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*J Immunol* published online 23 January 2013
http://www.jimmunol.org/content/early/2013/01/23/jimmunol.1202703

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/01/24/jimmunol.1202703.

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Inhibition of TLR4 Signaling by TRAM-Derived Decoy Peptides In Vitro and In Vivo

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Toll/IL-1R (TIR) domain-containing adapter-inducing IFN-β (TRIF)–related adapter molecule (TRAM) serves as a bridging adapter that enables recruitment of TRIF to activated TLR4 and thereby mediates the induction of TRIF-dependent cytokines. A library of cell-permeating decoy peptides derived from TRAM TIR domain has been screened for the ability of individual peptides to inhibit TLR4 signaling in primary murine macrophages. Peptides derived from TRAM TIR BB loop (TM4) and C helix (TM6) inhibited the LPS-induced activation of MyD88-dependent and TRIF-dependent cytokines, as well as MAPK activation. TM4 and TM6 did not block macropage activation induced by TLR2, TLR9, or retinoic acid–inducible gene 1–like receptor agonists. Both TM4 and TM6 blocked coimmunoprecipitation of TRAM and TLR4 ectopically expressed in HEK293T cells. Both peptides also blocked the LPS-induced recruitment of MyD88 to TLR4 in primary murine macrophages. In vivo examination of TRAM-derived peptides demonstrated that all peptides that were inhibitory in vitro profoundly suppressed systemic inflammatory response elicited in mice by a sublethal LPS dose, and protected mice against a lethal LPS challenge. This research identifies novel TLR inhibitors effective in vitro and in vivo and validates the approach taken in this study as a rational way for development of signaling inhibitors and lead therapeutics.

The Journal of Immunology, 2013, 190: 000–000.

Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, MD 21201

Received for publication September 26, 2012. Accepted for publication December 18, 2012.

This work was supported by National Institutes of Health Grants AI-082299 (to V.Y.T.) and AI-081797 (to S.N.V.).

Address correspondence and reprint requests to Dr. Vladimir Y. Toshchakov, Department of Microbiology and Immunology, University of Maryland School of Medicine, 685 West Baltimore Street, Room 380, Baltimore, MD 21201. E-mail address: vtolshchakov@som.umaryland.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: HA, hemagglutinin; ODN, oligonucleotide; P2C, S-[2,3-bis(2-carboxypropyl)oxy]-(2-RS)-propyl-N-palmitoyl-(R)-Cys-Ser-Lys-OH; poly(I:C), polyinosinic-polycytidylic acid; RIG-I, retinoic acid–inducible gene 1; TIR, Toll/IL-1R; TRAM, Mal or TIRAP; TIR domain-containing adapter protein, also known as MyD88-adapter-like; TRAM, TIR domain-containing adapter inducing IFN-β–related adapter protein; VIPER, viral inhibitory peptide of TLR4.

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of BB loop with E helix (18, 21) and TLR1/TLR2 heterodimer is formed by interaction of TLR1 BB loop and TLR2 DD loop (22).

The mechanism by which a decoy peptide inhibits signaling is presumed to be that the peptide blocks the docking site of its prototype protein and thereby prevents a functional protein–protein interaction (17). Therefore, inhibition of signaling by a decoy peptide often indicates that the inhibitory peptide represents a functional protein interface. In this study, we have screened a library of cell-permeable decoy peptides derived from the TRAM TIR for the ability of individual peptides to inhibit TLR4 signaling in vitro and identified two peptides that potently inhibit LPS signaling. One inhibitory peptide, TM4, represents the BB loop of TRAM TIR; the other, TM6, was derived from the third helical region of the TIR. Both peptides effectively inhibit induction of both MyD88-dependent and TRIF-dependent cytokines and activation of MAPKs by LPS. In accordance with these functional data, both peptides prevented coimmunoprecipitation of TLR4 with TRAM or MyD88. TM4 and TM6 and a truncated version of TM4, TM4–ΔC, effectively inhibit TLR4-driven inflammatory response in mice. All inhibitory TRAM peptides significantly diminished circulating cytokine levels induced by a sublethal dose of LPS, and dramatically improved survival of mice challenged with a lethal LPS dose.

Materials and Methods

Animals, cell culture, and treatment

C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Harvesting, culturing, and stimulation of peritoneal macrophages were described in our previous publications (17–19). Escherichia coli K235 LPS (23) was used at the final concentration of 100 ng/ml. The 2.3-bispalmitoyl-sn-glycerol-(2-RS)-propyl-N-palmitoyl-(R)-Cys-Ser-Lys4-OH (P2C) (EMC Microcollections, Tu¨bingen, Germany) were used at 500 and 50 ng/ml, respectively. Oligonucleotide (ODN) 1668 and low m.w. polyinosinic-polycytidylic acid [poly(I:C)] (Shandong University) were visualized and analyzed using DeepView/Swiss-pdbViewer (Swiss Institute of Bioinformatics).

Animal experiments

Eight-week-old C57BL/6J mice were injected with 1 or 17.5 K235 LPS i.p. Peptides reconstituted in PBS were administered by i.p. or i.v. Control groups received equivalent volume of solvent (PBS). The blood samples were collected 1, 2, 4, and 8 h, or 2, 8, and 24 h after LPS challenge. The plasma samples were obtained and kept frozen until TNF-α and IL-6 were measured by ELISA. Survival of animals was monitored every 6–16 h after LPS challenge. All animal experiments were carried out with institutional approval.

Results

Identification of inhibitory peptides

TRAM TIR decoy peptides were designed similarly to the libraries of TLR4- or TIRAP-derived peptides described previously (18, 20) by a program based on 10% SDS-PAGE and immunoblotting. The cell extracts containing equal amount of protein were incubated with 1 μg Ab for 2 h, followed by 4-h incubation with 25 μl protein G agarose beads (Roche); the beads were then washed four times with lysis buffer, boiled in 1× sample loading buffer, and analyzed by immunoblotting.

Quantitative real-time RT-PCR

Total RNA was isolated with Nucleospin RNA II kits (Macherey-Nagel, Bethlehem, PA), followed by DNase digestion. cDNA was synthesized from 1 μg RNA using Gosecript transcriptase (Promega), and subjected to real-time PCR with gene-specific primers for HPRT, TNF-α, IL-1β, IFN-β, and RANTES on H7900 ABI system using Fast SYBR Green master mix (Applied Biosystems, Foster City, CA).

Cell viability analysis

A total of 5 × 104 mouse peritoneal macrophages was plated into 96-well tissue culture plates, incubated overnight, and treated with peptides for 3 h. After the treatment, cells were incubated with MTT at 0.5 mg/ml (Sigma-Aldrich) for 3 h. Fifty microliters DMSO was added to cells before reading OD at 540 nm.

TRAM TIR model

The coordinate file for human TRAM TIR was provided by Dr. T. Wei (25) (Shandong University). The model was visualized and analyzed using DeepView/Swiss-pdbViewer (Swiss Institute of Bioinformatics).

Table I. Sequences of decoy peptides

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Peptide Sequence</th>
<th>Structural Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>TM1</td>
<td>GAGAEBQDEEEPLK</td>
<td>segment preceding βA</td>
</tr>
<tr>
<td>TM2</td>
<td>AEDDDTEALRQVDDL</td>
<td>AA and αA</td>
</tr>
<tr>
<td>TM3</td>
<td>QTNFGIRPG</td>
<td>αA, AB, and BB</td>
</tr>
<tr>
<td>TM4*</td>
<td>IFVABMPGRLHQL</td>
<td>BB and αB</td>
</tr>
<tr>
<td>TM4-N</td>
<td>IFVFAED</td>
<td>BB</td>
</tr>
<tr>
<td>TM4-M</td>
<td>EMPCDG</td>
<td>BB</td>
</tr>
<tr>
<td>TM4-C</td>
<td>RJLQH</td>
<td>αB</td>
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<td>TM4-ΔN</td>
<td>MPACRLHQL</td>
<td>BB and αB</td>
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<td>IFVFLRLHQL</td>
<td>BB and αB</td>
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<tr>
<td>TM4-ΔC</td>
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<td>BB</td>
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<tr>
<td>TM4-EA</td>
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</tr>
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<td>IFVFAEBCGRRLHQL</td>
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<tr>
<td>TM4-CH</td>
<td>IFVABMPGRHQL</td>
<td>BB and αB</td>
</tr>
<tr>
<td>TM5</td>
<td>NLDVNSGAMT</td>
<td>αB and BC</td>
</tr>
<tr>
<td>TM6</td>
<td>ENFLRQDWCNFBFY</td>
<td>αC and CD</td>
</tr>
<tr>
<td>TM7</td>
<td>TSLMSNVSQHKYNS</td>
<td>CD</td>
</tr>
<tr>
<td>TM8</td>
<td>RPLNLSLPRE</td>
<td>βD and DD</td>
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<td>TM8/9</td>
<td>PRERTPLAQTINA</td>
<td>DD and βE</td>
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<tr>
<td>TM9</td>
<td>RTPAQLQTINA</td>
<td>DD and βE</td>
</tr>
<tr>
<td>TM10/9/10</td>
<td>QITNALEEVE</td>
<td>βE and EE</td>
</tr>
<tr>
<td>TM11</td>
<td>LEESOSQFSTQVE</td>
<td>βE, EE, and αE</td>
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<tr>
<td>TM11</td>
<td>RIFRESVERQQS</td>
<td>αE</td>
</tr>
<tr>
<td>CP</td>
<td>SLMRGRDPMPEAT</td>
<td>Randomized sequence</td>
</tr>
</tbody>
</table>

*Structural regions of TIR domain are designated as follows: helices are designated by Greek α, for example, αA, helix A; strands are indicated by Greek β; loops are indicated by two capital letters, for example, AA, loop that connects strand A and helix A.

*This peptide was previously described as TRAM-BP (19).

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Each TRAM peptide represents a structural element(s) that comprises a nonfragmented patch of TIR surface with all peptides collectively encompassing the TIR domain. The sequences of the TRAM-derived decoy peptides and the corresponding structural regions are provided in Table I. The cell-permeating 16-aa-long sequence of Antennapedia homeodomain (RQIKIWFQNRRMKWKK) (27) was placed at the N terminus of each decoy sequence to render peptides cell permeable. Two additional peptides, TM8/9 and TM9/10, that represent the border area between regions 8 and 9, and 9 and 10, respectively, were additionally tested.

To evaluate peptide-inhibitory activities, we first measured LPS-induced cytokine mRNA expression. Murine macrophages were pretreated with peptides (5 or 20 μM) for 30 min, and then the cells were stimulated with LPS (100 ng/ml) for 1 h. Two peptides, TM4 and TM6, potently inhibited the TLR4-mediated cytokine mRNA transcription (Fig. 1A, 1B). Decoy peptide TM4 is derived from the BB loop of TRAM TIR; we previously identified this peptide as an effective TLR4 inhibitor (19). TM4, even at the low dose of 5 μM, significantly decreased mRNA expression of all cytokines examined (Fig. 1A). Another TRAM-derived peptide that strongly inhibited mRNA transcription is TM6. TM6 effectively blocked induction of IFN-β and RANTES, two TRIF-dependent genes, at both concentrations used (Fig. 1A). However, the effect of TM6 at 5 μM on the MyD88-dependent cytokines, IL-1β and TNF-α, was not statistically significant. Both TM4 and TM6 potently inhibited expression of all cytokine genes at 20 μM (Fig. 1A).

To determine the duration of the inhibitory effect preliminary, we measured cytokine expression after 5-h incubation. Two cytokines, RANTES and IL-6, have been selected for this test because these cytokines are strongly expressed at this time point, whereas expression of TNF-α and IFN-β is more transient and significantly decreased 5 h poststimulation (data not shown). Pretreatment of cells with 20 μM TM4 or TM6, but not with other peptides, profoundly suppressed expression of both cytokines, even at this late time point (Fig. 1B).

We next examined whether TRAM peptides inhibit the TLR4-mediated p38, ERK, and JNK MAPK activation. Murine macrophages pretreated with peptides for 30 min were challenged with LPS. Cell lysates were collected 30 min poststimulation and analyzed for MAPK phosphorylation by Western analysis. Consistent with peptide effects on steady-state mRNA, both TM4 and TM6 inhibited activation of all three MAPKs (Fig. 1C). The inhibition of MAPKs by TM4 or TM6 was long lasting; it persisted for at least 1 h after LPS stimulation (Supplemental Fig. 1A). To characterize further the effects of TRAM peptides on MAPKs, murine macrophages were treated with TRAM peptides, but not challenged by LPS. Without LPS stimulation, TM3 weakly activated p38 and ERK MAPK, whereas effect of other peptides was insignificant compared with the LPS-induced activation (Supplemental Fig. 1B).

We previously demonstrated that LPS stimulation leads to STAT-1-Y701 phosphorylation through autocrine activation of type I IFN receptor by IFN-β (28). Fig. 1D confirms this observation and shows that both TM4 and TM6 block LPS-induced STAT-1-Y701 phosphorylation. Inhibition of STAT1 phosphorylation is a consequence of the strong effect of both peptides on IFN-β transcription (Fig. 1A).

We next measured the effect of peptides on TNF-α, IL-6, IL-1β, IFN-γ, RANTES, and IFN-β secretion. Macrophage supernatants were collected 24 h after LPS stimulation, and cytokine contents were measured by ELISA. Fig. 2 shows that both TM4 and TM6 exert a strong and lasting inhibitory effect on secretion of all six cytokines examined (Fig. 2).

TM8, TM9, and TM10 peptides are derived from consecutive segments of TRAM primary sequence and represent adjacent areas on the TRAM TIR surface. To exclude the possibility of missing an inhibitory sequence due to arbitrary fragmentation of a peptide, we designed two additional peptides, TM8/9 and TM9/10, that overlap with TM8 and TM9, and TM9 and TM10, respectively (Table I). Neither TM8/9 nor TM9/10 inhibited the TLR4 signaling significantly (Supplemental Fig. 1D).

**FIGURE 1.** TRAM decoy peptides derived from BB loop and third helical region, TM4 and TM6, inhibit LPS-induced cytokine mRNA and activation of MAPKs and STAT1. Mouse peritoneal macrophages were preincubated with 5 or 20 μM (A, B) control or decoy peptides for 30 min prior to stimulation with LPS (100 ng/ml). Cells were lysed 1 (A) or 5 h (B) after LPS challenge. Cytokine mRNA expression is normalized to the expression of the housekeeping gene, Hprt. Data represent the means ± SEM of four independent experiments. *Data statistically different from the control group (p < 0.001). Cell lysates for immunoblotting were obtained 30 min (C) or 2 h (D) after LPS stimulation. GAPDH was used as a loading control. (C) and (D) show a representative blot of four separate experiments.
TRAM is an adapter protein selectively involved in TLR4 signaling (5, 9). We next studied whether the effects of TM4 and TM6 are specific and examined peptide effects on TLR2, TLR9, and retinoic acid–inducible gene 1 (RIG-I)/MDA-5 pathway. Mouse macrophages were stimulated with 500 ng/ml P3C or 50 ng/ml P2C, agonists that activate signaling through TLR2/TLR1 or TLR2/TLR6 heterodimers, respectively. In sharp contrast with the effect of these two decoy peptides on TLR4 signaling, TM4 and TM6 (20 μM) did not inhibit P3C- or P2C-induced p38, ERK, or JNK MAPK phosphorylation (Fig. 3A), or IL-1β and TNF-α mRNA induction (Fig. 3B). Neither peptide blocked cell activation induced by TLR9 or RIG-I/MDA-5 agonists, CpG oligonucleotide ODN 1668, or intracellularly delivered poly(I:C). The STAT1 Y701 residue was phosphorylated in macrophages in response to both agonists regardless of the presence of TRAM peptides, albeit the STAT1 activation by poly(I:C) occurred at later time points (Fig. 3C). TRAM peptides did not affect the induction of IFN-β mRNA statistically significantly (Fig. 3D).

Peptide effects on cell viability were evaluated using the MTT test. Neither inhibitory peptide affected cell viability in a significant way, even after 3-h incubation in the presence of TM4 or TM6 (Supplemental Fig. 1C).

Inhibitory activities of peptides derived from the third helical region of TLR adapters

We observed previously that peptides derived from the BB loop of TLRs and TLR adapters differ markedly in ability to inhibit TLR4 (19, 29). Our new data show that, in addition to the TRAM BB loop peptide, peptide derived from the third helical region of TRAM, TM6, strongly inhibits TLR4 signaling (Figs. 1, 2). Interestingly, the sequence of the region represented by TM6 is highly similar to the sequence of the structurally homologous region of TIRAP/Mal (26) (Fig. 3G). TIRAP/Mal peptide derived from this region, TR6, inhibited both TLR4 and TLR2 signaling (26). We next compared inhibitory efficiency of peptides derived from the homologous structural region of other TLR4 adapters. Both TRAM and TIRAP region 6 peptides, TM6 and TR6, potently inhibited the LPS-induced activation of p38 and ERK MAPKs (Fig. 3E). These peptides also blocked transcriptional activation of IL-1β and IFN-β genes (Fig. 3F). In contrast, the inhibitory effects of peptides derived from homologous region of TRIF and MyD88 TIR were less (Fig. 3E, 3F), especially on MAPK activation. Notably, region 6 in TRAM and TIRAP is the most similar region in these proteins. TIRAP and TRAM region 6 sequences are PGFLRDPWCKYQML and ENFLRDTWCFQFY, respectively, and have 7 identical aa (underlined). This is by margin the highest degree of local sequence conservancy that has been found in all four TLR4 adapters. Such a high degree of local conservancy of the surface-exposed residues strongly suggests that this region is functionally important. It is noteworthy that there is no notable similarity in the homologous regions of MyD88 and TRIF (Fig. 3G).

Inhibitory properties of truncated TM4 variants

TM4 is a very efficient overall inhibitor of TLR4 [Fig. 1 and our previous publications (17, 19)]. We next sought to identify amino acids that are critical for the inhibitory effects of TM4. We used three groups of modified peptides to address this question. First,
three short peptides each of which contained only 5 aa of TM4 were examined. TM4-N contains 5 N-terminal aa of TM4; TM4-M contains the TM4 middle 5-aa section; and TM4-C contains the 5 C-terminal aa (sequences are shown in Table I). The second group is comprised of three modified TM4 peptides that lack TM4-N, TM4-M, and TM4-C; these peptides are designated as TM4-DN, TM4-DM, and TM4-DC, respectively (Table I). Finally, we tested three peptides, TM4-E/A, TM4-P/H, and TM4-C/H, in which a single amino acid has been replaced (sequences are shown in Table I).

Among three short peptides, TM4-N was the strongest inhibitor. TM4-M demonstrated intermediate inhibition, whereas TM4-C was least active (Fig. 4A, 4B, Supplemental Fig. 2A). Consistently with these data, cells were stimulated with ODN 1668 for 5 h or with poly(I:C) in complex with Lipofectamine 2000 for 16 h (C). IFN-β mRNA induction by TLR9 and RIG-I-like receptor agonists was measured 5 h after stimulation (D). Other experimental details are as in Fig. 1. Data in (A) and (E) represent three independent experiments. Means ± SEM of three independent experiments are shown in (B), (D), and (F). *p < 0.01. (G) The residues that are conserved in TRAM and TIRAP peptides are underlined.

TM4 and TM6 block adapter recruitment to TLR4

We used coimmunoprecipitation assays to study peptide effects on TLR adapter recruitment. To study effect of peptides on TRAM recruitment, HEK293T cells were cotransfected with mouse TLR4 tagged with Cerulean fluorescent protein (TLR4-Cer) (18) and HA-tagged mouse TRAM (HA-TRAM). TM4 or TM6, but not a non-inhibitory TM3 peptide, blocked TLR4/TRAM coimmunoprecipitation (Fig. 5A). Interestingly, TR6, a TIRAP-derived inhibitory peptide that has sequence similar to TM6, also inhibited TLR4/TRAM association, although less efficiently than TM6 (Fig. 5A).

To study peptide effects on MyD88 recruitment, we used primary mouse peritoneal macrophages. In this cellular model, MyD88 co-immunoprecipitates with TLR4 in the agonist-dependent manner (30) (Fig. 5B). All inhibitory peptides tested, TM4, TM6, and TR6, efficiently prevented the LPS-induced TLR4/MyD88 association (Fig. 5B). TIRAP/Mal is necessary for MyD88 recruitment to TLR4 (3), whereas TRAM recruits TRIF and is not required for

FIGURE 3. TM4 and TM6 block TLR4, but not TLR2 (A, B), TLR9, or RIG-I-like receptor (C, D) signaling. Peptides derived from region 6 of TRAM and TIRAP TIR are strong inhibitors of TLR4-induced MAPK activation and cytokine transcription (E, F). Peptides were used at 20 μM. P2C and P3C were used at 50 and 500 ng/ml, respectively. Macrophages were treated with ODN 1668 at 2.5 μM or low m.w. poly(I:C) in complex with Lipofectamine 2000 at 400 ng/ml. MAPK phosphorylation was measured in whole-cell lysates collected 30 min after stimulation with LPS or P3C (A). To measure STAT1-Y701 phosphorylation, cells were stimulated with ODN 1668 for 5 h or with poly(I:C) in complex with Lipofectamine 2000 for 16 h (C). IFN-β mRNA induction by TLR9 and RIG-I-like receptor agonists was measured 5 h after stimulation (D). Other experimental details are as in Fig. 1. Data in (A) and (E) represent three independent experiments. Means ± SEM of three independent experiments are shown in (B), (D), and (F). *p < 0.01. (G) The residues that are conserved in TRAM and TIRAP peptides are underlined.
MyD88 recruitment and activation of MyD88-dependent genes (11). Yet, TIRAP- and TRAM-derived peptides cross-reacted in the sense that each peptide blocked the recruitment of both adapters, TRAM and MyD88. This observation agrees fully with the fact that the adapter-derived peptides are equally effective inhibitors of MyD88- or TRIF-dependent cytokines (Figs. 1, 2) (26). Collectively, these results suggest that TM4 and TM6 disrupt the assembly of TLR4 signaling complexes by blocking the TIR: TIR interactions required for adapter recruitment, possibly mediated by a common binding site for recruitment of TIRAP and TRAM to TLR4 TIR dimer.

Inhibitory peptides blunt LPS-induced cytokine response in mice

We next studied whether peptides identified as inhibitory in in vitro tests are effective in vivo. C57BL/6J mice were challenged i.p. with a sublethal LPS dose (1 μg/g animal weight), and plasma TNF-α and IL-6 levels were monitored for 8 h after the challenge. In the first series of experiments, we injected 10 nmol/g TM4 or TM6 i.p. 1 h before LPS challenge. The 10 nmol/g dose corresponds to 38, 41, and 32 mg/kg TM4, TM6, and TM4-ΔC, respectively. Pretreatment of mice with either inhibitory peptide dramatically decreased systemic IL-6 and TNF-α levels measured 2 h after LPS challenge; after the treatment, the plasma cytokine levels were only 5–15% of cytokine levels induced in the PBS-treated group (Fig. 6A). The effect was lasting; TM4 and TM6 significantly decreased circulating levels of both cytokines at every time point throughout the observation period (Fig. 6B).

Optimization of peptide dose. When used at a lower dose of 2.5 nmol/g, TM4 appears to affect the circulating TNF-α equally strongly as it does at 10 nmol/g (Supplemental Fig. 3A). However, the lower dose was less efficacious against the circulating IL-6 levels, especially at the later time points (Supplemental Fig. 3C). A similar decrease in the TM6 dose resulted in a less efficient inhibition of both cytokines (Supplemental Fig. 3B, 3D). Interestingly, these results, obtained in in vivo experiments, parallel very closely the effects of low peptide doses on macrophage expression of cytokine mRNA (Fig. 1A, 1B). Increasing the peptide dose to 25 nmol/g did not augment the efficiency of TM4 and TM4-ΔC with respect to circulating IL-6 at 4- or 8-h time points (Supplemental Fig. 3C, 3E). Based on these observations, the dose of 10 nmol/g was chosen for all subsequent experiments.

Effect of injection route. Efficient cellular uptake of cell-permeating peptides is well established for monolayers of cells in culture; however, the tissue permeability of peptides is expected to be lower. Diminished peptide permeability through the tissues of the body might limit their inhibitory efficacy in vivo. Results of experiments shown in Fig. 6B demonstrate that peptides effectively mitigate the TLR4-driven inflammatory symptoms when administered via the same route as the TLR agonist, that is, i.p. To understand whether peptides might be effective as systemic...
inhibitors of TLR4, we used separate routes of administration for the TLR4 agonist and TLR4 antagonists in the next series of experiments. Peptides (10 nmol/g) were injected i.v.; LPS was administered i.p. Intravenous injection of TM4 or TM4ΔC significantly lowered the levels of circulating IL-6 and TNF-α throughout the 8-h observation period following the LPS injection (Fig. 6C). However, the inhibitory effect of i.v. injection was less compared with that after i.p. administration of an inhibitory peptide (Fig. 6B, 6C, respectively). Although a statistically significant difference in cytokine levels after i.p. versus i.v. treatment was detected only for the effect of TM4 on IL-6 4 h after LPS injection (p < 0.01, data not shown), for all other time points, the p values of the difference between the untreated and i.v.-treated group were lower than between the untreated and i.p.-treated animals for both TM4 and TM4ΔC peptides (Fig. 6B, 6C, and data not shown). These data show that the i.v. injection of decoy peptide is less effective compared with i.p. administration in this model of inflammation; nevertheless, the results indicate that the peptides are efficient systemic TLR inhibitors.

Effects of timing of peptide treatment. TLR activation launches a complex chain of signaling events that ultimately result in production of multiple cytokines. Secreted cytokines act back on cells and activate a multitude of different signaling pathways, many of which help to sustain inflammation. In our previously described experiments, mice were pretreated with inhibitory peptides before activation of TLR signaling by LPS. We next wanted to determine whether the peptides administered after LPS are still effective for mitigation of TLR4-driven inflammatory symptoms. In this series of experiments, mice were first challenged i.p. with LPS (1 mg/g); peptides were injected at the dose of 10 nmol/g i.p. 30 min after LPS. The administration of inhibitory peptides 30 min after LPS did not affect levels of circulating TNF-α and IL-6 measured 1 h after LPS challenge (Fig. 6D, 6E, respectively). However, the peptide treatment significantly lowered the levels of TNF-α and IL-6 1 h after LPS challenge (p < 0.01, data not shown). These data show that the i.p. injection of decoy peptide is less effective compared with i.p. administration in this model of inflammation; nevertheless, the results indicate that the peptides are efficient systemic TLR inhibitors.

FIGURE 6. TM4, TM6, and TM4ΔC effectively suppress LPS-induced cytokine induction in vivo (A–E) and protect mice from lethal endotoxemia (F, G). (A–E) C57BL/6J mice were injected i.p. with purified E. coli K235 LPS (1 μg/g) or PBS. Peptides (10 nmol/g animal weight, i.e., 38, 41, 40, and 32 mg/kg for TM4, TM6, TR6, and TM4ΔC, respectively) were injected i.p. (A, B, D, E) or i.v. (C) 1 h before (A–C) or 30 min after (D, E) i.p. injection of LPS. Data in (A) and (D) show plasma IL-6 and TNF-α levels measured 2 h after LPS challenge. Peptide TM8/9 was used as a noninhibitory control peptide. Data represent the means ± SEM for 6–12 blood samples obtained in at least three independent experiments. (F and G) C57BL/6J mice were challenged i.p. with a lethal LPS dose (17.5 μg/g). Peptides (10 nmol/g) were injected i.p. 1 h before LPS challenge. (G) Also shows survival in the group treated with TM4ΔC 3 h after LPS challenge (n = 7). The statistical significance of changes in mortality and survival time was determined by the Mantel–Cox log-rank test using GraphPad Prism software (version 5.04). *p < 0.01.
after LPS administration (i.e., 30 min after administration of peptides) (Fig. 6E). However, 2 h after LPS challenge, that is, 1.5 h after injection of peptides, the plasma levels of both cytokines were markedly diminished in mice treated with any of the three inhibitory peptides tested, TM4, TM6, or TM-ΔC (Fig. 6D, 6E). The observed diminution in the cytokine levels at 2, 4, and 8 h after LPS was less than that in the experiments when mice were pretreated with inhibitory peptides (Fig. 6A, 6B, 6D, 6E).

TM8/9, a peptide that did not inhibit macrophage signaling in vitro, did not affect the LPS-induced cytokines in vivo 2 h after LPS challenge or thereafter (Fig. 6E). Our data suggest that both prophylactic and therapeutic administration of peptides mitigate the inflammatory response to LPS.

**Inhibitory peptides protect mice from a lethal LPS challenge**

TM4, TM6, and TM4-ΔC strongly suppress systemic cytokine response induced by a sublethal LPS dose. We next studied whether inhibitory peptides protect mice from a lethal LPS challenge. TR6, a previously described inhibitory peptide derived from the third helical region of TIRAP (26), was also included in this study as an additional control. C57BL/6J mice were injected i.p. with 17.5 μg/g LPS. This dose induced 100% mortality in the control group (Fig. 6F). Pretreatment of mice with 10 nmol/g TM4 or TM4-ΔC rescued all animals from the lethal LPS dose (Fig. 6F). TM6 also dramatically improves survival compared with the untreated group; however, 3 of 13 animals died after LPS challenge in this group. TR6, an inhibitory TIRAP peptide that shares significant sequence similarity with TM6, improved animal survival comparably to TM6 (Fig. 6F). In an additional control series of experiments, we used peptide TM8/9 as this peptide did not inhibit TLR4 in vitro tests. Although we had one survivor in the group of seven animals injected with 17.5 μg/g LPS following pretreatment with TM8/9, the difference in survival rate was highly significant between each of the control groups and the TM4-, TM6-, or TM4-ΔC-treated group (p < 0.0012, for all pairs). The peptide-treated survivors appeared active and healthy at the time the experiments were terminated, 7 d after injection of LPS.

We next studied whether the TRAM decoy peptides are effective when administered therapeutically, that is, in our model of the disease, after injection of LPS. TM4-ΔC was chosen for these tests. The peptide was administered i.p. 3 h after i.p. injection of LPS. Five of seven animals survived the lethal LPS challenge in this group, whereas no animals survived in the untreated group (0 of 12) and all animals (10 of 10) survived in the group pretreated with TM4-ΔC (Fig. 6G). These data demonstrate that inhibitory peptides have strong potential as therapeutics, because the difference in survival rate between the untreated and the group treated with TM4-ΔC therapeutically is highly significant (p = 0.009 as determined by the Gehan-Breslow-Wilcoxon test). The results presented strongly suggest that select decoy peptides effectively lessen the systemic inflammatory response induced by activation of TLR4 and, therefore, might be effective for treatment of septic shock (Fig. 7).

**Discussion**

Homotypic or heterotypic interactions of TIR domains mediate the early stages of TLR signaling complex assembly: agonist-induced dimerization of receptor TIR domains, followed by recruitment of TIR-containing adapters (13, 15, 16, 31). Blockage of any interface involved in the formation of the initial complex is predicted to abolish the TLR signaling because the molecular interactions that underlie the initial steps of TLR signaling complex assembly are highly cooperative (18). Therefore, molecular tools capable of blocking a specific TIR:TIR interaction potentially have a significant therapeutic value because excessive TLR signaling is a pathogenic mechanism in many inflammatory diseases. We previously screened libraries of decoy peptides derived from TIR domains of TLR4 and TIRAP/Mal (18, 26). This study expands our previous work and tests peptides derived from TRAM. Although previous studies have identified a number of TLR4-inhibitory peptides (18, 19, 26, 29, 32, 33), due to diversity of sequences that define the surface-exposed area of TIR domains (13, 34), identification of novel peptides would be valuable as it may yield insight into mechanisms of TLR complex assembly and recognize novel TLR targets and TLR-targeting agents. The library of TRAM peptides was designed similarly to TLR4 and TIRAP sets; each TRAM peptide represents a nonfragmented patch of the TRAM TIR surface with all peptides of the library encompassing the domain. The approach taken in these three studies has been highly successful in that a unique set of inhibitory peptides that block TLR4 signaling at low micromolar concentration has been identified in each TIR-specific library (18, 19, 26). In general, the sequences of identified inhibitory peptides are dissimilar, as are the surface-exposed residues of TIR domains. TIRAP and TRAM peptides derived from the third helical region, designated as TR6 (26) and TM6, were the most similar among identified inhibitors with 50% amino acid homology. In each set of the TIR peptides tested, the inhibitory peptides represent different structural regions of the TIRs, as illustrated by the observation that no TIR structural region would consistently produce an inhibitory decoy in all three TIR domains studied.

Two TRAM-derived peptides, TM4 and TM6, potently inhibit TLR4 signaling. TM4 is derived from the BB loop, whereas TM6 represents the short CC loop, together with the third helical region of the domain. TM4 and TM6 regions are located in close proximity on the TIR surface, although they are not juxtaposed (Fig. 7). The sufficient separation between the areas, especially if the C-terminal part of TM4 is excluded, together with significant cur-
nature of this part of the TIR (Fig. 7A), suggests that these regions interact with different TIR domains in the receptor/adapter complex.

Both peptides potentily inhibit all manifestations of TLR4 activation we examined in primary macrophages: the cytokine gene expression and secretion and activation of MAPKs and STAT-1 were all potently inhibited. Importantly, the TRAM peptides block not only the induction of cytokines traditionally associated with the TRIF-dependent signaling pathway, for example, IFN-β, RANTES, and IL-6 (9), but also the MyD88-dependent cytokines, for example, TNF-α and IL-1β. This finding parallels and expands the result of screenings of TLR4- and TIRAP-derived peptides that also have not identified an inhibitory peptide that would selectively block the expression of MyD88-dependent or TRIF-dependent cytokines (18, 26). This consistent pattern of specificity most likely indicates that all inhibitory peptides identified in these screenings target the very early, cooperative stages of the assembly of multi-TIR complex and that the adapter recruitment interfaces overlap significantly. Coimmunoprecipitation assays further confirmed that the inhibitory peptides block recruitment of adapters to TLR4. Importantly, each peptide tested, including TR6, a TIRAP-derived peptide that is similar to TM6, blocked recruitment of both adapters, TRAM and MyD88. This observation is also consistent with the absence of specificity of any of these peptides with respect to blockage of Myd88-dependent or TRIF-dependent cytokines. Interestingly, viral inhibitory peptide of TLR4 (VIPER), a TLR4-inhibitory peptide derived from viral protein A46 (33), demonstrates similar pattern of specificity as it blocks all TLR4 signaling by disrupting both TLR4-TIRAP/Mal and TLR4:TRAM interactions (35). Although effects of VIPER on TLR4 are similar to the effect of TRAM peptides, sequences of these peptides are dissimilar. Further studies are required to understand whether VIPER and TRAM peptides affect TLR4 via similar mechanism.

The TRAM peptides exerted a lasting inhibitory effect. A significant decrease in cytokine content was detected in macrophage supernatants collected 24 h poststimulation for every cytokine measured (Fig. 2). This level of stability is sufficient for both tested peptides to mitigate efficiently the systemic LPS-induced inflammatory response in vivo after a single-dose treatment.

Whereas TM4 and TM6 failed to inhibit MyD88-dependent and TRIF-dependent pathways selectively, they exhibited specificity for TLR4 as neither peptide blocked TLR2-, TLR9-, and RIG-I–dependent pathways selectively, they exhibited specificity for TLR4 as neither peptide blocked TLR2-, TLR9-, and RIG-I–dependent pathways selectively, they exhibited specificity for TLR4 as neither peptide blocked TLR2-, TLR9-, and RIG-I–dependent pathways selectively, they exhibited specificity for TLR4 as neither peptide blocked TLR2-,
TARGETING THE TLR4 SIGNALING COMPLEX ASSEMBLY


