

Targeting Toll-like Receptor (TLR) Signaling by Toll/Interleukin-1 Receptor (TIR) Domain-containing Adapter Protein/MyD88 Adapter-like (TIRAP/Mal)-derived Decoy Peptides*

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Background: Assembly of TLR signaling complexes is mediated by a cooperative interaction of TIR domains present in TLRs and TLR adapters.

Results: This work identifies several TIRAP/Mal-derived peptides that inhibit TLR4 and TLR2 signaling *in vitro* and *in vivo*.

Conclusion: TIRAP/Mal-derived inhibitory peptides block TLR signaling by interfering with signaling complex assembly.

Significance: Inhibitory peptides indicate TIRAP TIR interfaces and provide leads for development of TLR-targeting drugs.

Toll/interleukin-1 receptor (TIR) domain-containing adapter protein/MyD88 adapter-like (TIRAP/Mal) is an adapter protein that facilitates recruitment of MyD88 to TLR4 and TLR2 signaling complexes. We previously generated a library of cell-permeating TLR4 TIR-derived decoy peptides fused to the translocating segment of the *Drosophila* Antennapedia homeodomain and examined each peptide for the ability to inhibit TLR4 signaling (Toshchakov, V. Y., Szmazinski, H., Couture, L. A., Lakowicz, J. R., and Vogel, S. N. (2011) *J. Immunol.* 186, 4819–4827). We have now expanded this study to test TIRAP decoy peptides. Five TIRAP peptides, TR3 (for TIRAP region 3), TR5, TR6, TR9, and TR11, inhibited LPS-induced cytokine mRNA expression and MAPK activation. Inhibition was confirmed at the protein level; select peptides abolished the LPS-induced cytokine production measured in cell culture 24 h after a single treatment. Two of the TLR4 inhibitory peptides, TR3 and TR6, also inhibited cytokine production induced by a TLR2/TLR1 agonist, *S*-(2,3-bis(palmitoyloxy)-(2*R*,2*S*)-propyl)-*N*-palmitoyl-(*R*)-Cys-Ser-Lys₄-OH; however, a higher peptide concentration was required to achieve comparable inhibition of TLR2 *versus* TLR4 signaling. Two TLR4 inhibitory peptides, TR5 and TR6, were examined for the ability to inhibit TLR4-driven cytokine induction in mice. Pretreatment with either peptide significantly reduced circulating TNF- α and IL-6 in mice following LPS injection. This study has identified novel TLR inhibitory peptides that block cellular signaling at low micromolar concentrations *in vitro* and *in vivo*. Comparison of TLR4 inhibition by TLR4 and TIRAP TIR-derived peptides supports the view that structurally diverse regions mediate functional interactions of TIR domains.

Toll-like receptors (TLRs)² are a large family of type I transmembrane proteins that function as “pattern recognition receptors.” These receptors constitute an integral component of the innate immune system, as they are able to recognize both microbial products and pathogen-associated molecular patterns, as well as endogenous ligands associated with inflammation or danger-associated molecular patterns. Control of innate immunity in response to infection is vitally important, as an impaired response increases susceptibility to infection, whereas an uncontrolled response can lead to inflammatory disease (2).

All 10 germ line-encoded human TLRs are structurally similar, consisting of a leucine-rich repeat-containing extracellular domain, a membrane-spanning helix, and cytosolic Toll/IL-1 receptor (TIR) domain (2, 3). The TIR domain is a highly conserved structure that is found in both prokaryotic and eukaryotic species (4–6). Structurally, TIR domains consist of alternating β -strands and α -helices (7–9). TIR domains contain a five-stranded parallel β -sheet that is surrounded by five helices (8, 10). The most common nomenclature of TIR domain secondary structures is that strands and helices are alphabetized starting from the N terminus (10). Upon recognition of either a pathogen-associated or danger-associated molecular pattern ligand, the ectodomains of stimulated TLRs form an M-shaped structure in which the C termini of ectodomains converge, leading to dimerization of cytosolic TIR domains (7, 11–13). The TIR dimer provides a composite surface for recruitment of downstream TIR domain-containing adapter molecules that cause propagation of the signal.

Adapter recruitment is exceptionally important, as it governs the specificity of the TLR response (14). TIR domain-containing adapter protein (TIRAP), also known as MyD88 adapter-like (Mal), is a TIR domain-containing adapter utilized by both

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² The abbreviations used are: TLR, Toll-like receptor; TIR, Toll/IL-1 receptor; TIRAP, TIR domain-containing adapter protein; Mal, MyD88 adapter-like; TRIF, TIR domain-containing adapter-inducing interferon- β ; TRAM, TRIF-related adapter molecule; P3C, *S*-(2,3-bis(palmitoyloxy)-(2*R*,2*S*)-propyl)-*N*-palmitoyl-(*R*)-Cys-Ser-Lys₄-OH; TR, TIRAP region; RANTES, regulated on activation, normal T cell expressed and secreted; P2C, *S*-(2,3-bis(palmitoyloxy)-(2*R*,2*S*)-propyl)-(*R*)-Cys-Ser-Lys₄-OH.

Inhibition of TLR Adapter Recruitment

TLR2 and TLR4 to “bridge” MyD88 to the receptors and thus activate NF- κ B (15–18). In the case of TLR4, there are two signaling pathways: MyD88-dependent and MyD88-independent. The MyD88-dependent pathway utilizes TIRAP to bridge TLR4 and MyD88. The MyD88-independent pathway uses the TIR domain-containing adapter-inducing interferon- β (TRIF)-related adapter molecule (TRAM), which bridges TRIF and allows for activation of interferon regulatory factor 3. TIRAP and TRAM appear to bind the same site at the TLR4 TIR homodimer (11, 12). However, it has yet to be determined whether binding of one adapter precludes the binding of the other or whether TLR4 TIR dimerization provides two binding sites so that both adapters can bind the dimer simultaneously.

We have previously shown that cell-permeating peptides consisting of segments of the TLR4 TIR domain fused to the translocating segment of the *Drosophila* Antennapedia homeodomain can inhibit LPS-dependent TLR4 signaling (1, 19). In this study, we used decoy peptides based on the structure of the TIRAP TIR domain. Eleven decoy peptides were designed that together encompass the surface of the TIRAP TIR domain. Screening the peptide library for the ability to block TLR-mediated signaling has identified five peptides that inhibit LPS signaling through TLR4 and two peptides capable of inhibiting S-(2,3-bis(palmitoyloxy)-(2*R*,2*S*)-propyl)-*N*-palmitoyl-(*R*)-Cys-Ser-Lys₄-OH (P3C) signaling through TLR2. As only two of the TIRAP peptides inhibited both TLR2 and TLR4 signaling, this suggests that although the regions of the TIR domain involved in signaling through these different TLRs may overlap, there are also unique regions that are crucial to signaling. By identifying the specific protein sequences required for TLR signaling, we hope to identify both the areas of the TIR domain that are crucial for TIR-TIR interactions and new potential drug targets and leads that may be useful in the treatment of sepsis. We have tested two peptides that were inhibitory in the *in vitro* tests for the ability to inhibit LPS-induced signaling in mice. Both peptides profoundly decreased serum levels of TNF- α and IL-6 induced by intraperitoneal administration of LPS. These data show that the decoy peptide approach taken in this study identifies potent signaling inhibitors and provides very promising leads for development of TLR-targeting therapeutics.

EXPERIMENTAL PROCEDURES

Animals and Cell Culture—All animal experiments were conducted with institutional approval. C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Primary peritoneal macrophages were obtained by peritoneal lavage 4 days after intraperitoneal injection (3 ml) of sterile 3% thioglycolate broth (Remel). Washed cells were resuspended in RPMI 1640 medium that contained 2% FBS, 1% penicillin/streptomycin, and 2 mM L-glutamine. After plating, cells were incubated overnight at 37 °C and then washed with PBS to remove non-adherent cells. Cells were exposed to peptides 30 min before stimulation with a TLR agonist.

Eight-week-old C57BL/6J mice were intraperitoneally injected with TR5, TR6, or TR7 at a dose of 10 nmol/g of animal weight or mock-treated with PBS. LPS (1 μ g/g) was administered to animals intraperitoneally. Blood was collected 1, 2, and

4 h after LPS challenge. Plasma TNF- α and IL-6 were measured in culture supernatants or sera as described below.

Design and Synthesis of Peptides—Eleven decoy peptides representing the surface of the TIRAP TIR domain, as well as a control peptide (20), a random amino acid sequence, were synthesized jointly with the cell-permeating *Drosophila* Antennapedia homeodomain sequence (RQIKIWFQNRRMKWKK). The set of TIRAP-derived peptides was designed similarly to the TLR4 TIR-derived peptides that we used previously to identify the TLR4 TIR interaction sites (1) so that each peptide represents a non-fragmented patch of TIRAP TIR surface, and the entire set encompasses the TIR surface. The peptides were synthesized, purified, and verified by the Biopolymer and Genomics Core Facility at the University of Maryland, Baltimore. Peptides were synthesized on a Prelude peptide synthesizer (PTI Instruments, Boston, MA) using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) coupling. Peptide stocks were diluted in 0–25% Me₂SO/H₂O and quantified as described (21).

Isolation of mRNA and RT-PCR—Total cellular RNA was isolated using TriPure (Roche Applied Science) following the manufacturer's protocol. RNA was then reverse-transcribed using avian myeloblastosis virus reverse transcriptase (Promega) with poly(T) priming following the manufacturer's directions. cDNA was quantified by real-time PCR using Applied Biosystems Fast SYBR Green PCR Master Mix and an ABI Prism 7500HT cycler. Primers used for detection of hypoxanthine phosphoribosyltransferase (forward, GCTGACTGCTGATTACATTAA; and reverse, TGATCATTACAGTAGCTCTTCAGTCTGA), TNF- α (forward, GACCCTCACACTCAGATCATCTTCT; and reverse, CCACTTGGTGGT-TTGCTACGA), IL-1 β (forward, AAATACCTGTGGCCTTG-GGC; and reverse, CTTGGGATCCACACTCTCCAG), IFN- β (forward, CACTTGAAGAGCTATTACTGGAGGG; and reverse, CTCGGACCACCATCCAGG), and RANTES (forward, CTGCTTTGCTACCTCTCCCT; and reverse, GAGTGACAAACACGACTGCAAGAT) mRNAs were designed using Primer Express 2.0 (Applied Biosystems). With hypoxanthine phosphoribosyltransferase as a reference, the ΔC_t method was used to calculate relative gene expression.

Cytokine Detection—Cytokine secretion was measured in supernatant or plasma samples that had been stored at –80 °C. Samples were analyzed by a multiplex cytokine assay at the Cytokine Core Facility at the University of Maryland, Baltimore, using a Luminex 100 reader and SoftMax Pro software or with ELISA kits for mouse IL-6, IL-1 β , RANTES, IFN- γ , or TNF- α from BioLegend (San Diego, CA) and an LT-4000 microplate reader. IL-1 β was measured in cell lysates collected 24 h after LPS stimulation as described for the SDS-PAGE protocol, but not denatured.

Statistical Analysis—mRNA and cytokine data were statistically analyzed using GraphPad Prism 4 software. One-way analysis of variance was performed, as well as Dunnett's multiple comparison post hoc test with $p \leq 0.01$ selected as the level of significance.

SDS-PAGE and Western Analysis—Cellular protein extracts were isolated by the addition of 240 μ l/well cold lysate buffer (20 mM Tris, 50 mM NaCl, 1.5 mM MgCl₂, 2 mM EDTA, 10 mM NaF, 2 mM DTT, 1 mM Na₃VO₄, and 1% Triton). Cells were

incubated with lysis solution for 30 min on an agitator at 4 °C. Lysates were then collected and centrifuged at 14,000 × *g* for 10 min at 4 °C. After quantification using the Bio-Rad protein assay, protein samples were added to Laemmli buffer and boiled for 10 min. Samples were then resolved by SDS-10% PAGE in Tris/glycine/SDS buffer (25 mM Tris, 250 mM glycine, and 0.1% SDS) and transferred onto Immobilon P transfer membranes (100 V, 1.5 h, 4 °C; Millipore, Bedford, MA). Membranes were blocked for 1 h in TBS-T (20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20) containing 5% nonfat milk and probed overnight at 4 °C with the respective antibodies (1:1000 dilution in TBS-T with 2% BSA). After washing in TBS-T, membranes were incubated with secondary HRP-conjugated donkey anti-rabbit IgG (1:10,000 dilution in TBS-T with 5% nonfat milk) for 1 h at room temperature. Membranes were washed five times in TBS-T before development with ECL reagents (GE Healthcare) according to the manufacturer's directions.

Reagents—Protein-free *Escherichia coli* K235 LPS (22) was used at a final concentration of 10 ng/ml to stimulate cells. P3C and *S*-(2,3-bis(palmitoyloxy)-(2*R*,2*S*)-propyl)-(R)-Cys-Ser-Lys₄-OH (P2C) were purchased from EMC Microcollections GmbH (Tübingen, Germany) and used for cell stimulation at 500 and 50 ng/ml, respectively. Rabbit anti-phospho-ERK1/2 and anti-phospho-JNK1/2 antibodies were obtained from Cell Signaling Technology. Rabbit anti-β-actin antibody was from Santa Cruz Biotechnology.

RESULTS

TIRAP Peptides Inhibit LPS-induced Cytokine mRNA Expression—TIRAP/Mal functions to facilitate MyD88 recruitment to activated TLR4 and TLR2, presumably through a simultaneous interaction with receptor and MyD88 TIR domains. Therefore, multiple TIR-TIR interaction sites are expected in the TIRAP TIR domain. We previously used TLR4 TIR-derived decoy peptides to identify the TLR4 TIR dimerization interface (1). We sought to expand this work by applying the same “decoy peptide approach” to obtain leads on the functional protein-protein interfaces within TIRAP/Mal, a TLR adapter protein. To this end, 11 TIRAP decoy peptides were designed structurally homologous to the TLR4-derived peptides used previously (1) so that each peptide represents a non-fragmented patch of the TIRAP TIR surface, with all peptides encompassing the TIR domain. The TIRAP peptides were synthesized in tandem with the cell-permeating sequence of the Antennapedia homeodomain (23), and each peptide was tested for the ability to inhibit TLR4 and TLR2 signaling in primary mouse macrophages. Peptide sequences and the structural regions they represent are shown in Table 1. Because the TIRAP region between strand D and helix E is shorter than the corresponding TLR4 region and is composed of only 25 versus 35 amino acids in TLR4, three TIRAP peptides derived from this region, TR8, TR9, and TR10, overlap (Table 1).

We first examined the effects of TIRAP peptides on LPS-induced cytokine mRNA expression. The 1-h cytokine expression was measured in the initial peptide screening because our previous work suggested that the early cytokine mRNA expression provides the most direct and unbiased readout for overall evaluation of TLR inhibitory peptides (1, 20, 24). Five peptides,

TABLE 1
Sequences of TIRAP TIR-derived decoy peptides

Peptide name	Peptide sequence	Structural region
TR1	SSSSSSGRWSKD ^a YD ^a	N-terminal segment preceding βA ^b
TR2	SEEDLEAAQELVSY	AA, αA
TR3	EGSQASLR ^a CF	αA, AB, βB
TR4	QLRDAAPGGAI ^a VS	βB, BB
TR5	ELCQALS ^a RS ^a HC ^a R	αB, BC
TR6	PGFLRDPWCKYQML	CC, αC
TR7	QALTEAPASEGCT	αC, CD
TR8	LSGLSRAAYPPE	DD
TR9	AAYPPELR ^a RFMY ^a YVD	DD, αD, DE, βE
TR10	YYVDGRGKD	βE, EE
TR11	GGFYQVKEAVIHY	αE
TR45	GAIVSEL ^a CQALS ^a R	BB, αB
TR9-s	PPELR ^a RFMY	αD, DE
CP ^c	SLHGRGDPMEAFII	Randomized sequence

^a Residues that are conserved in TLR4 and TIRAP TIR domains are underlined.

^b Structural regions of the TIRAP TIR domain are indicated as follows. Helices are designated by α, e.g. αB, helix B; strands are indicated by β; and loops are indicated by two uppercase letters, e.g. CD, the loop that connects helix C and strand D.

^c CP, control peptide.

TR3, TR5, TR6, TR9, and TR11, significantly inhibited IFN-β and RANTES mRNA production at 5 μM (*p* < 0.01) and moderately inhibited IL-1β mRNA production (*p* < 0.001) (Fig. 1A). At 20 μM, these five decoy peptides exerted a stronger inhibition of the IL-1β, IFN-β, and RANTES mRNAs and were also able to inhibit potently TNF-α mRNA expression (*p* < 0.001) (Fig. 1B).

When used at a high concentration, the peptide derived from the BB loop of TIRAP, TR4, inhibited cytokine induction to some extent, although the inhibition was weaker than the effects of peptides derived from the neighboring TIRAP regions, TR3 and TR5 (Fig. 1B). TNF-α mRNA was the least affected by TR4 compared with the other cytokine mRNAs examined (Fig. 1B). These findings are in full agreement with our previously published observations that TR4 is the least potent inhibitor among the decoy peptides derived from the BB loops of the four TLR adapters (20) and that IFN-β induction is inhibited by this peptide (25).

We examined two additional peptides for the ability to inhibit LPS-induced TNF-α mRNA. Peptide TR45 comprised the C-terminal portion of TR4 and the N-terminal part of the TR5 decoy (Table 1), so the decoy part of TR45 is centered on the border region represented by TR4 and TR5. This modification did not enhance the inhibitory ability of the peptide compared with TR4 or TR5 (Fig. 1C). The second modified peptide tested was TR9-s, which included only the central portion of TR9 (Table 1). Deletion of both ends of TR9 markedly diminished the inhibition exerted by this peptide (Fig. 1C), thus suggesting that one or both ends of TR9 are important for the full inhibitory activity of this peptide.

TIRAP Peptides Inhibit LPS-induced MAPK Activation—All five peptides that inhibited LPS-induced cytokine mRNA expression (*i.e.* TR3, TR5, TR6, TR9, and TR11) potently inhibited ERK phosphorylation induced by a 30-min LPS stimulation (Fig. 1D). TR7, TR8, and TR10 also diminished ERK activation, although their effect was weaker (Fig. 1D and data not shown). None of the TIRAP peptides activated ERK in cells incubated without a TLR agonist (data not shown). However, JNK was moderately activated in macrophages after a 1-h incubation with 20 μM TR3, TR6, or TR7 (data not shown). This activation

Inhibition of TLR Adapter Recruitment

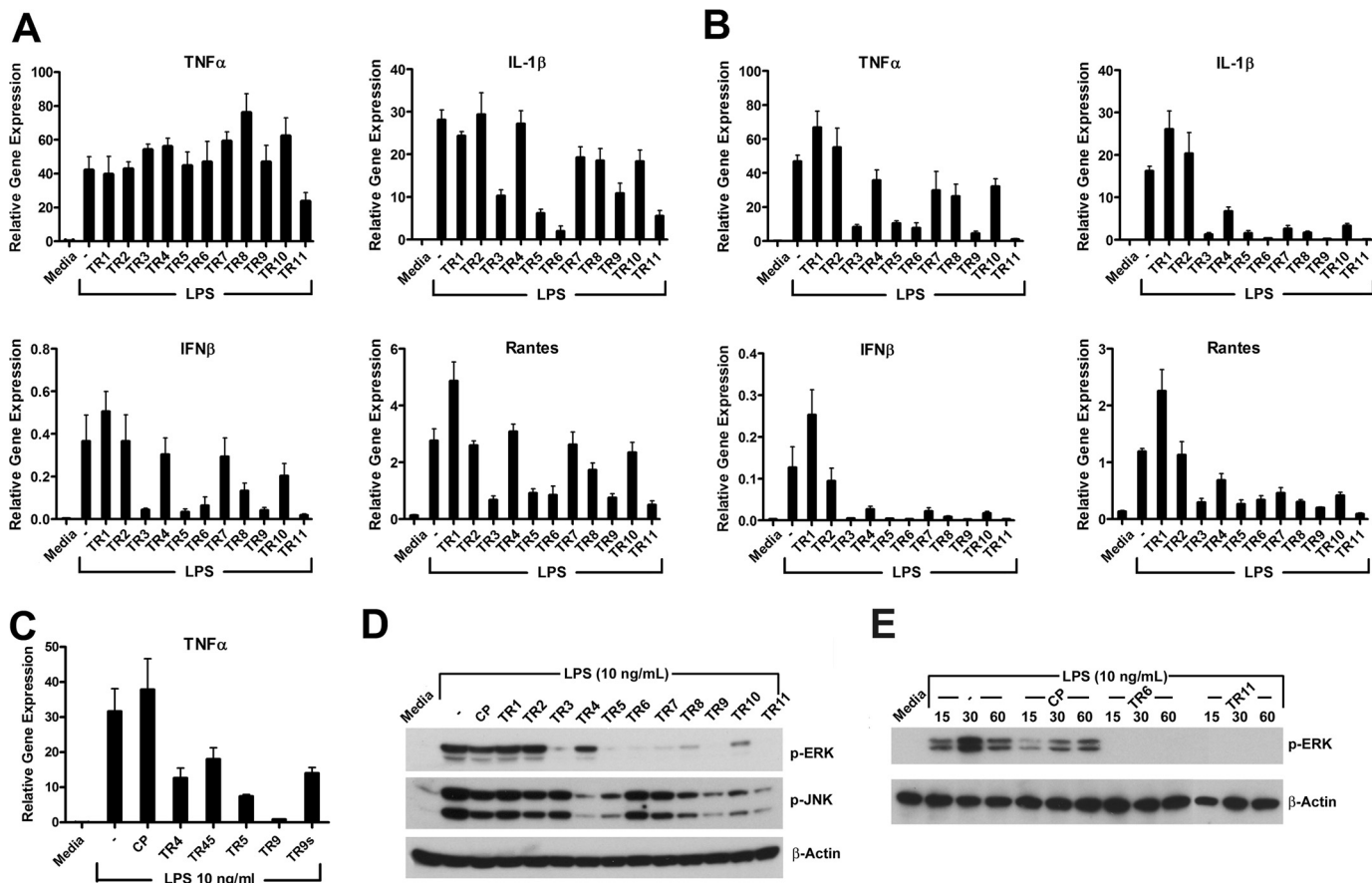


FIGURE 1. Effects of TIRAP TIR-derived decoy peptides on LPS-induced cytokine mRNA expression (A–C) and MAPK activation (D and E). Mouse macrophages were incubated in the presence of 5 (A) or 20 (B–E) μ M decoy peptide for 30 min prior to stimulation with LPS (10 ng/ml). Cytokine mRNA expression was measured 1 h after LPS challenge and is normalized to expression of the hypoxanthine phosphoribosyltransferase gene. mRNA expression data show the mean \pm S.E. for six separate experiments. Protein extracts for Western analysis were taken 30 min after LPS stimulation (D). Each Western blot image is representative of four separate experiments. CP, control peptide.

resulted in the apparent absence of JNK inhibition by the TR3 and TR6 peptides (Fig. 1D). Previously, we reported that the cell-permeable decoy peptide derived from the BB loop of TLR2 induces JNK and p38 MAPK phosphorylation but does not activate ERK (24). Although interesting, the mechanism of this differential MAPK activation by select peptides is not understood at this time. Peptides TR9 and TR11 inhibited both ERK and JNK MAPKs (Fig. 1D).

Peptide-induced inhibition of ERK persisted over a long period of time. Fig. 1E demonstrates that TR6 and TR11 prevented LPS-induced ERK activation for a duration of at least 1 h post-stimulation.

Inhibition of TLR4-mediated Cytokine Production by TIRAP Peptides—To evaluate further the translational potential of peptide TLR inhibitors, we studied the effects of inhibitory TIRAP peptides on LPS-induced cytokine production. Inhibition of cytokines by decoy peptides was confirmed at the protein level. All five inhibitory TIRAP peptides substantially diminished TNF- α and RANTES production and abolished the LPS-induced IL-6 measured in supernatants collected 5 or 6 h after stimulation (Fig. 2, A, C, and E). The cytokine production remained profoundly suppressed throughout 24 h of incubation after a single-dose treatment of macrophages with an inhibitory peptide for all measured cytokines (Fig. 2, B and D–H).

Importantly, TR3, TR5, TR6, TR9, or TR11 inhibited both early and late cytokine genes with comparable efficiency. In macrophages, IFN- γ is induced by LPS after a delay of a few hours (25). Accordingly, in our assay, the IFN- γ contents remained below the detection limit in 6-h macrophage supernatants (data not shown) and increased significantly overnight. Similar to IFN- γ , the RANTES concentration in culture supernatants continued to increase significantly during overnight incubation, thereby indicating the prolonged secretion of this cytokine by LPS-stimulated macrophages (Fig. 2, E and F). TNF- α was induced most transiently of all measured cytokines. The TNF- α mRNA profile suggests that the maximal TNF- α production occurs 1–3 h after LPS stimulation and decreases significantly thereafter. Accordingly, the TNF- α concentration slowly decreases in supernatants during overnight incubation. Typically, we observed a 10–20% reduction in TNF- α contents in the 24-h *versus* 5-h samples (Fig. 2, A and B). Peptides TR3, TR5, TR6, TR9, and TR11, but not the control peptide, TR4, or TR7, inhibited production of all cytokines measured (Fig. 2).

Effects of TIRAP Peptides on TLR2 Signaling—TIRAP/Mal was first identified as an adapter that facilitates the agonist-dependent recruitment of MyD88 to TLR4 (15, 16). TIRAP/Mal has also been implicated in TLR2 signaling because targeted mutation of the TIRAP gene affects both TLR4- and TLR2-mediated cellular responses (18, 26). More recent studies have

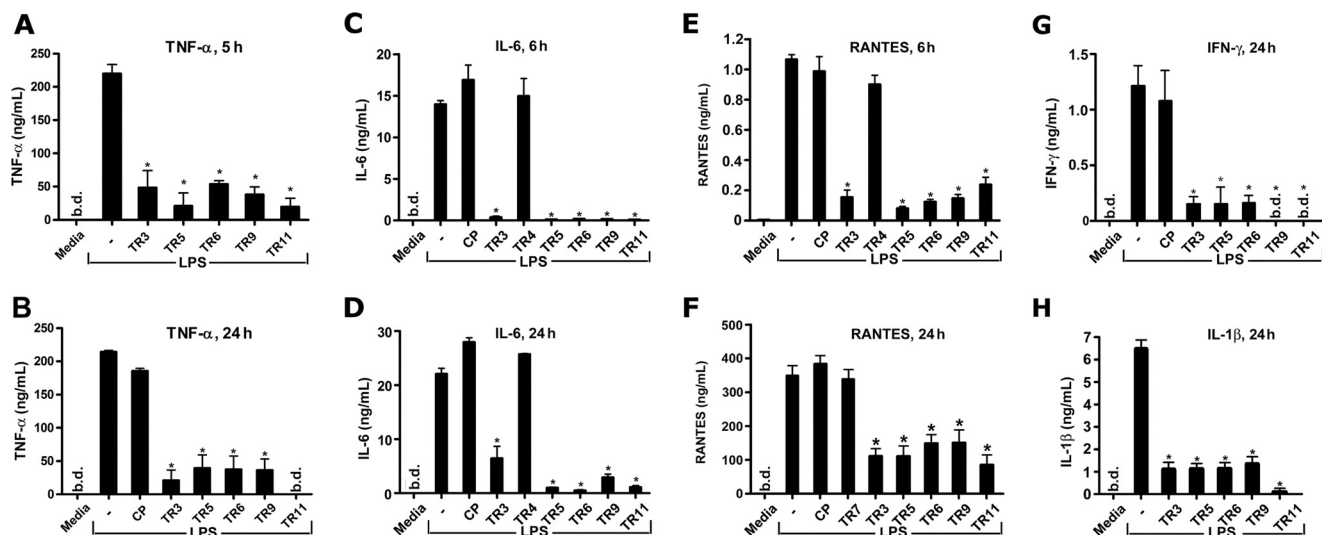


FIGURE 2. **Inhibition of LPS-induced cytokine secretion by cell-permeable decoy peptides derived from TIRAP TIR domain.** Mouse macrophages were stimulated with LPS (10 ng/ml) for 5 (A), 6 (C and E), or 24 (B, D, and F–H) h. Peptide treatments were carried out as described in the legend to Fig. 1. Data show the mean \pm S.E. for three separate experiments. The decrease in cytokine production induced by TR3, TR5, TR6, TR9, and TR11 is statistically significant ($p < 0.05$) in all eight panels. CP, control peptide.

suggested that, rather than playing an obligatory role in TLR2 signaling, TIRAP/Mal augments TLR2 signaling sensitivity, as TIRAP-deficient macrophages are still capable of responding to high doses of TLR2 agonists, although the response is diminished (27, 28).

Because of the role that TIRAP plays in TLR2 signaling, we next sought to examine if TIRAP-derived decoy peptides affect TLR2-induced cytokine mRNA expression. Primary mouse macrophages were stimulated either by P2C, an agonist that induces signaling through TLR2/TLR6 heterodimerization, or by P3C, a TLR2/TLR1 agonist, and the effects of TIRAP-derived decoy peptides on induction of cytokine mRNA were examined. At 5 μ M, TIRAP decoy peptides did not affect P2C- or P3C-induced TNF- α and IL-1 β mRNAs (data not shown). At 20 μ M, TR3 and TR6 inhibited TNF- α and IL-1 β mRNAs induced by P3C (Fig. 3A); however, the effect of these peptides on the P2C-induced cytokines was significantly less (Fig. 3B). The TR3 and TR6 peptides, which showed moderate inhibition of TLR2 signaling at 20 μ M, were examined at a higher concentration. Both TR3 and TR6 profoundly inhibited P3C-induced TNF- α and IL-1 β mRNAs at 40 μ M (Fig. 3A). In sharp contrast to the P3C-activated induction, these peptides did not significantly affect P2C-induced TNF- α and IL-1 β mRNAs, even at this higher dose (Fig. 3B). To confirm the inhibitory effect of TR3 and TR6 on P3C-induced signaling, we measured IL-6 and TNF- α concentrations in macrophage supernatants collected 5 h after stimulation of cells with P3C. Secretion of both cytokines was profoundly inhibited after treatment of the cells with TR3 or TR6 (Fig. 3C). Consistent with this observation, both TR3 and TR6 diminished the P3C-induced ERK phosphorylation (Fig. 3D).

In Vivo Evaluation of Peptide Inhibitors—We next studied if the inhibitory peptides are effective TLR inhibitors *in vivo*. TR5 and TR6 were chosen for this study because they were highly inhibitory *in vitro*. TR7 was used as a control peptide, as our *in vitro* tests suggested that this peptide is not an effective inhibitor of LPS-induced cytokine mRNA.

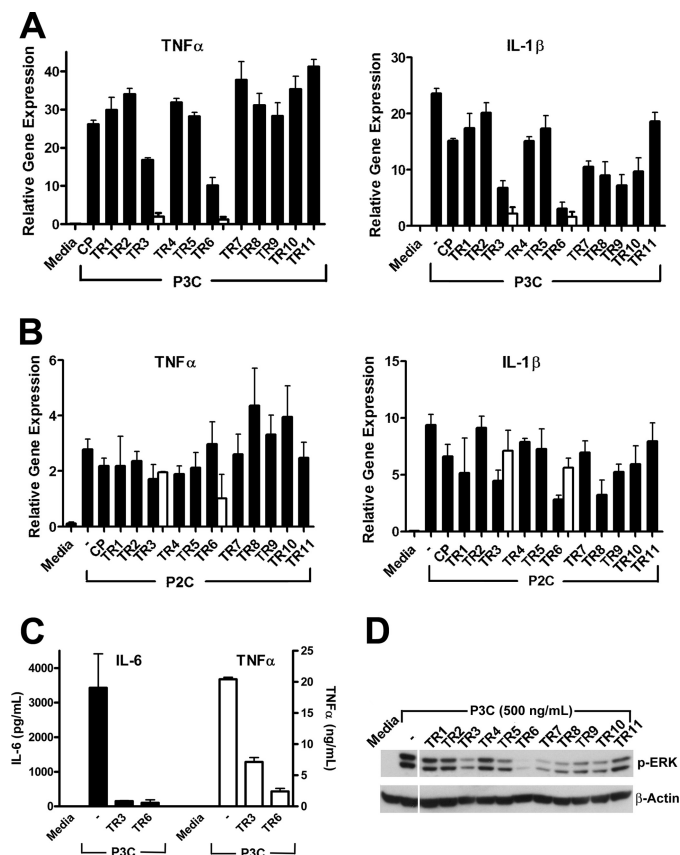


FIGURE 3. **Effect of TIRAP decoy peptides on TLR2 signaling.** Mouse macrophages were incubated in the presence of 20 (black bars) or 40 (white bars) μ M decoy peptide for 30 min prior to stimulation with P3C (500 ng/ml; A and C) or P2C (50 ng/ml; B). Cytokine mRNA expression was measured 1 h after TLR2 stimulation and is normalized to expression of the hypoxanthine phosphoribosyltransferase gene. C, IL-6 and TNF- α were measured by ELISA in macrophage supernatants collected 5 h after P3C (500 ng/ml) stimulation. D, ERK phosphorylation was measured by Western analysis in primary macrophage stimulated with P3C for 30 min. CP, control peptide.

Inhibition of TLR Adapter Recruitment

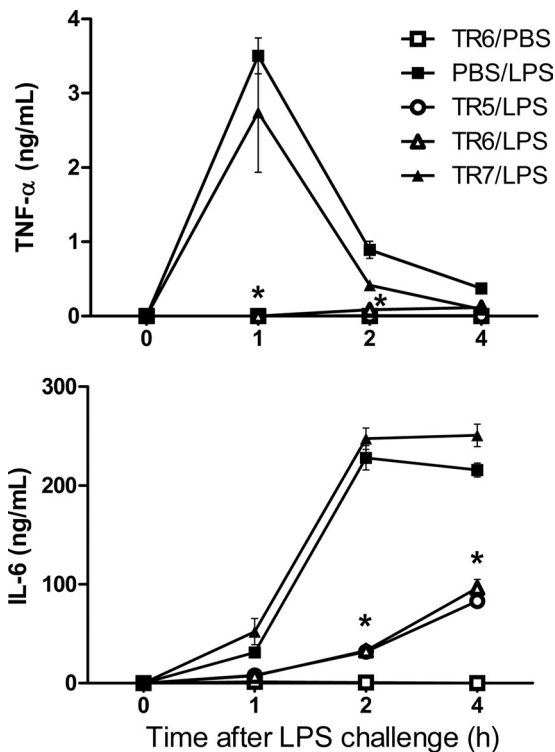


FIGURE 4. Effect of select TIRAP decoy peptides on TNF- α and IL-6 in mouse blood following intraperitoneal injection of LPS. C57BL/6J mice were injected with peptide TR5, TR6, or TR7 or mock-treated 1 h before injection of purified *E. coli* LPS. Peptides and LPS were injected intraperitoneally at 10 nmol/g or 1 μ g/g of animal weight, respectively.

C57BL/6J mice were mock-treated with PBS or treated with a peptide administered to the animals intraperitoneally (10 nmol/g). One hour after peptide treatment, mice were challenged with LPS administered intraperitoneally at a sublethal dose of 1 μ g/g. TNF- α and IL-6 were then measured in serum samples collected 1, 2, and 4 h after LPS challenge.

LPS induced a sharp increase in plasma TNF- α (Fig. 4). This increase was transient, with the maximum observed 1 h after LPS injection. Two hours after LPS challenge, the TNF- α concentration was <25% of the maximum and continued to decrease in the next 2 h (Fig. 4). Both inhibitory peptides tested, TR5 and TR6, abolished the LPS-induced TNF- α response, whereas TR7 failed to block TNF- α induction (Fig. 4).

The concentration of IL-6 peaked later compared with TNF- α . Although significantly increased 1 h after LPS challenge, the concentration of circulating IL-6 increased further and peaked 2–4 h after LPS challenge (Fig. 4). The circulating IL-6 concentration decreased significantly 8 h post-LPS in untreated animals (data not shown). TR5 and TR6, but not TR7, blunted LPS-induced IL-6 production. Peptides blocked IL-6 more effectively at the early (1 and 2 h) time points. Although circulating IL-6 increased between 2 and 4 h in the TR5- or TR6-treated animals, it remained substantially reduced compared with that in the untreated or TR7-treated mice (Fig. 4). These data clearly show that decoy peptides are effective inhibitors of the systemic TLR4-driven response.

In summary, we have identified five TIRAP decoy peptides that inhibit TLR4 signaling. Two of the TLR4 inhibitory peptides, TR3 and TR6, also inhibited TLR2/TLR1-mediated signaling, but not

the signaling initiated through the TLR2/TLR6 dimer. Inhibition of TLR2-mediated signaling by TIRAP peptides was less effective than inhibition of TLR4, as a higher concentration of peptides was required to achieve a similar degree of inhibition. Two peptides that demonstrated strong inhibition in the cell culture assays were further examined *in vivo*; both peptides effectively reduced systemic cytokines induced by LPS.

DISCUSSION

TIR domains mediate transient interactions of signaling proteins involved in inflammatory signaling and host defense. TIR domains tend to interact with other TIR domains; yet functional TIR-TIR interactions are specific, as exemplified by the recruitment of specific TIR domain-containing adapters in response to activation of a particular receptor. Some TIR domain-containing adapters participate in multiple signaling pathways, whereas others interact with a smaller set of proteins. For example, MyD88 is a necessary adapter for all members of the IL-1R family and all TLRs with the exception of TLR3, whereas TIRAP/Mal participates only in TLR2 and TLR4 signaling. Despite considerable effort, the molecular mechanisms that determine specificity of TIR-TIR interactions are not understood.

Previously, we examined a set of 11 TLR4 TIR-derived decoy peptides for the ability to inhibit TLR4 signaling (1). The TIRAP peptides tested in this study were designed similarly to the TLR4 peptides so that each peptide represents a region that is structurally homologous to the corresponding TLR4 TIR region (Table 1) (1). Five TIRAP-derived peptides, TR3, TR5, TR6, TR9, and TR11, potently inhibited TLR4 signaling. This set is different from the set of inhibitory peptides derived from the TLR4 TIR domain, in which peptides from regions 1, 3, 4 (BB loop), 9, and 11 inhibited. Peptides derived from regions 3, 9, and 11 of both TLR4 and TIRAP TIR domains were inhibitory. It is noteworthy that the sequences of these TIRAP and TLR4 regions are very dissimilar (Table 1) (1); for example, the inhibitory peptides derived from region 3 of TIRAP and TLR4 TIR domains have sequences EGSQASLRFC and EEGVPRF-HLC, respectively. The absence of local sequence conservancy, especially in the surface-exposed segments of TIR domains, is well documented (*e.g.* Ref. 29). Peptides derived from regions 5 and 6 of TIRAP/Mal potently inhibited LPS signaling, whereas peptides from the structurally homologous regions of TLR4 were poor inhibitors. Regions 5 and 6 represent surface-exposed amino acids that are adjacent to strand C, the most central strand of the TIR β -sheet that spans the core of the domain. Therefore, regions 5 and 6 are not contiguous on the TIR surface (Fig. 5) and are unlikely to represent one TIRAP interface. Instead of the peptide derived from TR5, the peptide derived from the BB loop of TLR4, which is juxtaposed to region 5, inhibited LPS signaling. The BB loop of TLR4 and several other TIR domains is involved in TIR homodimerization (1, 10, 30). Poor inhibitory activity of the TIRAP BB peptide may indicate that the TIRAP/Mal homodimerization is not important for the function of this protein.

Although TIRAP/Mal is necessary for recruitment of MyD88, but not TRIF, to the TLR4 signaling complex, all inhibitory TIRAP peptides blocked both MyD88-dependent and MyD88-independent cytokine genes induced by LPS. This find-

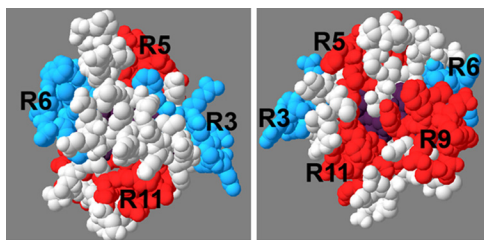


FIGURE 5. **Relative positions of inhibitory peptides on TIRAP TIR surface.** Regions represented by peptides that potently inhibited TLR4 signaling are shown in red. Peptides that inhibited both TLR4 and TLR2 signaling are shown blue. This model of the mouse TIRAP TIR domain was kindly provided by Drs. Gregory Snyder and Sam Xiao (Laboratory of Immunology, NIAID).

ing agrees with and expands our previous reports that TIR-derived peptides do not preferentially affect MyD88-dependent cytokine production (1, 20, 24). The ability of TIRAP-derived peptides to inhibit both MyD88-dependent and MyD88-independent cytokines suggests that TLR4 recruits adapters of MyD88-dependent or MyD88-independent pathways either through the same or significantly overlapping sites. This notion is amenable with the concept that the recruitment of adapters of MyD88-dependent and MyD88-independent pathways occurs sequentially at different cellular locations and is controlled by TLR4 trafficking (31, 32).

Two TIRAP peptides, TR3 and TR6, inhibited TLR2/TLR1-mediated signaling. Interestingly, TR3 and TR6 inhibited P3C-induced, but not P2C-induced, signaling. This finding suggests that these peptides target TLR1. However, regions 3 and 6 are located on opposite sides of the TIRAP TIR domain (Fig. 5). Therefore, it is unlikely that they target the same molecule. Interestingly, both TR3 and TR6 induced activation of JNK, but not ERK MAPK. This “unspecific” effect of TR3 and TR6, together with findings that both peptides target TLR2 in addition to both MyD88-dependent and TRIF-dependent arms of TLR4 signaling, suggests that TR3 and TR6 may target a wider set of proteins. Further studies will be required to understand the molecular mechanisms that underlie the specificity and strength of inhibition by these decoy peptides.

TR3 represents the AB loop of TIRAP/Mal. The highly surface-exposed residues of this loop include two charged amino acids, Glu-128 and Arg-135, and a polar amino acid, Gln-131. Interestingly, the same motif (-ExxQxxxR-) formed by Glu-152, Gln-155, and Arg-159 is also present in TR5 (Table 1). Because these TIRAP regions have different secondary structure and the residues are therefore spaced differently on the TIRAP surface, it is unlikely that these regions would have the same docking sites. Nevertheless, the corresponding peptides have higher conformational flexibility and may be predicted to target the same binding site. A counterargument for the statement that the -ExxQxxxR- sequence is solely responsible for inhibition by both TR3 and TR5 is that the TR45 peptide, which is not a strong inhibitor of TLR4 (Fig. 1C), also has this motif. In addition to TLR4, TR3 inhibits TLR2 signaling, whereas TR5 does not. This finding suggests that the -ExxQxxxR- motif does not play a major role in inhibiting the TLR2 pathway.

New data confirm our previous finding that the peptide derived from the BB loop of TIRAP, TR4 (QLRDAAPGGAI^{VS}), is not a particularly strong inhibitor of TLR4 (20). Interestingly,

the peptide derived from the corresponding region of the TLR4 TIR domain, 4BB (LHYRDFIPGV^{AI}AA; here and below, the underlined residues are identical in TLR4 and TIRAP peptides), is a quite potent inhibitor of TLR4 that targets the TLR4 TIR dimerization surface (1, 24). A comparison of TR4 and 4BB sequences suggests that hydrophobic amino acids of 4BB at positions 6, 7, and 10 might be important for the binding of 4BB to its target.

Region 6 represents the N-terminal part of the third helical region of the TIRAP TIR domain. The peptide derived from region 6 of TIRAP, TR6 (PGFLRDPW^{CKY}QML), inhibited both TLR4 and TLR2 signaling, whereas the peptide derived from the structurally homologous region of TLR4 (RHFIQSR^{WC}IFEYE) did not inhibit TLR4 as strongly. Sequence conservancy between TR6 and the homologous TLR4 region is less than that in the BB loop region, and it has yet to be determined which residues of TR6 are more important for the inhibitory properties of this peptide.

Peptides derived from region 9, the extended fourth helical region, and region 11, the fifth TIR helix, of both TIRAP and TLR4 TIR domains inhibited LPS signaling, yet there is very little sequence similarity in the corresponding regions of TIRAP and TLR4. TR9 represents the region that has been identified as the TRAF6-binding site (33). Further studies are required to elucidate if TR9 indeed targets TRAF6.

An important and practical addition to the tests we used in the past to characterize TIR-derived decoy peptides (1, 24) is that, in this study, we investigated in more detail the peptide effects on cytokine secretion after a prolonged 24-h incubation of macrophages with LPS. Every peptide that has been identified as inhibitory based on the manifestations of early TLR4 signaling (*i.e.* 1-h mRNA induction and MAPK activation) profoundly suppressed secretion of every cytokine measured over the 24-h time course. Importantly, our study included an “immediate-early” and transiently induced cytokine (*i.e.* TNF- α), a cytokine that is induced with some delay (*i.e.* IL-6), and two cytokines whose secretion significantly increases several hours after LPS stimulation (RANTES and IFN- γ). The ability of decoy peptides to inhibit cytokine secretion over a long period after a single treatment implies the high translational potential of these substances as TLR inhibitors or lead therapeutics.

Finally, an *in vivo* examination of two inhibitory peptides demonstrated that the cell-permeable decoy peptide inhibitors effectively block systemic manifestations of TLR signaling. Although preliminary, this result is very exciting, as it provides a new avenue for development of novel TLR-targeting therapeutics.

TLR signaling is a significant therapeutic target because uncontrolled TLR signaling is a pathogenic mechanism in many inflammatory diseases, including sepsis. This study has identified several novel TLR inhibitors effective *in vitro* and *in vivo*, each of which can be used for further refinement as a lead substance in development of TLR-targeting pharmaceuticals.

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