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Viral Inhibitory Peptide of TLR4, a Peptide Derived from Vaccinia Protein A46, Specifically Inhibits TLR4 by Directly Targeting MyD88 Adaptor-Like and TRIF-Related Adaptor Molecule

Tatyana Lysakova-Devine,* Brian Keogh,† Barry Harrington,* Kamalpreet Nagpal,‡ Annett Halle,‡ Douglas T. Golenbock,‡ Tom Monie,§ and Andrew G. Bowie*

TLRs are critical pattern recognition receptors that recognize bacterial and viral pathogen-associated molecular patterns leading to innate and adaptive immune responses. TLRs signal via homotypic interactions between their cytoplasmic Toll/IL-1R (TIR) domains and TIR domain-containing adaptor proteins. Over the course of evolution, viruses have developed various immune evasion strategies, one of which involves inhibiting TLR signaling pathways to avoid immune detection. Thus, vaccinia virus encodes the A46 protein, which binds to multiple TIR-domain containing proteins, ultimately preventing TLRs from signaling. We have identified an 11-aa-long peptide from A46 (termed viral inhibitor peptide of TLR4, or VIPER), which, when fused to a cell-penetrating delivery sequence, potently inhibits TLR4-mediated responses. VIPER was TLR4 specific, being inert toward other TLR pathways, and was active in murine and human cells and in vivo, where it inhibited LPS-induced IL-12p40 secretion. VIPER also prevented TLR4-mediated MAPK and transcription factor activation, suggesting it acted close to the TLR4 complex. Indeed, VIPER directly interacted with the TLR4 adaptor proteins MyD88 adaptor-like (Mal) and TRIF-related adaptor molecule (TRAM). Viral proteins target host proteins using evolutionary optimized binding surfaces. Thus, VIPER possibly represents a surface domain of A46 that specifically inhibits TLR4 by masking critical binding sites on Mal and TRAM. Apart from its potential therapeutic and experimental use in suppressing TLR4 function, identification of VIPER’s specific binding sites on TRAM and Mal may reveal novel therapeutic target sites. Overall, we demonstrate for the first time disruption of a specific TLR signaling pathway by a short virally derived peptide. The Journal of Immunology, 2010, 185: 4261–4271.
of type I IFN in response to vaccinia virus (VACV) in vivo, whereas TLR4-mediated signaling limited viral replication and increased animal survival during a VACV infection (21, 22). Given the importance of such antiviral responses, it comes as no surprise that many pathogens have developed ways to evade recognition and/or signaling by TLRs (23). VACV encodes for many immunomodulatory proteins, such as A46, A52, N1, B14, and K7, which target various components of innate immune signal transduction pathways (24–28). For example, A46 inhibits TLR signaling by interacting with the TIR domain-containing proteins Mal, TRAM, MyD88, and TRIF, resulting in inhibition of TLR-induced NF-κB, MAPK, and IRF3 activation (29).

Apart from their roles in antipathogen responses, the number of autoimmune and inflammatory diseases, in which TLRs play a key role, is rapidly growing. Thus, TLR4 has been shown to participate in the development of rheumatoid arthritis, atherosclerosis, septic shock, and many others (30, 31). In light of this, TLRs rather than the effector molecules they induce have become a new target for drug development to fight inflammatory and autoimmune conditions. Further, by inhibiting signaling or ligand recognition by a specific TLR, the degree of inflammation can be significantly reduced while other pattern recognition receptors remain functional, leaving the host immunocompetent.

The design of decoy cell-penetrating peptides (CPPs) is one rational approach to developing TLR inhibitors, whereby peptides derived from signaling proteins act as a dominant negative of the parental protein to prevent signal transduction by a specific signaling pathway. Such peptides were derived from the BB loop of TLR4. Inhibition of TLR-induced NF-κB by TLR4 signaling proteins has been reported in both in vitro and in vivo models (32, 33).

Given that A46 likely represents an evolutionary refined strategy to inhibit TLR signaling, understanding how it functions at the molecular level will help to further explain the molecular interactions underlying TLR signaling and also may lead to the development of specific therapeutic agents. Based on this, and the success of BB loop CPPs, using peptides derived from refined viral TLR inhibitory proteins may be an optimal approach. McCoy et al. (37) previously reported that a peptide derived from the VACV protein A52, named P13, inhibited signaling by several TLRs and inner ear inflammation in vivo in mice. However, P13, like other reported CPPs, lacked specificity for distinct TLRs and also failed to inhibit in human cells (see Fig. 3C). Furthermore, no host target for P13 was identified. In this study, we describe a potent TLR4-specific inhibitory peptide named viral inhibitory peptide of TLR4 (VIPER) derived from A46, which inhibits multiple TLR4-mediated responses. VIPER was active in murine and human cells and in vivo, being inert toward other TLR pathways. Furthermore, we identify host targets for VIPER as the TLR4 adaptors Mal and TRAM. To our knowledge, VIPER is the first pathway-specific TLR viral inhibitory peptide identified, and its discovery increases our understanding of the molecular interactions involved in TLR signaling, whereas identification of VIPER’s specific binding sites on TRAM and Mal may reveal novel therapeutic target sites.

**Materials and Methods**

**Cell culture**

The human embryonic kidney (HEK) cell line 293 (HEK293) and HEK293 cells stably transfected with IL-1R (HEK293 R1) were a gift from Tulairk (San Francisco, CA). HEK293 cells stably transfected with TLR2, -3, -4, or -8 (HEK293 TLR) were a gift from Dr. K. Fitzgerald (University of Massachusetts Medical School, Worcester, MA). The mouse leukemia monocye-macrophage cell line RAW264.7 and the human acute monocytic leukemia cell line THP-1 were obtained from the European Collection of Animal Cell Cultures (Salisbury, U.K.). Immortalized murine wild-type (wt) and MyD88−/−, Mal−/−, TRIF−/−, and TRAM−/− bone marrow–derived macrophages (bMDMs) were generated from corresponding knockout mice using J2 recombinant retrovirus carrying v-myel and v-raf/mi oncogenes as previously described (38, 39). Human PBMCs were purified from the buffy coat of heparinized whole-blood preparations from healthy volunteers by density centrifugation on low-endotoxin Ficoll-Hypaque. Isolated PBMCs was washed three times in sterile PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM NaH2PO4) counted, and seeded at a density of 1 × 106 cells/ml in complete RPMI 1640 medium.

**Peptide synthesis and reconstitution**

Peptides were synthesized by GenScript (Piscataway, NJ) and were >95% pure as identified by HPLC. Lyophilized peptides were reconstituted aseptically with molecular biology-grade water to a concentration of 10 mM and stored at −80˚C. Working stocks of 0.2 or 1 mM were stored at −20˚C or kept at 4˚C for a maximum of 2 wk. See Table I for the sequences of the peptides used.

**Receptor agonists**

Ultrapure LPS from Gram-negative bacteria (Eschericia coli) (>99.9% pure in respect to contaminating protein, DNA, and TLR2 agonists) was purchased from Alexis Biochemicals (Plymouth Meeting, PA). Polyinosinic-polyctydicytic acid (poly-IC) was purchased from Amersham Biosciences (Piscataway, NJ). IL-1α was obtained from the National Cancer Institute (Frederick, MD), and TNF-α was a gift from Zenea Pharmaceuticals (Macclesfield, U.K.). CpgG was purchased from Eurotins MWG Operon (85560; Ebersberg, Germany), and PMA was purchased from Sigma-Aldrich (Dublin, Ireland). MALP2 was purchased from Alpha Technologies (Wicklow, Ireland), and PAM-CSK was purchased from Autojen Bioclear (Nottingham, U.K.).

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Cell viability analysis by MTT

MTT was purchased from Sigma-Aldrich and reconstituted at 1 mg/ml in PBS. Cells were seeded into 96-well plates and treated as described in the figure legends. Medium was removed, and the cells were washed once with PBS. A total of 200 μl/well of 1 mg/ml MTT solution was added directly to the cells, and the plates were incubated at 37°C for 2 h in the dark. Postincubation, the MTT solution was discarded, and 200 μl/well DMSO solution was added for 20 min at 37°C in the dark and the absorbance read at λ = 595 nm.

Reporter gene activation, mRNA, and cytokine analysis

Unless otherwise stated, peptides were added at required concentrations 1 h prestimulation with agonists for 6 h. HEK293 cells were seeded at 1 × 10^5 cell/ml and reporter gene assays performed as previously described (29). Induction of mRNA was assayed in wt iBMDMs by quantitative real-time PCR. Cells were seeded at 2 × 10^5 cell/ml 24 h pretreatment. RNA was isolated using High Pure RNA isolation kits from Roche Applied Science (Burgess Hill, U.K.) according to the manufacturer’s instructions. RT-PCR was performed using Quagen’s One-Step RT-PCR Kit (Qiagen, Valencia, CA) according to manufacturer’s instructions. Quantitative real-time PCR was done using GoTaq qPCR Master Mix (Promega, Valencia, CA) according to the manufacturer’s instructions. For cytokine production, RAW264.7 cells and iBMDMs were seeded at 2 × 10^5 cell/ml. THP-1 cells were differentiated with 100 nM PMA and seeded at 5 × 10^5 cell/ml, and PBMCs seeded at 1 × 10^6 cell/ml in 96-well plates 24 h prior to treatment. The supernatants were collected and assessed for different cytokines by ELISA (R&D Systems, Minneapolis, MN).

Immunoblotting

For analysis of IκB degradation and MAPK activation, iBMDMs were seeded at 2 × 10^5 cell/ml in six-well plates 24 h pretreatment. The peptides were added at 5 μM 1 h before stimulating with 20 ng/ml LPS. After 30 min, supernatants were removed and cells washed with ice-cold PBS. Cells were lysed in 100 μl SDS sample buffer [62.5 mM Tris (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue, 50 mM DTT] and boiled for 5 min. Thirty microliters cooled lysate was resolved on 10% SDS-PAGE, transferred onto Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA), and probed with either mouse mAb against IκBa (a kind gift from Prof. R. Hay, Dundee University, Dundee, U.K.), rabbit anti-p38 phosphospecific Ab (Cell Signaling Technology, Danvers, MA), or rabbit anti-JNK phosphospecific Ab (Biosource International, Camarillo, CA). To control for protein loading, the membranes were reprobed with anti–β-actin Ab (Sigma-Aldrich), rabbit anti-p38 Ab (Cell Signaling Technology), or rabbit anti-JNK Ab (Bio-

FIGURE 1. Identification of VIPER as TLR4-specific inhibitor in murine cells. A, RAW264.7 cells were treated with 1 or 5 μM A46 peptides 1 h prestimulation with 10 ng/ml LPS. Supernatants were collected 6 h poststimulation and assayed for TNF-α by ELISA. B–E, iBMDMs were treated with 1 or 5 μM VIPER and CP7 peptides 1 h prestimulation with 20 ng/ml LPS. Supernatants were collected 6 h poststimulation and assayed for TNF-α, MIP-2, RANTES, and IL-6 by ELISA. iBMDMs were treated with 1 or 10 μM VIPER and CP7 peptides 1 h before stimulating with 20 ng/ml PAM3csk4 (F), 20 nM MALP2 (G), 25 μg/ml poly-IC (H), or 1 μg/ml CpG (I). Supernatants were collected 6 h poststimulation and assayed for TNF-α by ELISA. The data are mean ± SD of triplicate samples and are representative of at least three experiments. *p < 0.05 compared with LPS only.
Expression of GST-tagged proteins

The GEX.4T2 plasmid containing the TIR domains of Mal, TRAM, or TLR4 or empty GEX.4T2 were transformed into *Escherichia coli* Rosetta-Gami B Host Strains (Novagen, EMD Chemicals, Darmstadt, Germany) and grown in Terrific Broth. Protein expression was induced with 0.7 mM isopropyl β-D-thiogalactoside at 18˚C for GST-TLR4 and 30˚C for GST, GST-Mal, and GST-TRAM for 24 h. Cells were lysed in low-salt extraction buffer (300 mM NaCl, 1% Triton X-100, PBS). Whole-cell lysates were cleared and levels of protein expression confirmed by SDS-PAGE and Coomassie staining of the gel.

Peptide-pulldown assays

HEK293 cells were seeded at 1 × 10^5 cell/ml in 100-mm dishes 24 h prior to transfection. A plasmid encoding Flag-tagged Mal, TRAM, or TRIF or HA-tagged MyD88 was transfected into cells (2 ng/transfection) using GeneJuice (Novagen). After 24 h, supernatants were removed, and cells were washed with ice-cold PBS. Cells were lysed in 900 μl 1% NETN lysis buffer [100 mM NaCl, 50 mM HEPES (pH 7.5), 1 mM EDTA, 1% Nonidet P-40, 10% glycerol, and 50 mM imidazole] and incubated on ice for 40 min. A total of 300 μl whole-cell lysate was incubated with 25 μM polyhistidine (6xHis)-tagged VIPER or CP7 peptides (containing no delivery sequence) and 40 μl Ni-agarose beads for 2 h at 4˚C with rolling to avoid sedimentation of the beads. To exclude nonspecific binding, lysates were also incubated with the Ni-agarose beads alone. Postincubation, beads were washed five times in 1% Nonidet P-40 lysis buffer with 50 mM imidazole. After the final wash, the buffer was completely removed, and the beads were resuspended in 35 μl SDS sample buffer. Samples were boiled for 5 min and resolved with SDS-PAGE. The resolved proteins were transferred to polyvinylidene difluoride membrane and immunoblotted for the corresponding protein.

Determination of the effect of VIPER in vivo

Groups of five female BALB/c mice were injected i.v. with a single bolus of one of the following: PBS, 1 μg LPS, 1 μg LPS with 0.1 mg/kg 9 arginine (9R)-VIPER, 1 μg LPS with 0.3 mg/kg 9R-VIPER, 1 μg LPS with 0.1 mg/kg 9R-CP7, or 1 μg LPS with 0.1 mg/kg 9R-CP7. Four hours later, blood was harvested and serum derived. The serum was assayed for IL-12p40 by ELISA.

Statistical analysis

Statistical analysis was carried out using paired Student t test. Two-tailed p values were obtained comparing groups treated with peptide and LPS versus LPS only.

Structural modeling

Structural homologs to A46 were retrieved using the 3D-Jury metaserver (38). Alignments were manually adjusted and a model built using Modeler 9v7 with A52 as a template (University of California San Francisco, San Francisco, CA). All structural figures were generated using PyMOL (PyMOL Molecular Graphics System, version 1.2r3pre, Schrödinger, Camberley, U.K.). Electrostatic potentials were calculated using a nonlinear Poisson-Boltzmann equation with APBS tools in PyMOL.

Results

The A46-derived peptide VIPER is a potent TLR4-specific inhibitor in murine cells

VACV protein A46 was previously identified as an inhibitor of TLR signaling (29). To test the ability of regions of A46 to recapitulate TLR inhibition, we designed peptides from two regions of A46 protein that were found to be important for inhibition of TLR signaling by using truncation mutants of A46 (data not shown).

**FIGURE 2.** VIPER inhibition of TLR4 is independent of the nature or position of the delivery sequence. iBMDMs were treated with 1 or 5 μM His-tagged peptides VIPER and CP7 with (B) or without (A) the 9R delivery sequence for 1 h prestimulation with 10 ng/ml LPS. iBMDMs were treated with peptides VIPER and CP7 with the 9R delivery sequence at the N terminus (C), with the α-enantiomer of the peptides with the 9R delivery sequence at either C (D) or N termini (E) or with the β-enantiomer of the peptides with a TAT delivery sequence at the N terminus (F). The peptides were added at 1, 5, and 25 μM for 1 h prestimulation with 10 ng/ml LPS. In all cases, supernatants were collected 6 h poststimulation and assayed for TNF-α by ELISA. The data are mean ± SD of triplicate samples and are representative of at least three experiments. *p < 0.05 compared with LPS only.
The designed peptides contained 11 aa from A46, and each proceeding peptide was designed to overlap with the previous one by 5 aa. These peptides were linked to a 9R homopolymer delivery sequence at their C termini, as this was shown to be an efficient way to deliver peptides into cells (37, 40). The peptides were aseptically reconstituted in water, and only water-soluble peptides were assayed for biological activity using the murine macrophage cell line RAW264.7. Cells were treated with 1 and 5 μM peptide 1 h prestimulation with LPS. Among the 12 peptides tested, peptide #4 (KYSFKLILAEY-9R) was identified as the only peptide that inhibited LPS-induced TNF-α production (Fig. 1A). In this study, the peptide #4 was termed VIPER. VIPER also inhibited LPS-induced TNF-α, MIP-2, RANTES, and IL-6 in iBMDMs, whereas an inert control peptide, CP7 (Table I), had no effect (Fig. 1B–E). Neither VIPER nor CP7 affected cell viability at the concentrations used (data not shown).

Next, we investigated the effect of VIPER on other murine TLRs and found that in contrast to the ability of full-length A46 to inhibit all TLRs (29), VIPER at concentrations of 1 and 10 μM did not inhibit secretion of TNF-α in response to PAM3CSK4 (which signals via a TLR2/TLR1 heterodimer), MALP2 (via TLR2/TLR6), poly-IC (via TLR3), nor CpG (via TLR9) (Fig. 1F–I). This identified VIPER as the first TLR4-specific inhibitor derived from a viral protein.

To investigate whether VIPER required internalization to inhibit TLR4, we used the VIPER peptide with and without the 9R delivery sequence. For this experiment, both VIPER and CP7 contained a polyhistidine tag (6xHis) fused to the N termini, and therefore peptides without the delivery sequence were termed His-VIPER or His-CP7, and the peptides with the 9R delivery sequence were termed His-VIPER-9R or His-CP7-9R. Inhibition of LPS-induced TNF-α secretion by VIPER required the delivery sequence to be present (Fig. 2A, 2B). In contrast, the position of the delivery sequence was not important, as VIPER inhibited equally well when the 9R sequence was linked to either the C terminus (Fig. 1B) or the N terminus (9R-VIPER; Fig. 2C). We also tested a D-form of VIPER (D-VIPER), composed of D-enantiomers of the L-amino acids of the parental sequence. This modification prolongs the t1/2 of peptides both in vitro and in vivo by rendering them resistant to proteosomes, which often increases a peptide’s biological activity (41, 42). D-VIPER still retained its inhibitory properties (Fig. 2D), with slightly reduced potency when the delivery sequence was fused to the N terminus (Fig. 2E). Another commonly used delivery sequence, TAT 49–57, which is derived from the HIV-1 protein TAT, was tested to ensure the inhibitory properties of VIPER did not depend on the polyarginine sequence.

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TAT49–57 was linked to the N terminus of VIPER, and the D-iso-
mer of TAT-VIPER was tested (Fig. 2F). This peptide still retained
the ability to inhibit LPS-induced TNF-α secretion, but with
a slightly decreased potency, which likely was due to less efficient
delivery of peptides into cells by TAT 49–57 when compared with
9R, as previously reported (40).

Together, these data indicate that VIPER specifically inhibits
murine TLR4, that inhibition requires internalization of the pep-
tide, and that inhibition is independent of the nature or position of
the delivery sequence.

VIPER inhibits TLR4 in primary human cells

We next tested the inhibitory properties of VIPER in human cells.

The array of the A46-derived peptides was reassayed in the hu-
man monocyte cell line THP-1 at 5 μM and similar to murine
cells, only VIPER was found to inhibit LPS-induced TNF-α production (Fig. 3A). A peptide derived from the Vaccinia virus
A52, termed P13, was previously reported to inhibit various
TLR including TLR4 in murine cells (37). However, compared
with VIPER, P13 was ineffective in human cells, because even at
40 μM P13 failed to inhibit LPS-induced TNF-α production in
THP-1s (Fig. 3B) and in PBMCs (data not shown). In contrast,
VIPER dose dependently inhibited LPS-induced TNF-α in pri-
mary human PBMCs between 1 and 25 μM (Fig. 3C) without
affecting the cells’ viability as determined by MTT assay (Fig.
3D). The IC₅₀ for TNF-α production in PBMCs was determined
as 5 μM (Fig. 3E).

VIPER inhibits TLR4-dependent cytokine induction in vivo

To examine the ability of VIPER to inhibit cytokine production
in vivo, we tested the effect of the peptide on LPS-induced cytokine
production in BALB/c mice. As seen in Fig. 4, injection of mice
with LPS induced a 4-fold increase in serum levels of IL-12p40.
Coinjection of VIPER, but not CP7, with LPS resulted in a sig-
nificant and dose-dependent suppression of IL-12p40 levels (Fig.
4). Thus, the VIPER peptide was found to be able potentally inhibit
the induction of the proinflammatory cytokine IL-12p40 upon LPS
administration in vivo.

VIPER inhibits TLR4 signaling at the receptor-adaptor level

To investigate the mechanism of inhibition of TLR4-induced cy-
kotive production by VIPER, we examined its effect on TLR4-
induced transcription and signaling. Quantitative RT-PCR showed
that VIPER inhibited induction of both LPS-induced TNF-α mRNA
(Fig. 5A) and LPS-induced IFN-β mRNA (Fig. 5B).

TLR4 is the only known receptor that signals via both the
MyD88-dependent pathway to activate NF-κB and MAPKs and
the TRIF-dependent pathway to activate IRF3 and late NF-κB
(43–45), whereas other IL-1/TLR family members use either
MyD88 (e.g., IL-1, TLR8) or TRIF (e.g., TLR3). To investigate
which of the two pathways is targeted by the peptide, we per-
formed reporter gene assays in HEK293 cells stably expressing
TLR4, TLR8, IL-1R, or TNF-α receptor 1. VIPER completely
prevented activation of NF-κB in TLR4-expressing cells upon
LPS stimulation (Fig. 5C), but had no effect on NF-κB activation

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**FIGURE 5.** VIPER inhibits TLR4 signaling pathways. A and B, iBMDMs were treated with 5 μM peptides 1 h prestimulation with 10 ng/ml
LPS for 6 h. Induction of TNF-α and IFN-β mRNA expression was assayed by quantitative RT-PCR. HEK293 cells stably expressing TLR4 (C), TLR8
(D), IL-1R (E), TLR3 (F), or nothing (G) and transfected with the NF-κB luciferase reporter gene were treated with 5 and 10 μM VIPER prestimulation
with 10 ng/ml LPS, 3 μg/ml CL075, 50 μg/ml IL-1α, 25 μg/ml poly-IC, or 50 ng/ml TNF-α, respectively, for 6 h, prior to luciferase assay. I, 293HEK
cells stably expressing TLR4 and transfected with IRF3-Gal4 and pFR-luciferase reporter gene were stimulated with 10 ng/ml LPS for 6 h prior to
luciferase assay. For A–G and I, the data are mean ± SD of triplicate samples and are representative of at least three experiments. H, J, and K, A total of
5 μM VIPER and CP7 were added to iBMDMs prestimulation with 20 ng/ml LPS for 30 min. Lysates were immunoblotted for IκB (H), p-JNK (J), and
p-p38 (K) with loading controls of β-actin, total JNK, and total p38, respectively. Each immunoblot is representative of at least three experiments. *p < 0.05
compared with LPS only.
via the MyD88-dependent pathway in TLR8 and IL-1R–expressing cells (Fig. 5D, 5E) nor on TRIF-mediated TLR3-induced NF-κB activation (44) (Fig. 5F). Also, VIPER did not affect TNF-induced activation of NF-κB via TNF-α receptor 1 (Fig. 5G). Thus VIPER inhibits NF-κB activation, but only in response to TLR4. In addition, VIPER completely prevented degradation of IκB upon LPS treatment of RAW264.7 cells (Fig. 5H).

Next, the effect of VIPER on IRF3 activation via the TRIF-dependent pathway (44) was measured, and it was established that LPS-induced activation of IRF3 was completely prevented by the peptide (Fig. 5I). Finally, LPS-induced activation of the MAPKs JNK and p38 were assayed. VIPER inhibited TLR4-mediated phosphorylation of JNK and p38 after 30 min of stimulation with LPS (Fig. 5J, 5K).

**FIGURE 6.** VIPER directly targets the TLR4 adaptors Mal and TRAM. A, HEK293 cells were transfected with Flag-Mal, HA-MyD88, Flag-TRAM, or Flag-TRIF for 24 h and cell lysates generated. B, GST protein or GST-tagged TIR domains of Mal, TRAM, and TLR4 were expressed in Rosetta-Gami E. coli prior to generation of lysates, as described in Materials and Methods. In A and B, lysates were incubated with 25 μM 6xHis-tagged VIPER or CP7 peptides (containing no delivery sequence) and Ni-agarose beads as described in Materials and Methods. Proteins were eluted from the beads with SDS sample buffer, resolved by SDS-PAGE, and immunoblotted for the corresponding protein. C–F, Immortalized MyD88+/−, TRIF+/−, Mal−/−, or TRAM−/− BMDMs were treated with 1 and 5 μM VIPER or CP7 for 1 h and stimulated with 20 ng/ml LPS for 6 h. Supernatants were collected and assayed for TNF-α. The data are mean ± SD of triplicate samples. G and H, A total of 5 μM VIPER and CP7 were added to TRAM−/− and Mal−/− BMDM for 1 h prestimulation with 20 ng/ml LPS for 30 min. Lysates were immunoblotted for p-JNK with loading control of total JNK. All data are representative of at least three experiments. *p < 0.05; **p < 0.005 compared with LPS only.
Collectively, these data suggest that VIPER prevents activation of all TLR4-induced signaling pathways by targeting TLR4 receptor-adaptor complex upstream of its adaptors MyD88 and TRIF.

**VIPER directly targets the TLR4 adaptors Mal and TRAM**

Upon ligand binding, TLR4 forms a homodimer that recruits MyD88 and TRIF via two bridging adaptors Mal and TRAM, respectively (14, 18). To date, TRAM was considered to be involved only in TLR4 signaling (18) and would therefore make a likely target for the peptide to explain its TLR4 specificity. However, VIPER also inhibits the TLR4/MyD88-dependent pathway without affecting other TLR-MyD88–dependent responses (Fig. 5), and therefore, Mal would be another possible target for the peptide (46). Also, it was possible the peptide targets TLR4 itself, preventing its homodimerization or interaction with the adaptors. To identify which, if any, of the aforementioned scenarios were possible, we examined the ability of VIPER to interact with the TIR proteins in cells by a pulldown assay using VIPER and CP7 peptides linked to a 6xHis tag on the N termini. Flag-tagged Mal, TRAM, and TRIF and HA-tagged MyD88 were overexpressed in HEK293 cells, and 25 μM peptide was incubated with the whole-cell lysate in the presence of Ni²⁺ agarose beads at 4°C. Interestingly, Mal and TRAM but not MyD88 nor TRIF were detectable in the His–VIPER complex isolated (Fig. 6A). Importantly, the noninhibiting peptide CP7 did not complex with any of the TLR4 adaptors. To investigate whether VIPER binds to TLR4 directly, we expressed the GST-tagged TIR domain of TLR4 in a bacterial system, thus excluding the possibility of indirect interaction between the peptide and TLR4 due to presence of endogenous Mal or TRAM in mammalian lysates. As a positive control, we included GST-tagged TIR domains of Mal and TRAM and also overexpressed GST protein alone to rule out nonspecific binding. In this system, the interaction between VIPER and Mal or TRAM observed in mammalian cell lysates was recapitulated for GST-Mal and GST-TRAM, strongly suggesting a direct peptide-adaptor interaction, whereas GST-TLR4 failed to interact with VIPER (Fig. 6B).

In support of the dual targeting of Mal and TRAM, we found that the ability of VIPER to inhibit LPS responses was not dependent on the presence of one specific TLR4 adaptor, because LPS-induced TNF-α secretion was still inhibited in iBMDMs lacking MyD88, TRIF, Mal, or TRAM (Fig. 6C–F). The iBMDMs lacking either MyD88, TRIF, Mal, or TRAM used in the assay showed impaired levels of TNF-α secretion upon LPS treatment compared with the wt iBMDMs (Fig. 1B). Also, VIPER inhibited activation of JNK equally well in the absence of either Mal or TRAM (Fig. 6G, 6H).

Together, these data show that VIPER directly targets the TIR domains of both Mal and TRAM.

**Identification of the residues in VIPER critical for TLR4 inhibition**

We next attempted to identify which amino acids within the VIPER sequence were critical for the inhibition of TLR4 via Mal and TRAM targeting. From the initial scan of all A46-derived peptides, we noted that the neighboring peptides #3 and #5 that also overlap with either end of VIPER had no inhibitory effect on TLR4 signaling. Thus, four VIPER peptides with deletions of terminal amino acids were synthesized, with the 9R delivery sequence at their N termini: N-1 (9R-YSFKLILAEY), N-2 (9R-SFKLILAEY), C-3 (9R-KYSFKLLIL), and C-6 (9R-KYSFK). These peptides were assayed for TLR4 inhibition in iBMDMs, and it was found that only the peptide with the deletion of the 6 aa from the C terminus had significantly reduced ability to inhibit LPS-induced TNF-α production compared with full-length VIPER (Fig. 7A). The same results were found in PBMCs (data not shown). Thus, the sequence SFKLIL within VIPER was especially important for TLR4 inhibition. Furthermore, we performed an alanine scan, which involved synthesis of a series of peptides, each with a substitution of one of the amino acids in the VIPER peptide sequence for alanine. Thus, 10 new peptides were designed (Table I) and assayed for inhibition of LPS-induced TNF-α in iBMDMs compared with VIPER. As a result, we identified leucine at position 6 (KYSFKLILAEY) as a critical residue for inhibition of TLR4 because L6A was the only peptide that lost TLR4 inhibitory potential (Fig. 7B).

**Position of VIPER on an A46 structural model**

To further examine the mechanism whereby VIPER inhibits TLR4 signaling via targeting Mal and TRAM and the relevance of this to how the VACV protein A46 antagonizes TLR4 function, the structure of full-length A46 protein was modeled. Homology modeling for A46 produced a Bcl-2–like fold consisting of seven α helices from aa 87–212, which includes the VIPER sequence. The most significant structural homology was found with the VACV Bcl-2–like proteins A52 and B14 (47). A52 was used as the final model template, and the alignment of the resulting structures and model is shown in Fig. 8A. The VIPER motif is located at the very N terminus of the first helix (Fig. 8B). Consistent with its critical inhibitory role, leucine 6 in the VIPER motif is surface exposed and would be available for interaction with other proteins. Interestingly, the VIPER motif sits in an electropositive patch on the modeled surface of A46 (Fig. 8C). This complements the proposed surfaces used by the TIR domains of Mal and TRAM to engage with TLR4 (9), both of which are predicted to be electronegative (Fig. 8D, 8E). Consequently, electrostatic attraction may play a key role in initiating inhibitory protein contacts between A46 and Mal or TRAM.

Together, these data indicate that VIPER represents a critical surface used by the poxviral protein A46 to antagonize TLR4 function via interaction with TRAM and Mal.
**Discussion**

The fact that VACV has developed a number of effective strategies for inhibiting TLRs attests to the importance of these host receptors in antiviral immunity. VACV encodes a number of proteins that have been proven or predicted to adopt a Bcl-2-like fold and shown to manipulate TLR signaling pathways (48). Thus, N1 prevents activation of NF-κB and IRF3 by interacting with the IkB kinase complex and TANK-binding kinase 1 (28). K7 inhibits TLR-mediated IRF3/7 activation by interacting with DEAD box protein 3 (49). A52 inhibits activation of NF-κB by TLRs by interacting with IL-1R-associated kinase 2 (24, 50) and enhances MAPK activation via TNFR-associated factor 6 (51). Unlike any of the aforementioned proteins, A46 inhibits TLR signaling by interacting directly with TIR domain-containing proteins (29). Indeed, A46 has been shown to be able to associate with the TIR domains of TLR4, IL-1R accessory protein, MyD88, Mal, TRIF, and TRAM, consistent with its ability to inhibit multiple IL-1 and TLR pathways (29). However, how exactly A46 antagonizes TIR function remains to be determined.

The development of decoy peptides derived from the BB loop of the TIR domains of TLR2/4/1/6, Mal, and MyD88, which were shown to inhibit TLR signaling (32–36), led to the idea of designing peptides derived from A46 that might inhibit TLRs and thus help to understand the molecular basis whereby A46 functions. Furthermore, such peptides might act as more potent TLR inhibitors than previous ones, because viral immunosuppressive proteins have been finely tuned and honed by evolution to target the host immune system with maximal effectiveness. This is analogous to a naturally occurring drug development program, whereby the protein has already undergone cycles of modification due to natural selection, leading to enhanced inhibitory function. Thus, the identification of such virally derived inhibitory peptides would lead to insights at the molecular level as to how TLRs function, how they are antagonized by viruses, and also may form the basis of therapeutics either based on the peptides themselves or the sites on host proteins they optimally antagonize.

Given the ability of A46 to inhibit multiple TLR signaling pathways, it came as a surprise to identify VIPER, an A46-derived peptide that specifically inhibited TLR4 and not IL-1 nor other TLRs. This suggests that A46 does not have a generic interaction site for all the TIR proteins it antagonizes but rather has specific sites for interaction with different proteins, with VIPER representing the region of A46 important for TLR4 inhibition. Consistent with this, VIPER was found to directly interact with two adaptor proteins essential for TLR4 signaling, namely TRAM and Mal, and not MyD88 nor TRIF, and thus VIPER is likely derived from a region on the A46 surface that interacts with Mal and TRAM.

Because A46 was originally shown to contain some conserved sequence motifs of a TIR domain (29), it was assumed that A46 would adopt a TIR fold and thus bind to TIR proteins by engaging in homotypic interactions with them. However, since then, the crystal structure of poxviral proteins with some shared sequence similarity to A46 has been determined and shown to adopt a Bcl-2-like fold, leading to the suggestion that A46 also adopts such a Bcl-2-like fold (47, 48, 52). Consistent with this, the VIPER sequence is predicted to be on the surface of A46 when modeled as a Bcl-2-like fold.
fold using the A52 structure (Fig. 8). Furthermore, the amino acid critical for inhibition of TLR4 within VIPER, leucine 6, was shown in this model to be surface exposed and thus would be available for interaction with host TIR proteins. Thus, VIPER most likely represents a surface on a Bcl-2–folded viral protein that is capable of specific antagonism of TLR4. From a mechanistic perspective, one can envisage that leucine 6 may insert into a hydrophobic pocket on the TIR domain of the target proteins Mal and TRAM, thereby preventing access to the TLR4 TIR domain. Interestingly, the VIPER motif sits in an electropositive patch on the modeled surface of A46 (Fig. 8C). This complements the proposed surfaces on the TIR domains of Mal and TRAM thought to interact with TLR4 (9), which are predicted to be electronegative (Fig. 8D, 8E). Consequently, electrostatic attraction may play a key role in initiating inhibitory protein contacts between A46 and Mal or TRAM. This trapping of a host signaling protein by VACV has been recently dramatically illustrated at the molecular level by the structural determination of a complex of VACV protein K7 with a peptide derived from the N-terminal motif of DDX3 responsible for its interactions with IκB kinases (27).

The targeting of TRAM by VIPER is consistent with the specific role for TRAM in TLR4 signaling (53). In contrast, the second target of VIPER, Mal, is involved in both TLR4 and TLR2 responses. However, antagonism of Mal by VIPER without an effect on TLR2 could be explained by the suggested model in which Mal binds to TLR4 and TLR2 via two different interfaces (54). In TLR4 signaling, Mal was predicted to interact with the surface formed by the BB loops of the TLR4 homodimer via the region near its own BB loop (9). In TLR2 signaling, in contrast, the DD loop of Mal seemed to play the more important role in the TLR2–Mal interaction as the S180L polymorphism of Mal prevents this interaction (55, 56). Therefore, it is possible that VIPER targets the interface of Mal responsible for interactions with TLR4, leaving the TLR2-binding surface intact. In addition, there are recent reports suggesting that Mal is redundant in TLR2 signaling (15–17). Furthermore, Mal has been reported to have a negative role in TLR3 signaling (16), which may explain why VIPER enhanced TLR3-mediated NF-κB activation (Fig. 5F).

Apart from these insights into TLR4 complex formation and disruption by a virus, VIPER itself represents a highly potent and specific species-independent antagonist of TLR4. In contrast, the previously reported viral peptide P13, derived from another VACV TLR-signaling inhibitory protein A52, was shown not to have TLR inhibitory capacity in human cells (Fig. 3B and data not shown). Furthermore, in murine cells, P13 is less potent than VIPER, and its target(s) is unknown (37, 57). VIPER also inhibits LPS-induced activation of transcription factors and induction of mRNA at much lower concentrations than the host-derived BB loop peptides and is a smaller molecular entity (32, 33). Importantly, when tested in mice, VIPER successfully inhibited LPS-induced IL-12/23 p40, one of the key cytokines involved in the pathogenesis of autoimmunity (58, 59) at a dose of 0.1 mg/kg when coadministered with LPS, demonstrating rapid and potent TLR4 antagonism in vivo.

Indeed, given the role of TLR4 in disease pathogenesis, the development of specific TLR4 inhibitors is an important goal. The described properties of VIPER combined with its relatively small size lend it to further development into peptidomimetic compounds. Bartfai et al. (60) reported the successful development of a low m.w. compound based on the tripeptide sequence from the MyD88 BB loop that was specific for IL1R1 signaling. A similar approach may be applied to the VIPER peptide to develop a TLR4–specific small molecule inhibitor. TLR4 inhibitors are of particular interest for use in a number of conditions including severe sepsis, sterile inflammation (61), ischemia/reperfusion injury (62–64), atherosclerosis (31), rheumatoid arthritis (31), acute lung injury (65), etc. Traditionally, attempts to control sepsis or autoinflammation have centered on blockade of proinflammatory cytokines such as TNF-α, a presumed critical effector of TLR4-mediated inflammation and LPS toxicity; however, TLR4 itself may be a much more effective target for intervention, as it would prevent actual initiation of inflammation.

In summary, we have identified VIPER, a specific TLR4 inhibitor that acts by directly targeting Mal and TRAM. VIPER likely represents a surface domain of A46 that specifically inhibits TLR4 by masking critical binding sites on Mal and TRAM. Apart from its potential therapeutic and experimental use in suppressing TLR4 function, identification of VIPER’s specific binding sites on TRAM and Mal may reveal novel therapeutic target sites. Overall, we demonstrate for the first time, to our knowledge, disruption of a specific TLR signaling pathway by a short virally derived peptide, leading to a molecular explanation as to how a poxviral Bcl-2–like protein antagonizes TLR4 function.

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Disclosures

A patent has been filed on VIPER as an inhibitor of TLR4.

References


