simulation snapshot depicting the anti-septic mode of action of a TCP when complexed with CD14. The TCP, HVF18, is bound to the N-terminal hydrophobic pocket of CD14, and this blocks the entry of an LPS molecule. Each molecule is colored differently, and labeled.

(NH_2-HVFRLKKWIQKVIDQFGE-COOH) ([69,70];), sequences included in the prototypic TCP GKY25 (NH_2-GKYGFYTHVFRLKKWIQKVIDQFGE-COOH) [67,71].

TCPs have antimicrobial activity, but also serve anti-endotoxic functions in vitro and in vivo [67,71–73], presumably as a result of LPS scavenging [70]. Lending support to this, NMR was recently employed to determine the conformation of a TCP (HVF18) in complex with LPS [42]. NMR-derived restraints were subsequently incorporated into atomic-resolution simulations of a single amphipathic TCP bound to an LPS micelle, revealing that the curved, positively charged N-terminal segment interacts with lipid A phosphate groups, whilst hydrophobic residues in the C-terminal helix contact the lipid interfacial and acyl tail regions [42]. CG models were developed to assess the capacity for LPS neutralization; a series of ˜40 μs simulations revealed spontaneous assembly and dispersion of multiple peptides into LPS aggregates, with the basic N-terminal region competing with calcium ions cross-linking lipid A phosphates, thereby loosening the interface. A comparison between GKY25, HVF18, and a truncated construct, VFR12, also disclosed a graduated ability of TCPs with different lengths to shield LPS tails from solvent, which correlated with their experimentally measured potency for LPS neutralization [42].

Interestingly, proteolysis of thrombin has also been shown to form a C-terminal fragment of 11 kDa (TCP96) which likely precedes the emergence of the smaller TCPs described above. TCP96 results in amorphous, amyloid-like aggregates in wounds in the presence of both LPS and bacteria, aiding microbial clearance and LPS scavenging [41]. To rationalize this aggregation at the molecular level, atomic-resolution simulations of TCP96 were employed, revealing the presence of an exposed, amphipathic region that forms a hydrophobic cluster with a nearby twisted – and hence strained – β-sheet motif. Extended CG simulations subsequently confirmed that the tails of individual LPS molecules intercalate into this hydrophobic region to form extended aggregates (Fig. 3), and the resultant relaxation of the twist in the associated sheet likely explains the propensity for increased β-content upon aggregation [41]. This aggregation-based host defense mechanism represents an interesting link with amyloidogenic proteins, some of which have been reported to exhibit antimicrobial and anti-inflammatory activities [74].

7. Alternative routes to immunomodulation

Whilst LPS scavenging is undoubtedly a dominant mode for blocking TLR4 signaling, it is likely not the only mechanism. There are numerous reports in the literature of peptides that can bind LPS without blocking sepsis [75]; for example, a variant of GKY25 in which the sequence is scrambled retains high LPS affinity but is no longer anti-endotoxic [67,76]. Pre-incubation of cells with TCPs, followed by washing and subsequent addition of LPS is still effective in preventing downstream NF-κB activation, whilst electron microscopy studies have demonstrated that TCPs co-localize with LPS on the surface of monoctyes [72]. Collectively, these observations hint at specific interactions with targets at host cell membranes. Consistently, we recently used computational modelling approaches, guided by our multiscale characterization of the TLR4 relay and the mapped lipid transfer pathway [40], to uncover a previously undisclosed mode of interaction between CD14 and HVF18 [42]. The TCP was predicted to bind to the hydrophobic pocket of CD14 via its N-terminal tail, with the C-terminal helix bound to polar residues surrounding the pocket that have been shown to be important in LPS capture [56,57]. Microscale thermophoresis measurements revealed a low-micromolar affinity of HVF18 and GKY25 for CD14, and competitive inhibition of LPS binding, whilst chemical cross-linking and mass spectrometry analysis confirmed the predicted TCP-CD14 binding mode [42]. Based on subsequent atomic-resolution simulations of the CD14-HVF18 complex, the solvent-exposed hydrophobic tails of an LPS molecule placed near to the CD14 N-terminus are unable to spontaneously enter the high-affinity binding pocket, consistent with the notion that TCPs competitively impede LPS capture (Fig. 4). Nevertheless, it cannot be ruled out that such peptides may also

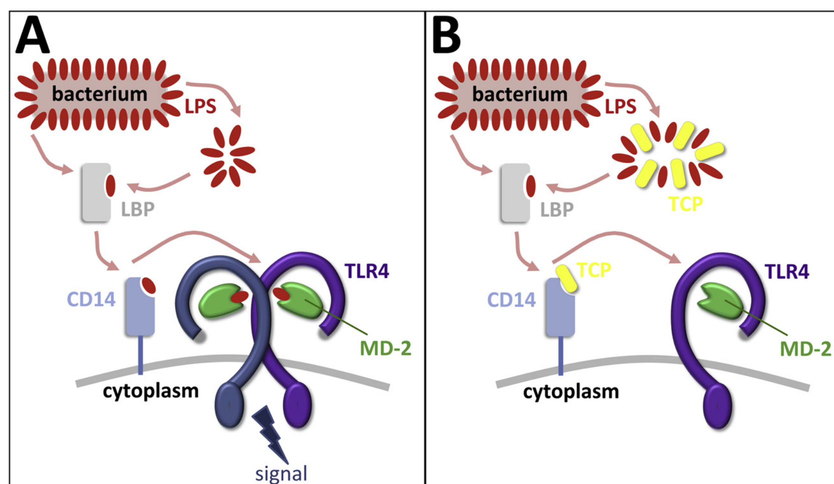

Fig. 5. Summary of TLR4 pathway and anti-septic mechanisms of TCP peptides. (A) LPS is bound by CD14, extracted from aggregates or the bacterial membrane, facilitated by LBP. LPS is then transferred to TLR4/MD-2; agonistic LPS structures stabilize the dimeric $(TLR/MD\text{-}2)_2$ complex, resulting in productive downstream signaling. **(B)** Proteolysis of thrombin during the clotting cascade leads to the formation of multiple TCPs. These peptides inhibit the TLR4 pathway, either by aggregating/scavenging free LPS molecules, or by direct binding to CD14 to prevent LPS transfer to TLR4/MD-2.

interfere with complexation of CD14 with the TLR4/MD-2 complex, and/or block lipid transfer between them.

Thus, multiple orthogonal approaches indicate that TCPs effect their anti-endotoxic activity both indirectly, through LPS sequestration, and directly, via antagonistic binding to CD14. These different mechanisms are summarized in Fig. 5. It is worth noting that multiple TCP variants with variable activities are observed endogenously (Saravanan, 2017), and targeted proteolysis under different conditions may help to fine-tune the innate response to infection and clearance, as required ([67,71]). We have also recently shown that TCPs exhibit a decreased affinity to CD14 and an increase in LPS-binding, with decreasing pH (Holdbrook et al), factors that may affect the modulatory roles of TCPs in the control of bacteria and endotoxin-induced inflammation during infection. The observed micromolar affinities and multitude of interactions, further fine-tuned by the local microenvironment, are suggestive of transient interactions that help to modulate the host immune response. Such transient interactions are observed throughout nature, and are a promising avenue for the development of novel anti-septic drugs [77].

Declaration of Competing Interest

Artur Schmidtchen is a founder of in2cure AB, a company developing therapies based on thrombin-derived host defense peptides. The peptide GKY25 and variants are patent-protected.

Acknowledgements

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