VIPER, a peptide derived from vaccinia protein A46, specifically inhibits TLR4 by directly targetting Mal and TRAM

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TLR4 inhibition by a viral peptide

Abstract

TLRs are critical pattern recognition receptors, which 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-1 receptor (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 in order to avoid immune detection. Thus, vaccinia virus (VACV) encodes the A46 protein, which binds to multiple TIR-domain containing proteins, ultimately preventing TLRs from signaling. We have identified an 11-amino acid long peptide from A46 (termed VIPER), which when fused to a cell-penetrating delivery sequence potently inhibits TLR4-mediated responses. VIPER was TLR4-specific, being inert towards 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 Mal and 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.

Introduction

Cells of the innate immune system express a number of pattern recognition receptors amongst which are the retinoic acid-inducible gene I-like helicases, nucleotide-oligomerisation domain (NOD)-like receptors and TLRs (1-3). There are 13 mammalian TLRs reported to date which recognise a wide array of pathogen associated molecular patterns (4). TLRs are members of the larger TLR/IL1-receptor (TIR) domain-containing family of proteins, which also includes IL-1 receptors, IL-18 receptors and a group of orphan receptors (5).

The TLRs are type I transmembrane receptors, that consist of a globular TIR domain-containing intracellular domain, a single trans-membrane spanning section and an extracellular domain mainly made up of 19 to 25 leucine-rich repeats (LRRs), which mediate the specificity of the ligand-receptor interaction (6). Most TIR domains contain a protruding BB-loop, which is essential for homo- and hetero-dimerisation (7-10). Such dimerisation interactions occur upon ligand binding to the TLR and are believed to be triggered by conformational changes in the TIR domain of the receptors, which promotes recruitment of the TIR domain-containing adaptor proteins (11, 12). There are five such adaptor proteins known. Thus, all TLRs, except TLR3, signal via MyD88 (13), TIR domain-containing adaptor inducing interferon-β ((TRIF)) is employed by TLR3 and TLR4 and MyD88-adaptor-like (Mal, or TIRAP) is involved in TLR4 and TLR2 signalling where it plays the role of a bridging adaptor between the receptor and MyD88 (14). However, recent publications suggest that Mal is redundant in TLR2 signalling under certain circumstances (15, 16, 17). TRIF-related adaptor molecule (TRAM) is recruited by TLR4 only and like Mal is a bridging adaptor, here between TLR4 and TRIF (18). The fifth adaptor, sterile α and HEAT-armadillo motifs protein (SARM), was shown to negatively regulate

TRIF-dependent signalling for TLR3 and TLR4 (19). The formation of receptor-adaptor complexes triggers stimulation of signal transduction pathways which ultimately leads to the activation of mitogen-activated protein kinases (MAPK), such as p38 and JNK, and transcription factors such as NF-κB, IFN regulatory factor 3 (IRF3), IRF5 and IRF7, leading to induction of pathway-specific gene transcription (4). Thus, TLR3, 7, 8 and 9, which recognise viral nucleic acids, mount a potent antiviral response by inducing type I IFNs (20). TLR4 and 2, well-known to recognise bacterial ligands, are also involved in recognition of viral glycoproteins, leading to the induction of pro-inflammatory responses (4). Also, recently TLR2 was found to be a key receptor for the induction of type I IFN in response to VACV in vivo, while TLR4-mediated signalling 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 signalling 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 signalling 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 anti-pathogen responses, the number of autoimmune and inflammatory diseases, in which TLRs play a key role, are rapidly growing. Thus, TLR4 has been shown to participate in the development of rheumathoid arthitis (RA), atherosclerosis, septic shock and many others (30, 31). In light of this, TLRs rather then the effector molecules they induce have become a new target for drug development to fight inflammatory and autoimmune conditions. Further, by inhibiting

signalling or ligand recognition by a specific TLR the degree of inflammation can be significantly reduced while other PRRs 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 signalling proteins act as a dominant negative of the parental protein to prevent signal transduction by a specific signalling pathway. Such peptides were derived from the BB-loop of the TIR domains of TLR2, 4, 1, 6, Mal and MyD88 and were shown to interfere with homodimerisation of the TIR domains, thus blocking signal transduction (32-36).

Given that A46 likely represents an evolutionary refined strategy to inhibit TLR signalling, understanding how it functions at the molecular level will help to further explain the molecular interactions underlying TLR signalling, 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. previously reported that a peptide derived from the VACV protein A52, named P13, inhibited signalling by several TLRs and inner ear inflammation *in vivo* in mice. (37). However, P13 like other reported CPPs lacked specificity for distinct TLRs, and also failed to inhibit in human cells (see Fig 3*C*). Furthermore no host target for P13 was identified. Herein we describe a potent TLR4-specific inhibitory peptide named VIPER (for viral inhibitory peptide of TLR4) derived from A46, which inhibits multiple TLR4-mediated responses. VIPER was active in murine and human cells and *in vivo*, being inert towards other TLR pathways. Furthermore we identify host targets for VIPER as the TLR4 adaptors Mal and TRAM. VIPER is the first pathway-specific TLR viral inhibitory peptide identified and its discovery increases our understanding of the molecular interactions

involved in TLR signalling, while 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 cell line 293 (HEK293) and HEK293 cells stably transfected with interleukin-1 receptor (HEK293_R1) were a gift from Tularik Inc (San Francisco, CA 94080, USA). HEK293 cells stably transfected with TLR2, TLR3, TLR4 or TLR8 (HEK293_TLR) were a gift from Dr K. Fitzgerald (University of Massachusetts Medical School, Worcester, MA, USA). The mouse leukaemia monocyte-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 (ECACC, Salisbury, UK). Immortalised murine wild type (wt) and $MyD88^{-/-}$, $Mal^{-/-}$, $TRIF^{-/-}$ and $TRAM^{-/-}$ bone marrow-derived macrophages (iBMDMs) were generated from corresponding knockout mice using J2 recombinant retrovirus carrying v-myc and v-raf/mil 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 3x in sterile PBS (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM NaH_2PO_4), counted and seeded at density $1x10^6$ cells/ml in complete RPMI medium.

Peptide synthesis and reconstitution

Peptides were synthesised by GenScript Corporation (Piscataway, New Jersey, USA) and were > 95% pure as identified by HPLC. Lyophilised peptides were reconstituted aseptically with molecular biology grade water to 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 weeks. See Table I for the sequences of the peptides used.

Receptor Agonists

Ultra-pure LPS from Gram-negative bacteria (*Eschericia coli*) (>99.9% pure in respect to contaminating protein, DNA and TLR2 agonists) was purchased from Alexis Corporation. Poly I:C was purchased from Amersham Biosciences. IL-1α was obtained from the National Cancer Institute (Frederick, WA, USA) and TNFα was a gift from Zeneca Pharmaceutics (Macclesfield, UK). CpG was purchased from Eurofins MWG Operon (85560 Ebersberg, Germany) and PMA was purchased from Sigma-Aldrich Ireland Ltd. (Dublin, Ireland). MALP2 was purchased from Alpha Technologies Ltd. (Blessington, Co. Wicklow, Ireland) and PAM3Csk4 purchased from Autogen Bioclear (Nottingham, UK).

Cell viability analysis by MTT

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

Reporter gene activation, mRNA and cytokine analysis

Unless otherwise stated, peptides were added at required concentrations 1 hour before stimulation with agonists for 6 h. HEK293 cells were seeded at $1x10^5$ 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 $2x10^5$ cell/ml 24 hours before treatment. RNA was isolated using High Pure RNA isolation kits from Roche Applied Science according to manufacturer's instructions. RT-PCR was performed using QIAGENs One-Step RT-PCR Kit according to manufacturer's instructions. Quantitative real time PCR was done using GoTaq® qPCR Master Mix according to manufacturer's instructions. For cytokine production, RAW264.7 cells and iBMDMs were seeded at $2x10^5$ cell/ml, THP-1 cells were differentiated with 100 nM PMA and seeded at $5x10^5$ cell/ml, and PBMCs seeded at $1x10^6$ cell/ml in 96-well plates 24 hour prior to treatment. The supernatants were collected and assessed for different cytokines by ELISA (R&D Systems).

Immunoblotting

For analysis of IκB degradation and MAP kinase activation iBMDMs were seeded at $2x10^5$ cell/ml in 6 well-plates 24 h before treatment. The peptides were added at 5 μM one hour before stimulating with 20 ng/ml LPS. After 30 min supernatants were removed and cells washed with ice-cold 1x PBS. Cells were lysed in 100 μl of SDS sample buffer (62.5mM Tris pH 6.8, 2% (w/v) SDS, 10% (v/v) Glycerol, 0.1% (w/v) Bromophenol Blue, 50mM DTT) and boiled for 5 min. 30 μl of cooled lysates was resolved on 10% SDS-PAGE, transferred onto Immobilon™ polyvinylidene difluoride (PVDF) membrane and probed with either mouse mAb against IκBα (a kind gift from Prof. R. Hay (Dundee University, UK)), rabbit anti-p38 phosphospecific Ab (Cell Signalling Inc.(Danvers, MA01923, USA)) or rabbit anti-JNK phosphospecific Ab (Biosource). In order to control for protein loading, the membranes were re-probed with either anti-β-actin Ab (Sigma), rabbit anti-p38 Ab

(Cell Signalling) or rabbit anti-JNK Ab (Biosource). Anti-GST Ab was a kind gift from Dr. T. Mantle (Trinity College Dublin, Ireland).

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) and grown in Terrific Broth. Protein expression was induced with 0.7 mM IPTG at 18°C for GST-TLR4 and 30°C for GST, GST-Mal and GST-TRAM for 24 hours. 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 $1x10^5$ cell/ml in 100 mm dishes 24 hours 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®. After 24 hours supernatants were removed and cells were washed with ice-cold PBS. Cells were lysed in 900µl 1% NETN lysis buffer (100mM NaCl, 50mM HEPES (pH 7.5), 1mM EDTA, 1% NP-40, 10% Glycerol, 50 mM imidazole) and incubated on ice for 40 min. 300 µl of the whole-cell lysates were incubated with 25 µM of 6xHistidine-tagged VIPER or CP7 peptides (containing no delivery sequence) and 40 µL of Ni-agarose beads for 2 h at 4°C with rolling to avoid sedimentation of the beads. To exclude non-specific binding lysates were also incubated with the Ni-agarose beads alone. After incubation beads were washed five times in 1% NP-40 lysis buffer with 50 mM imidazole. After the final wash the buffer was completely removed and the

beads were resuspended in 35 μl of SDS sample buffer. Samples were boiled for 5 min and resolved with SDS-PAGE. The resolved proteins were transferred to PVDF membrane and immunoblotted for the corresponding protein.

Determination of the effect of VIPER in vivo.

Groups of 5 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 0.1mg/kg 9R-VIPER, 1 μg LPS with 0.3mg/kg 9R-VIPER, 1 μg LPS with 0.1mg/kg 9R-CP7 or 1 μg LPS with 0.1mg/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's t-test. Two-tailed p values were obtained comparing groups treated with peptide and LPS versus LPS only.

Structural modeling

Structural homologues to A46 were retrieved using the 3DJury Metaserver (38). Alignments were manually adjusted and a model built using Modeller 9v7 with A52 as a template. All structural figures were generated using PYMOL (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC). Electrostatic potentials were calculated using a non-linear 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 signalling (29). In order to test the ability of regions of A46 to recapitulate TLR inhibition, we designed peptides from 2 regions of A46 protein, which were found to be important for inhibition of TLR signalling by using truncation mutants of A46 (data not shown). The designed peptides contained 11 amino acids from A46 and each proceeding peptide was designed to overlap with the previous one by 5 amino acids. These peptides were linked to a 9 arginine (9R) homopolymer delivery sequence at their C-termini as this was shown to be an efficient way to deliver peptides into cells (41) (37). The peptides were numbered depending on the order each appeared on the protein sequence (Table I). 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 hour before stimulation with LPS. Among the 12 peptides tested peptide #4 (KYSFKLILAEY-9R) was identified as the only peptide that inhibited LPS-induced TNFα production (Fig. 1*A*). Herein the peptide #4 was termed Viral Inhibitory Peptide of Toll-like Receptor 4 (VIPER). VIPER also inhibited LPS-induced TNFα, MIP-2, RANTES and IL-6 in iBMDM, while an inert control peptide, CP7 (Table I), had no effect (Fig. 1*B-E*). Neither VIPER nor CP7 affected cell viability at the concentrations used (data not shown).

Next we investigated the effect of the 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 TNFα in response to

Pam3CSK4 (which signals via a TLR2/TLR1 heterodimer), MALP2 (via TLR2/TLR6), Poly(I:C) (via TLR3) nor CpG (via TLR9) (Fig. 1*F-I*). This identified VIPER as the first TLR4-specific inhibitor derived from a viral protein.

To investigate whether VIPER required internalisation 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 TNFα secretion by VIPER required the delivery sequence to be present (Fig. 2*A*, 2*B*). On the other hand, 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. 1*B*) or the N-terminus (9R-VIPER, Fig. 2*C*). 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 half-life of peptides both *in vitro* and *in vivo* by rendering them resistant to the proteosomes, which often increases a peptide's biological activity (42, 43). D-VIPER still retained its inhibitory properties (Fig. 2*D*), with slightly reduced potency when the delivery sequence was fused to the N-terminus (Fig. 2*E*). Another commonly used delivery sequence, TAT $_{49\text{-}57}$, which is derived from the HIV-1 protein TAT, was tested to ensure the inhibitory properties of VIPER did not depend on the poly-arginine sequence. TAT $_{49\text{-}57}$ was linked to the N-terminus of VIPER and the D-isomer of TAT-VIPER was tested (Fig. 2*F*). This peptide still retained the ability to inhibit LPS-induced TNFα secretion, but with a slightly decreased potency, which likely was due to less efficient delivery of peptides into cells by TAT $_{49\text{-}57}$ when compared to 9R, as previously reported (41).

Together, these data indicate that VIPER specifically inhibits murine TLR4, that inhibition requires internalisation of the peptide, 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 re-assayed in the human monocyte cell line THP-1 at 5 μM, and similar to murine cells, only VIPER was found to inhibit LPS-induced TNFα production (Fig. 3*A*). A peptide derived from the VACV protein A52, termed P13, was previously reported to inhibit various TLRs including TLR4 in murine cells (37). However, compared to VIPER, P13 was ineffective in human cells, since even at 40 μM P13 failed to inhibit LPS-induced TNFα production in THP-1s (Fig. 3*B*) and in PBMCs (data not shown). In contrast, VIPER dose-dependently inhibited LPS-induced TNFα in primary human PBMC between 1-25 μM (Fig. 3*C*) without affecting the cells' viability as determined by MTT assay (Fig. 3*D*). The IC_{50} for TNFα production in PBMC was determined as 5 μM (Fig. 3*E*).

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. Co-injection of VIPER, but not CP7, with LPS resulted in a significant and dose-dependent suppression of IL-12p40 levels (Fig. 4). Thus, the VIPER peptide was found to be able potently inhibit the induction of the pro-inflammatory cytokine IL-12p40 upon LPS treatment *in vivo*.

VIPER inhibits TLR4 signalling at the receptor-adaptor level

To investigate the mechanism of inhibition of TLR4-induced cytokine production by VIPER we examined its effect on TLR4-induced transcription and signalling. Quantitative RT-PCR showed that VIPER inhibited induction of both LPS-induced TNFα mRNA (Fig. 5*A*), and LPS-induced IFNβ mRNA (Fig. 5*B*).

TLR4 is the only known receptor that signals via both the MyD88-dependent pathway to activate NFκB and MAP Kinases, and the TRIF-dependent pathway to activate IRF3/7 and late NFκB (44-46), while other IL-1/TLR family members use either MyD88 (e.g. IL-1, TLR8) or TRIF (e.g. TLR3). In order to investigate which of the two pathways is targeted by the peptide we performed reporter gene assays in HEK293 cells stably expressing TLR4, TLR3, TLR8, IL-1R or TNFα receptor (TNFR1). VIPER completely prevented activation of NFκB in TLR4-expressing cells upon LPS-stimulation (Fig. 5*C*), but had no effect on NFκB activation via the MyD88-dependent pathway in TLR8 and IL-1R-expressing cells (Fig. 5*D*, 5*E*), nor on TRIF-mediated TLR3-induced NFκB activation (45) (Fig. 5*F*). Also, VIPER did not affect TNF-induced activation of NFκB via TNFR1 (Fig. 5*G*). Thus VIPER inhibits NFκB activation, but only in response to TLR4. In addition, VIPER completely prevented degradation of IκB upon LPS treatment of RAW264.7 cells (Fig. 5*H*).

Next the effect of VIPER on IRF3 activation via the TRIF-dependent pathway (45) was measured, and it was established that LPS-induced activation of IRF3 was completely prevented by the peptide (Fig. 5*I*). Finally, LPS-induced activation of the MAP kinases Jun-N-terminal Kinases (JNK) and p38 were assayed. VIPER inhibited

TLR4-mediated phosphorylation of JNK and p38 after 30 min of stimulation with LPS (Figure 5*J* and *K*).

Collectively these data suggest that VIPER prevents activation of all TLR4-induced signalling 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 which 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 signalling (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 (47). Also, it was possible the peptide targets TLR4 itself, preventing its homodimerisation 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 pull-down assay using VIPER and CP7 peptides linked to a poly-histidine tag (6x His) on the N-termini. Flag-tagged Mal, TRAM, TRIF and HA-tagged MyD88 were over-expressed in HEK293 cells and 25 μM of the peptide was incubated with the whole cell lysate in the presence of Ni^{2+} agarose beads at 4°C. Interestingly, Mal and TRAM but not MyD88 nor TRIF were detectable in the His-VIPER complex isolated (Fig. 6*A*). Importantly, the non-inhibiting peptide CP7 did not complex with any of the TLR4 adaptors. To investigate whether VIPER binds to TLR4 directly we expressed 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 non-specific 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, while GST-TLR4 failed to interact with VIPER (Fig. 6*B*).

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, since LPS-induced TNFα secretion was still inhibited in iBMDMs lacking either 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 TNFα secretion upon LPS treatment compared to the wt iBMDM (Fig. 1*B*). Also VIPER inhibited activation of JNK equally well in the absence of either Mal or TRAM (Fig. 6*G*, 6*H*).

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-KYSFKLIL) 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 amino acids from the C-terminus had significantly reduced ability to inhibit LPS-induced TNFα production compared to full length VIPER (Fig. 7*A*). 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 (see Table I) and assayed for inhibition of LPS-induced TNFα in iBMDM compared to VIPER. As a result, we identified leucine at position 6 (KYSFKLILAEY) as a critical residue for inhibition of TLR4 since L6A was the only peptide that lost TLR4 inhibitory potential (Fig. 7*B*).

Position of VIPER on an A46 structural model

In order 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 alpha helices from amino acids 87-212, which includes the VIPER sequence. The most significant structural homology was found with the VACV Bcl-2 like proteins A52 and B14 (48). A52 was used as the final model template and the alignment of the resulting structures and model is shown in Fig. 8*A*. The VIPER motif is located at the very N-terminus of the first helix (Fig. 8*B*). 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. 8*D* and *E*). 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 anti-viral 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 (49). Thus, N1 prevents activation of NFκB and IRF3 by interacting with the IKK complex and TANK-binding kinase 1 (TBK1) (28). K7 inhibits TLR-mediated IRF3/7 activation by interacting with DEAD-box protein 3 (50). A52 inhibits activation of NFκB by TLRs though interacting with IL-1 receptor associated kinase 2 (IRAK2) (24, 51) and enhances MAP kinase activation via TNF receptor associated factor 6 (TRAF6) (52). Unlike any of the aforementioned proteins, A46 inhibits TLR signalling 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-1 receptor 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 signalling (32-36) led to the idea of designing peptides derived from A46 which 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, since 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 programme’, 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 antagonises 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 signalling, 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.

Since 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 have been determined and shown to adopt a bcl-2 fold, leading to the suggestion that A46 also adopts such a Bcl-2-like fold (48, 49, 53). Consistent with this, the VIPER sequence is predicted to be on the surface of A46 when modelled as a bcl-2 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. 8*C*). This complements the proposed interfaces between the TIR domains of Mal and TRAM (9) both of which are predicted to be electronegative (Fig. 8*D* and *E*). Consequently electrostatic attraction may play a key role in initiating inhibitory protein contacts between A46 and Mal or TRAM. This trapping of a host signalling 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 IKKs (27).

The targeting of TRAM by VIPER is consistent with the specific role for TRAM in TLR4 signalling (54). 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 (55). In TLR4 signalling 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 signalling on the other hand the DD-loop of Mal seems to play the more important role in the TLR2-Mal interaction as the S180L polymorphism of Mal prevents this interaction (56, 57). 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 signalling (15-17).

Furthermore, Mal has been reported to have a negative role in TLR3 signalling (16), which may explain why VIPER enhanced TLR3-mediated NFκB activation (Fig. 5*F*).

Apart from these new insights into TLR4 complex formation and disruption by a virus, VIPER itself represents a highly potent and specific novel species-independent antagonist of TLR4. In contrast, the previously reported viral peptide P13 derived from another VACV TLR-signalling inhibitory protein A52, was shown not to have TLR inhibitory capacity in human cells (Fig. 3*B* and data not shown). Furthermore, in murine cells P13 is less potent than VIPER, and its target(s) are unknown (37, 58). 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 (59, 60) at a dose 0.1 mg/kg when co-administered 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 lends it to further development into peptidomimetic compounds. Bartfai et al reported the successful development of a low molecular weight compound based on the tripeptide sequence from the MyD88 BB-loop, that was specific for IL1RI signalling (61). 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 (62), ischemia/reperfusion injury (63-65), atherosclerosis (31), rheumatoid arthritis (31), acute lung injury (66), etc.

Traditionally, attempts to control sepsis or auto inflammation have centered on blockage of pro-inflammatory cytokines such as TNFα, a presumed critical effectors 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 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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Abbreviations used: CPP, cell penetrating peptides; Mal, MyD88-adaptor-like; TIR domain, TLR/IL1-receptor domain; TRAM, TRIF-related adaptor molecule; TRIF, TIR domain-containing adaptor inducing interferon-β; VACV, vaccinia virus; VIPER, Viral Inhibitory Peptide of Toll-like Receptor 4; iBMDMs, immortalised wild type murine bone marrow derived macrophages.

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Figure legends

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 hr before stimulation with 10 ng/ml LPS. Supernatants were collected 6 hrs after stimulation and assayed for TNFα by ELISA. *B-E*, **i**BMDMs were treated with 1 or 5 μM VIPER and CP7 peptides 1 hr before stimulation with 20 ng/ml LPS. Supernatants were collected 6 hrs after stimulation and assayed for TNFα, MIP-2, RANTES and IL-6 by ELISA. *F-I*, **i**BMDMs were treated with 1 or 10 μM VIPER and CP7 peptides 1 hr before stimulating with 20 ng/ml PAM3csk4 (*F*), 20 nM MALP2 (*G*), 25 μg/ml Poly(I:C) (*H*) or 1 μg/ml CpG (*I*). Supernatants were collected 6 hrs after stimulation and assayed for TNFα by ELISA. The data are mean ± SD of triplicate samples and are representative of at least three experiments. * $p<0.05$, ** $p<0.005$ compared to LPS only.

Figure 2. VIPER inhibition of TLR4 is independent of the nature or position of the delivery sequence.

A,B, **i**BMDMs were treated with 1 or 5 μM His-tagged peptides VIPER and CP7 with (*B*) or without (*A*) the polyarginine (9R) delivery sequence for1 hr before stimulation with 10 ng/ml LPS. *C-F*, iBMDMs were treated with peptides VIPER and CP7 with the 9R delivery sequence at the N-terminus (*C*), with the D-enantiomer of the peptides with the 9R delivery sequence at either C- (*D*) or N-termini (*E*) or with the D-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 hr before stimulation with 10 ng/ml LPS. In all cases supernatants were collected 6 hrs after stimulation and assayed for TNFα

by ELISA. The data are mean ± SD of triplicate samples and are representative of at least three experiments. * $p<0.05$ compared to LPS only.

Figure 3. VIPER inhibits TLR4 in primary human cells.

*A***,** THP-1 cells were treated with 5 μM A46 peptides for 1 hr before stimulation with 10 ng/ml LPS. *B*, Peptides P13, VIPER and CP7 were added to the THP-1 cells at 20 and 40 μM 1 h before LPS stimulation. *C***,** Human PBMCs were treated with 1, 5 and 25 μM peptide 1 hr before stimulation with 10 ng/ml LPS. *D***,** The cells were assayed for viability using the MTT assay**.** *E***,** PBMC were treated with 0, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5 and 25 μM VIPER 1 hr before stimulation with 10 ng/ml LPS. In *A*, *B*, *C* and *E*, supernatants were collected 6 hrs after stimulation with LPS and assayed for TNFα by ELISA. In *E*, the concentration of TNFα measured was expressed as a percentage of that seen for LPS alone. The data are mean ± SD of triplicate samples and are representative of at least three experiments. * $p<0.05$ compared to LPS only.

Figure 4. VIPER inhibits LPS-induced IL-12/23 p40 secretion in vivo.

BALB/c mice were injected i.v. with PBS, 1 μg LPS, 1 μg LPS with 0.1mg/kg 9R-VIPER, 1 μg LPS with 0.3mg/kg 9R-VIPER, 1 μg LPS with 0.1mg/kg 9R-CP7 or 1 μg LPS with 0.1mg/kg 9R-CP7. Blood was harvested after 4h and serum was assayed for IL-12p40 by ELISA. Statistical analysis was carried out using paired Student's t-test. * $p<0.05$, ** $p<0.005$ compared to LPS only. The data is representative of two experiments.

Figure 5. VIPER inhibits multiple TLR4 signalling pathways

A, B, iBMDMs were treated with 5 μM peptides 1 hr before stimulation with 10 ng/ml LPS for 6 hrs. Induction of TNFα and IFNβ mRNA expression was assayed by qRT-PCR. *C-G*, HEK293 cells stably expressing TLR4 (C), TLR8 (D), IL-1R (E), TLR3 (F) or nothing (G) and transfected with the NFκB-luciferase reporter gene were treated with 5 and 10 μM VIPER before stimulation with 10 ng/ml LPS, 3 μg/ml CL075, 50 ng/ml IL-1α, 25 μg/ml Poly(I:C) or 50 ng/ml TNFα respectively for 6 hrs, 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, K*, 5 μM of VIPER and CP7 were added to iBMDMs before stimulation 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. * $p<0.05$, ** $p<0.005$ compared to LPS only.

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 hr 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 Methods. In *A* and *B*, lysates were incubated with 25 μM of 6xHistidine-tagged VIPER or CP7 peptides (containing no delivery sequence) and Ni-agarose beads as described in Methods. Proteins were eluted from the beads with SDS sample buffer, resolved by SDS-PAGE, and immunoblotted for the corresponding protein. *C-F*, Immortalised *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 TNFα. The data are mean ± SD of triplicate samples. *G-J*, 5 μM of VIPER and CP7 were added to *TRAM*$^{-/-}$ and *Mal*$^{-/-}$ BMDM for 1 h prior to stimulation with 20 ng/ml LPS for 30 min. Lysates were immunoblotted for *p*-JNK (*G*, *H*) with loading control of total JNK. All data are representative of at least three experiments. * $p<0.05$, **$p<0.005$ compared to LPS only.

Figure 7. Identification of residues in VIPER critical for TLR4 inhibition

A, **i**BMDMs were treated with 1, 5 and 25 μM of peptides lacking amino acids from the N- or C-termini (N-1, N-2, C-3 and C-6, see Table I for sequences), or 5 μM VIPER for 1 h. *B*, For the alanine scan 5 μM peptides (see Table I for sequences) were added for 1 h. In *A* and *B* cells were stimulated with 10 ng/ml LPS for 6 h and supernatants then collected and assayed for TNFα by ELISA. The data are mean ± SD of triplicate samples and are representative of at least three experiments. * $p<0.05$, **$p<0.005$ compared to LPS only.

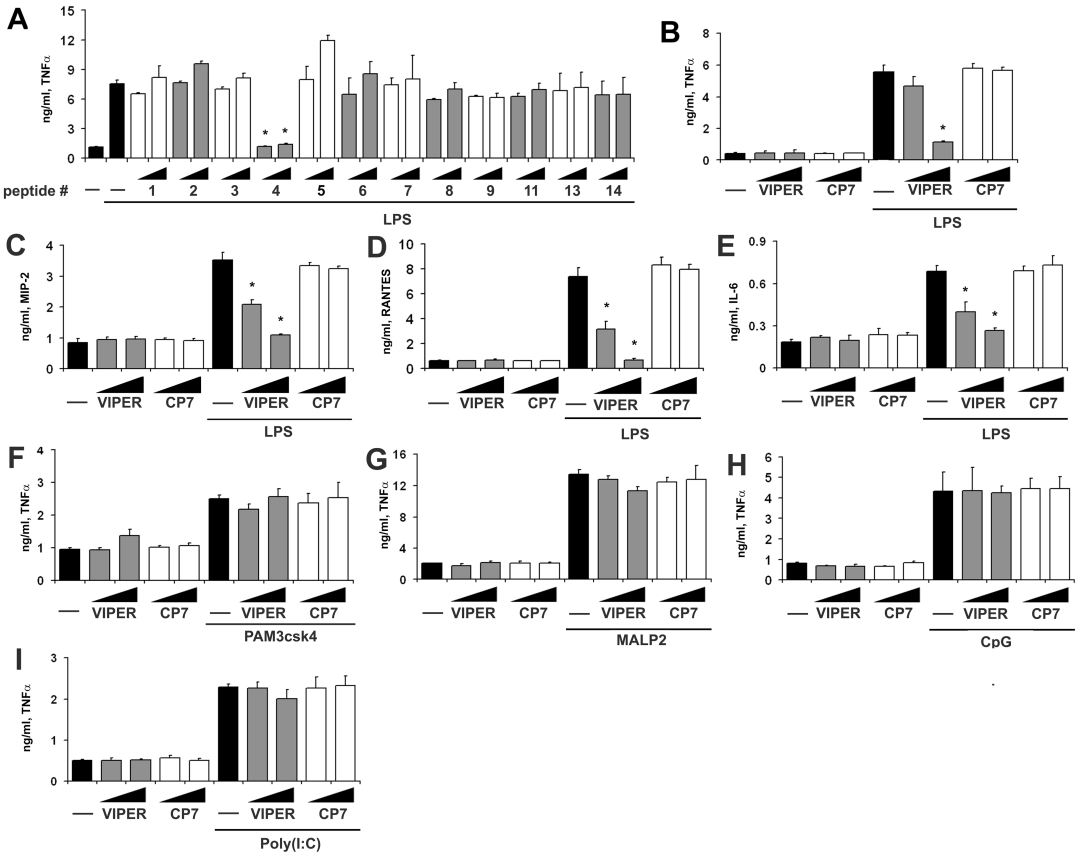
Figure 8: Structural model of A46 showing position of VIPER in the protein.

A, Structure based alignment of A52 and B14 (PDB IDs 2VVW and 2VVY) with the modelled A46 fold. Helices are highlighted grey. The VIPER motif is in bold and underlined. *B*, Cartoon representation of A46 with a Bcl-2 like fold. The VIPER motif at the start of helix 1 is coloured purple and shown as a stick representation. *C-E*, Electrostatic potential of the molecular surface of A46 (C), Mal (D) and TRAM (E). Blue is electropositive and red is electronegative. Leucine 6 from VIPER is coloured purple (C). A46 (C) is in the same orientation as (B). Mal and TRAM are orientated

to show the interface predicted to contact the TIR domain of TLR4. Structural models were generated using PYMOL.

Table I. ***List of the A46-derived peptides and their modifications***

No.	Name	Sequence	No.	Name	Sequence
1	**#1**	GCAVNTPVSMT-9R	21	**D-9R-VIPER**	9x{dR}- {G}-d{ KYSFKLILAEY}
2	**#2**	TPVSMTYLYNK-9R	22	**D-CP7**	d{RNTISGNIYSA}-{G}-9x{dR}
3	**#3**	TYLYNKYSFKL-9R	23	**D-9R-CP7**	9x{dR}-{G}-d{RNTISGNIYSA}
4	**VIPER**	KYSFKLILAEY-9R	24	**D-TAT-VIPER**	d{GRKKRRQRRR}-{G}-d{ KYSFKLILAEY}
5	**#5**	LILAEYIRHRN-9R	25	**D-TAT-CP7**	d{GRKKRRQRRR}-{G}-d{RNTISGNIYSA }
6	**#6**	YIRHRNTISGN-9R	26	**P13**	DIVKLTVYDCI-9R
7	**CP7**	RNTISGNIYSA-9R	27	**N-1**	YSFKLILAEY-9R
8	**#8**	GNIYSALMTLD-9R	28	**N-2**	SFKLILAEY-9R
9	**#9**	DSGLFDFVNFV-9R	29	**C-3**	KYSFKLIL-9R
10	**#10**	DFVNFVKDIMC-9R	30	**C-6**	KYSFK-9R
11	**#11**	VKDIMCCDSRI-9R	31	**K1A**	AYSFKLILAEY-9R
12	**#13**	VALSSLVSKHW-9R	32	**Y2A**	KASFKLILAEY-9R
13	**#14**	LVSKHWELTNK-9R	33	**S3A**	KYAFKLILAEY-9R
14	**His-VIPER**	6xHis- KYSFKLILAEY	34	**F4A**	KYSAKLILAEY-9R
15	**His-VIPER-9R**	6xHis- KYSFKLILAEY-9R	35	**K5A**	KYSFALILAEY-9R
16	**His-CP7**	6xHis- RNTISGNIYSA	36	**L6A**	KYSFKAILAEY-9R
17	**His-CP7-9R**	6xHis- RNTISGNIYSA-9R	37	**I7A**	KYSFKLALAEY-9R
18	**9R-VIPER**	9R- KYSFKLILAEY	38	**L8A**	KYSFKLIAAEY-9R
19	**9R-CP7**	9R-RNTISGNIYSA	39	**E10A**	KYSFKLILAAY-9R
20	**D-VIPER**	d{KYSFKLILAEY}-{G}-9xd{R}	40	**Y11A**	KYSFKLILAEA-9R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8
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VIPER
CP7
LPS
C
ng/ml, MIP-2
D
ng/ml, RANTES
E
ng/ml, IL-6
F
ng/ml, TNFα
PAM3csk4
G
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H
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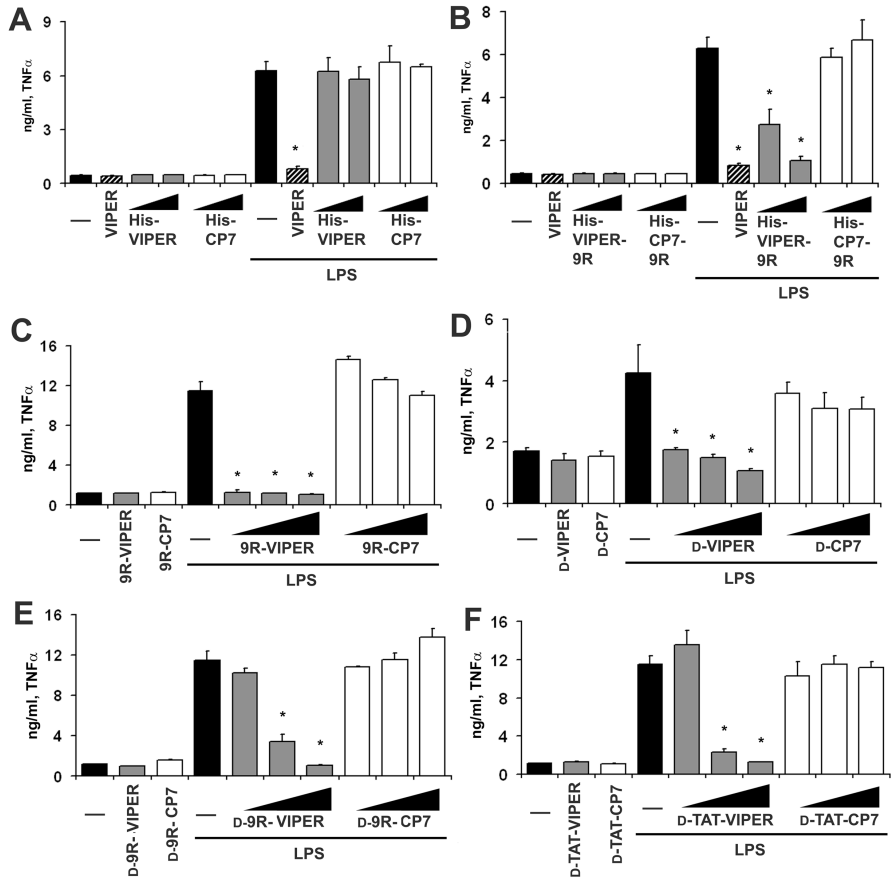
CpG
I
ng/ml, TNFα
Poly(I:C)

Figure 1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B
C
D
E
F
ng/ml, TNFα
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His-CP7
His-VIPER-9R
His-CP7-9R
9R-VIPER
9R-CP7
D-VIPER
D-CP7
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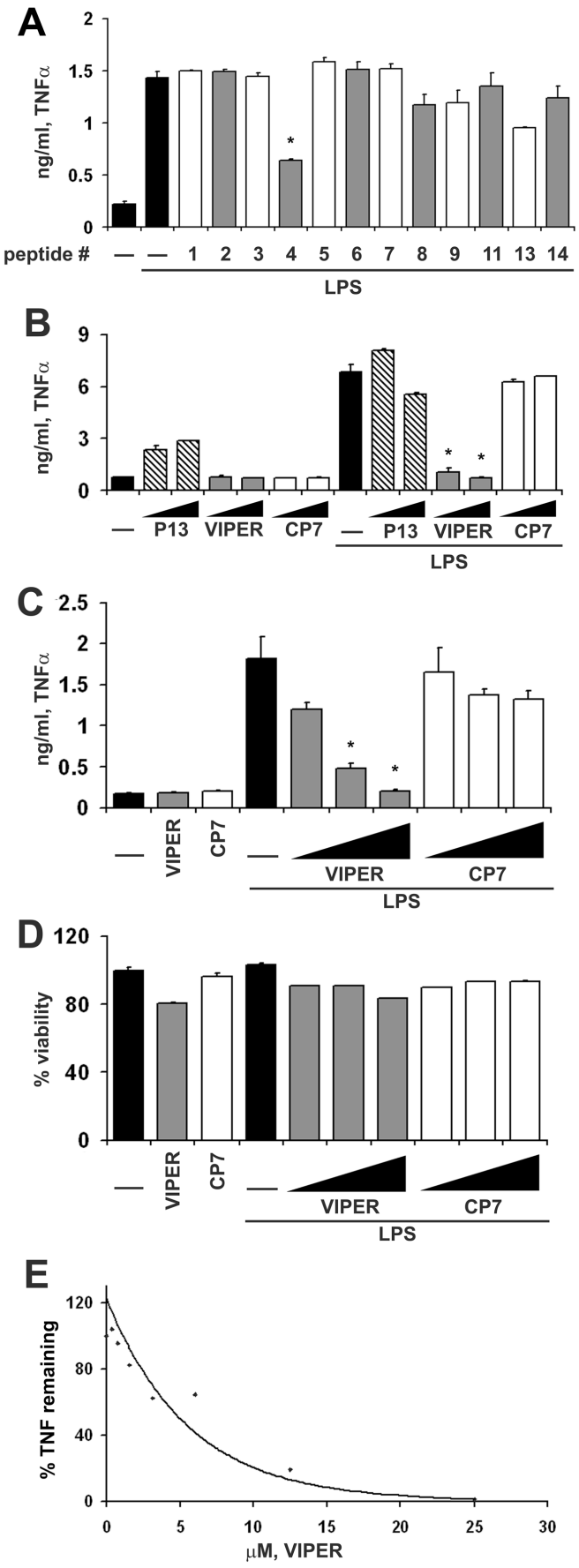
D-TAT-VIPER
D-TAT-CP7
LPS

Figure 2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1
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0
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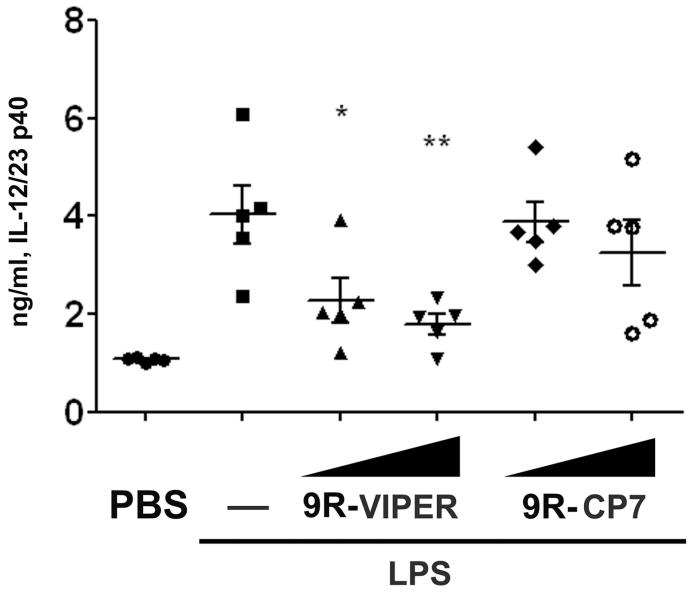
B
ng/ml, TNFα
9
6
3
0
* *
— P13 VIPER CP7 — P13 VIPER CP7
LPS
C
ng/ml, TNFα
2.5
2
1.5
1
0.5
0
* *
— VIPER CP7 — VIPER CP7
LPS
D
% viability
120
80
40
0
— VIPER CP7 — VIPER CP7
LPS
E
% TNF remaining
120
80
40
0
0 5 10 15 20 25 30
μM, VIPER

Figure 3



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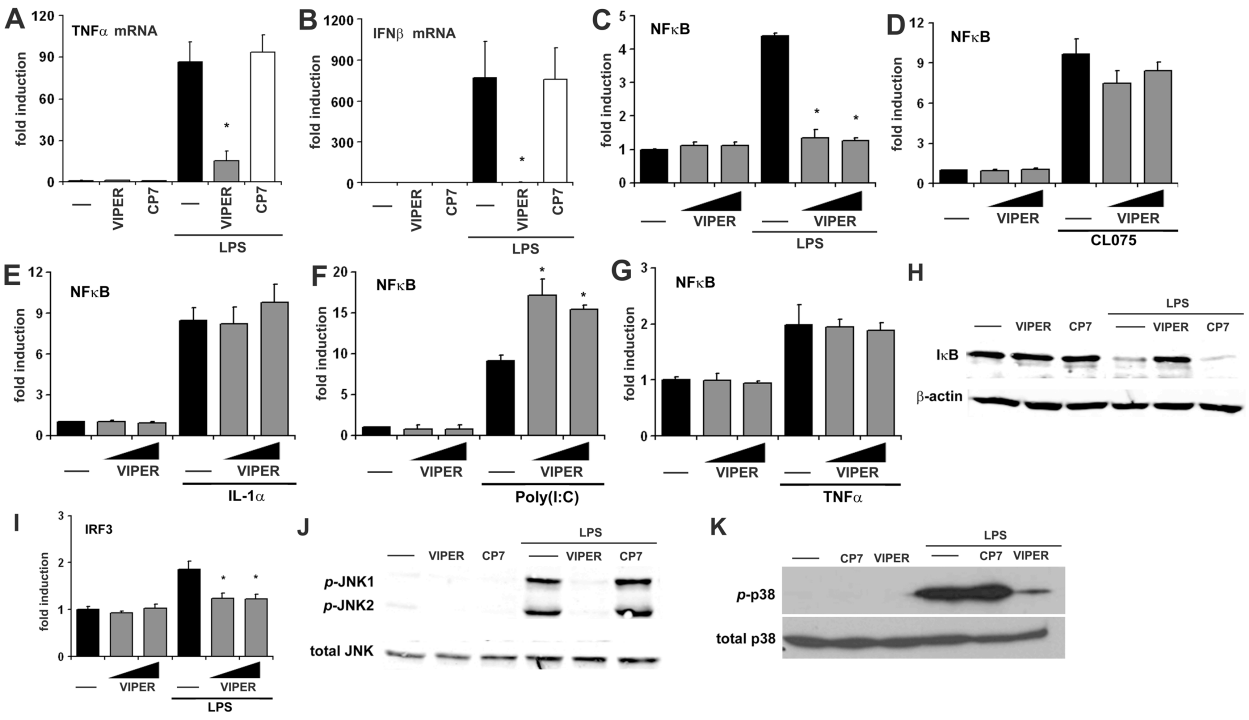

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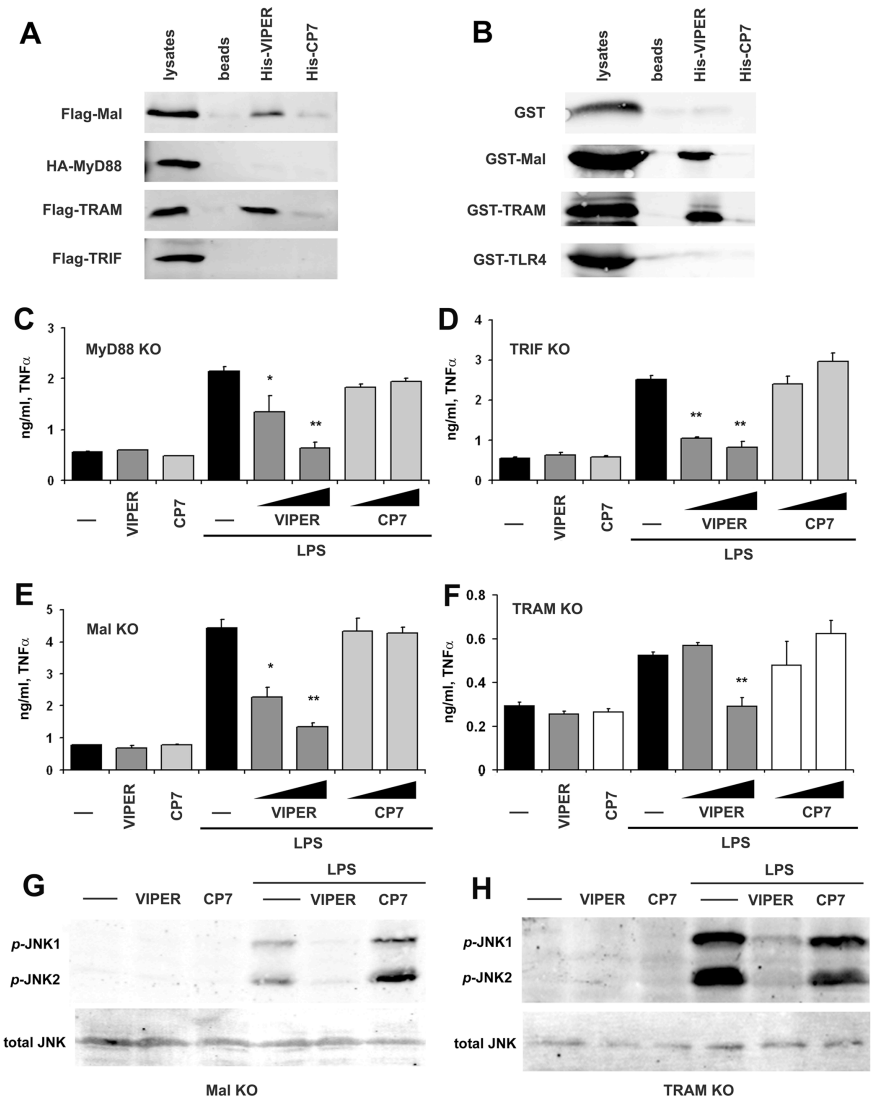
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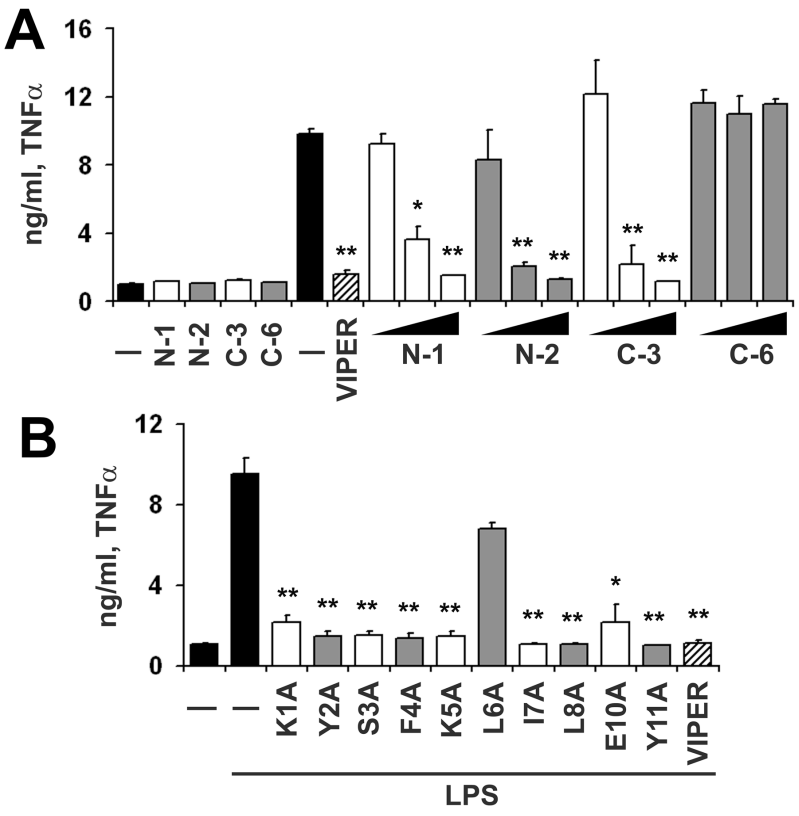
His-VIPER
His-CP7
GST
GST-Mal
GST-TRAM
GST-TLR4
C
MyD88 KO
ng/ml, TNFα
D
TRIF KO
ng/ml, TNFα
VIPER
CP7
LPS
E
Mal KO
ng/ml, TNFα
F
TRAM KO
ng/ml, TNFα
G
LPS
VIPER
CP7
p-JNK1
p-JNK2
total JNK
Mal KO
H
LPS
VIPER
CP7
p-JNK1
p-JNK2
total JNK
TRAM KO

Figure 6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VIPER
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N-2
C-3
C-6
B
ng/ml, TNFα
12
8
4
0
—
—
K1A
Y2A
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E10A
Y11A
VIPER
LPS

Figure 7

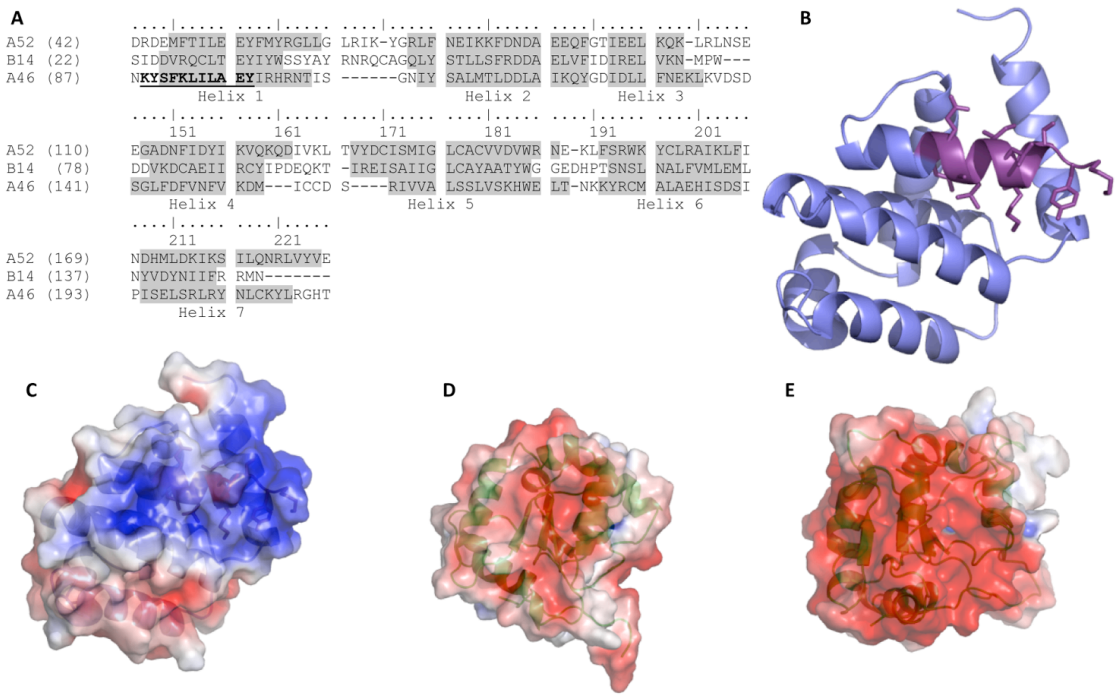

Figure 8