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TLR4/MD2 specific peptides stalled *in vivo* LPS-induced immune exacerbation

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Negative regulation of Toll-like receptor-4 (TLR4) is anticipated to control the pathogen-induced exaggerated immune response. However, effective TLR4 antagonists with scarce off-target effects are yet to be developed. To fill this void, we sought to design small peptide-inhibitors of the TLR4/MD2–LPS interaction. Here we report novel TLR4-antagonistic peptides (TAP), identified through phage display, endowed with the LPS-induced proinflammation inhibition, and confirmed in mice. TAPs-attributed TLR4-antagonism were initially evaluated through NF-κB inhibition in HEK-blue hTLR4 and RAW264.7 cells, and further reinforced by the downregulation of MAPKs (mitogen-activated protein kinases), NF-κB, interleukin 6, and suppression of the oxidative-stress products and iNOS in macrophages and human peripheral blood mononuclear cells (hPBMCs). Among these, TAP2 specifically halted the TLR4, but not other TLRs signaling, which was further confirmed by the biophysical kinetic assay. Finally, TAP2 diminished LPS-elicited systemic cytokine response *in vivo*, suggesting that TAPs, specifically TAP2, have the potential to treat TLR4-mediated immune ailments.

1. Introduction

TLR4 is endowed with the capacity to activate the innate immune signaling cascade via both myeloid differentiation primary response protein 88 (MyD88)-dependent and MyD88-independent pathways. TLR4 is mainly activated by lipopolysaccharide (LPS) recognition through accessory molecules such as LPS-binding protein (LBP), the cluster of differentiation 14 (CD14), and myeloid differentiation factor 2 (MD2) [1]. Stimulation of TLR4 through the MyD88-dependent signaling pathway leads to the early phase activation of nuclear factor κB (NF-κB) and mitogen-activated protein kinases (MAPKs); these events result in the secretion of proinflammatory cytokines such as interleukin 1β (IL-1β), IL-6, and tumor necrosis factor α (TNF-α) [2,3]. Unlike MyD88-dependent pathway, the TRAM/TRIF-dependent route of TLR4 signaling leads to late-phase activation of interferon-regulatory factors (IRFs) and NF-κB, resulting in the secretion of type I interferons (IFN) [4,5]. Besides, LPS-induced TLR4 activation is also reported to cause oxidative stress in macrophages via nitric oxide (NO) and reactive oxygen species (ROS) [6,7]. These signaling pathways, if regulated in their threshold, provide a crucial defensive role and contribute to the host survival by engaging the dangers. However, if dysregulated or over activated and left unchecked, this system put host in extreme danger by activating multiple immune-related disorders [8]. Therefore, countering uncontrolled TLR-mediated signaling has been a major subject of research in the last decade or more [9].

Disturbance in the activation of TLR4 may lead to the initiation or further aggravation of various human illnesses, such as autoimmune diseases, inflammatory diseases, and cancers [10–13]. To address this problem, TLR4-targeting compounds have been actively developed, and some are in clinical trials as agents that can reduce the severity of TLR4-related diseases [14,15]. Many TLR4 agonists and antagonists have been obtained by modifying the main scaffold structure of lipid A [16,17]. For instance, the number of acyl chains or phosphate groups of lipid A switches between TLR4/MD2 agonistic (six chains) [18] and antagonistic activity (lipid IVa) [19]. Eritoran, lipid A, and *Rhodobacter sphaeroides* lipid A (RsLA) can block the interaction of LPS with MD2 and prevent LPS-

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induced shocks in mice [20,21]. Recently a new type of molecule that bears no structural similarity to lipid A or lipid A-derived compounds has been reported. This peptidomimetic compound, Neoseptin-3, activates mouse TLR4 in MyD88- and TRIF-dependent manner [22]. Most TLR4 (ant)agonists have been reported to interact with the ectodomain or MD2 and modulate downstream signaling. However, significant progress has been made in the search for inhibitors of TLR4, and cytoplasmic domain (TIR domain)-targeting molecules and small peptides that block the downstream TLR4 pathway have also been identified (as reviewed in our previous studies [14,15]). As one example, TAK-242 is a molecule that inhibits the protein-protein interaction between TLR4 and TIRAP/TIRAM and blocks downstream signaling [23,24].

Small peptides are structurally versatile elements that have been identified through various techniques, such as yeast two-hybrid assay and phage display (Ph.D.); small peptides are often designed to mimic or oppose the activity of PAMPs and to complimentarily interact with the target proteins [25–27]. These peptides have attracted interest in the field of peptide therapeutics and vaccine adjuvants. The Ph.D. technology is generally used to identify novel peptides in phage libraries that can bind to the proteins of choice [28]. Ph.D. has been widely utilized in finding reagents, diagnostics, and therapeutics since the establishment of this technique by George P. Smith [29–31]. A modified phage is constructed in this method by fusing the corresponding gene fragments with phage coat-expressing gene, thereby allowing the peptides to be displayed on the phage surface. Engineering of the M13 coat protein has substantially moved the Ph.D. platform forward [30]. Alizadeh et al. have recently reported small peptides through Ph.D. that bind to TNF-α and neutralize its cytotoxicity [32]. Similarly, a 13-mer peptide has been selected through Ph.D. that binds to Clec9a on mouse DCs and activates $CD8^+$ cells and IFNγ secretion. This peptide has been reported to reduce lung metastasis and could work as an antigen delivery caries in cancer immunotherapy [33]. Ph.D. technique has also been invested to develop peptide-based nanosensors that can detect pentaerythritol- tetranitrate (PETN) and its surrogate PETNH [34]. Peptides are thought to have fewer side effects than regular drugs do and are easy to modify, which can elicit or suppress TLR4 signaling in a more specific manner [25,35]. Multiple studies have been conducted with an aim to standardize screens for TLR4-antagonistic peptides that bind to the ecto- or cytoplasmic domain of TLR4. One study utilized a computational method and derived some peptides from MD2. These peptides are believed to block TLR4-MD2 interaction and inhibit LPS-induced TLR4 signaling [26]. Another group used a decoy approach and selected small TRAM-derived peptides that effectively block TLR4 signaling [36].

Nonetheless, the issues with the pharmacodynamics and pharmacokinetics of peptide drugs stress the importance of their stability. Compared to that of small molecule and monoclonal antibody therapeutics, one of the drawbacks of peptide drugs is their vulnerability to proteolytic degradation [37]. However, this could be overcome by altering the amide bonds and side chains of the amino acids, to generate proteolytic-resistant peptidomimetics [38]. Cyclization and structural constrains can also improve the stability of the peptides [39]. Crossing biological barriers is another issue for peptide drugs that can be resolved by the addition of carrier molecules [40]. For example, cell and tissue penetration can be facilitated by adding positively charged amino acids to the peptide terminus [41]. In addition, covalent modifications, like macro- and nanoparticle-mediated peptide assemblies have also improved targeting efficiencies [40]. In some cases, the oral availability of the peptides can be disregarded. For example, the peptide hormones amylin, insulin, somatostatin, and human growth hormone can be administered subcutaneously. The development of peptide drugs and their therapeutic potentials are increasing rapidly. This overburdens chemical biologists to improve the suboptimal parameters of these peptides and educate biotechnology investors and commercial drug developers about the misconceptions related to peptide drugs.

Considering that small peptides are more convenient to bind to their target, we employed the same technique and identified three novel peptides that halt the LPS induced TLR4 pathway. The selected TLR4-antagonizing peptides (TAPs) suppress the LPS-induced secretion of IL-6, inhibit the production of oxidative-stress markers (NO and ROS) and block the downstream signaling pathway of TLR4 in RAW264.7 and human peripheral blood mononuclear cells. Biophysical and molecular docking analysis suggested that these peptides preferentially bind to TLR4/MD2 and likely hinder the LPS access to MD2. The TLR4 antagonistic effect of TAP also confirmed in mouse model, which inhibited the LPS elicited systemic cytokines response.

2. Materials and methods

2.1. Construction and enrichment of the TLR4/MD2 binding peptide library

A 15-mer peptide library was synthesized with forward primer 5′-TTG ATC GCA AGG ATC GGC TAG C-3′ and reverse primer 5′-AA GGC CTT GGT ACC GCT GCC ACC $(MNN)_{15}$ GCT AGC CGA TCC TTG CGA TCA A-3′. These two primers were annealed by incubation for 10 min at 95 °C, 30 s at 90 °C, 30 s at 85 °C, 30 s at 80 °C, 30 s at 75 °C, 30 s at 70 °C, 30 s at 65 °C, 30 s at 60 °C, 30 s at 55 °C, 30 s at 50 °C, 30 s at 45 °C, 30 s at 40 °C, and 30 s at 35 °C, and then elongated using *Pfu* DNA polymerase (SolGent, Daejeon, Korea) for 30 min at 68 °C. The DNA product was digested with *Nhe*I/*Kpn*I and cloned into the fUSE55 vector with T4 DNA ligase (New England Biolabs, Inc., Ipswich, MA, USA). The DNA library was transformed into electrocompetent *Escherichia coli* (*E. coli*) DH10B cells, generating 6.6×10^7 distinct clones. The phage library DNA was transformed into electrocompetent *E. coli* TG-1 cells and recovered in 10 ml of SOC. The recovered TG-1 cells were transferred to 1 l of 2 × YT medium containing 20 μg/ml tetracycline and incubated at 37 °C overnight. After centrifugation (9300 × *g*, 15 min, 4 °C), the supernatant was transferred to a clean bottle. Then, 1/5 volume of 20% PEG/2.5 M NaCl was added to the supernatant and incubated at 4 °C overnight. The phage pellet was collected after centrifugation (9300 × *g*, 15 min, 4 °C) and resuspended in 15 ml of TBS, and then centrifuged again (12000 × *g*, 10 min, 4 °C) to remove residual cells. Then, 1/5 volume of 20% PEG/2.5 M NaCl was added to the supernatant and incubated on ice for 1 h. Finally, the phage pellet was collected after centrifugation (12000 × *g*, 10 min, 4 °C) and resuspended in 3 ml of TBS. The 12-mer peptide library was synthesized with forward primer 5′-GCC CAG CCG GCC ATG GCC $(NNK)_{12}$ TCG AGT GGT GGA GGC GGT TCA G-3′ and reverse primer 5′-GCC AGC ATT GAC AGG AGG TTG AG-3′. The two primers were annealed and elongated as described above. Then, the DNA product was digested with *Nco*I/*BamH*I and cloned into the pHEN2 phagemid vector with T4 DNA ligase. The library was then transformed into electrocompetent *E. coli* DH10B cells, generating 2.0×10^9 distinct clones. The phage library was transformed into *E. coli* XL1-Blue cells and recovered in 10 ml of SOC. Recovered XL1-Blue cells were transferred to 1 l of 2 × YT medium containing 100 μg/ml ampicillin and incubated overnight at 37 °C. The culture was transferred to 500 ml of fresh 2 × YT medium and grown at 37 °C until the OD_{600} reached 0.5. The hyperphage, M13K07ΔpIII (PROGEN Biotechnik GmbH, Heidelberg, Germany), was then added to 25 ml of culture (final concentration, 1×10^{12} Pfu/ml). After incubation at 37 °C for 30 min, the cell pellet was collected by centrifugation (3300 × *g*,