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Identification of a Peptide Derived from Vaccinia Virus A52R Protein That Inhibits Cytokine Secretion in Response to TLR-Dependent Signaling and Reduces In Vivo Bacterial-Induced Inflammation¹

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TLRs recognize and respond to conserved motifs termed pathogen-associated molecular patterns. TLRs are characterized by an extracellular leucine-rich repeat motif and an intracellular Toll/IL-1R domain. Triggering of TLRs by pathogen-associated molecular patterns initiates a series of intracellular signaling events resulting in an inflammatory immune response designed to contain and eliminate the pathogen. Vaccinia virus encodes immunoregulatory proteins, such as A52R, that can effectively inhibit intracellular Toll/IL-1R signaling, resulting in a diminished host immune response and enhancing viral survival. In this study, we report the identification and characterization of a peptide derived from the A52R protein (sequence DIVKLTVYDCI) that, when linked to the nine-arginine cell transduction sequence, effectively inhibits cytokine secretion in response to TLR activation. The peptide had no effect on cytokine secretion resulting from cell activation that was initiated independent of TLR stimulation. Using a mouse model of otitis media with effusion, administration of heat-inactivated *Streptococcus pneumoniae* into the middle ears of BALB/c mice resulted in a significant inflammatory response that was dramatically reduced with peptide treatment. The identification of this peptide that selectively targets TLR-dependent signaling may have application in the treatment of chronic inflammation initiated by bacterial or viral infections. *The Journal of Immunology*, 2005, 174: 3006–3014.

The innate immune system, involved in both the detection and control of infection, recognizes conserved motifs from pathogens termed pathogen-associated molecular patterns (PAMPs)³ (1, 2). TLRs recognize PAMPs, and their interaction triggers a series of intracellular signaling events that culminates in expression of cell surface molecules, secretion of proinflammatory cytokines, and induction of acquired immunity (2–9). TLRs are characterized by an extracellular leucine-rich repeat motif and an intracellular Toll/IL-1R (TIR) domain. Pathogens are detected by the innate immune system, with recognition of the bacterial PAMPs LPS, CpG DNA, flagellin, and peptidoglycan mediated by TLR4, TLR9, TLR5, and TLR2, respectively (4, 10–13). Recognition of viral infections are mediated primarily by TLR3 in response to viral dsRNA (14). Cell activation in response to different PAMPs involves a number of intracellular molecules

common to all TLRs, including MyD88, members of the IL-1R-associated kinase (IRAK) proteins, TNFR-associated factor (TRAF)6, and NF- κ B (1).

Vaccinia virus, a member of the poxvirus family, is a DNA virus that has been demonstrated to encode immunomodulatory proteins (15–18). One of these proteins, A52R, has been shown to inhibit NF- κ B activation following initiation of the TIR signaling cascade (15, 18). Recent studies have demonstrated that A52R inhibits TIR signaling and contributes to the virulence of vaccinia virus. Inhibition of TIR signaling by A52R is mediated by binding of the protein to both TRAF6 and IRAK2 (18).

In this study, we report the identification and characterization of a peptide, derived from the A52R protein, that significantly inhibits in vitro cytokine production in response to both bacterial and viral PAMPs. This peptide has characteristics consistent with a reagent that inhibits intracellular signaling triggered by TLR activation. Cytokine secretion induced by non-TLR stimulation was not inhibited by the peptide. The in vivo activity of this peptide was demonstrated by dramatically reducing middle ear inflammation in mice injected with heat-inactivated *Streptococcus pneumoniae*. We speculate that this peptide may have application in the treatment of this and other inflammatory conditions that result from ongoing TLR activation.

Materials and Methods

Peptide synthesis

Peptides were synthesized containing an 11- to 18-aa sequence from the vaccinia virus A52R protein and a nine-residue arginine cell transduction sequence positioned at the C-terminal end to allow for cell internalization. Each peptide was constructed both with and without a FITC label. FITC-labeled peptides were used for FACS analysis. The peptides lacking the FITC label were used for in vitro inhibition assays and in vivo treatment studies.

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³ Abbreviations used in this paper: PAMP, pathogen-associated molecular pattern; TIR, Toll/IL-1R; IRAK, IL-1R-associated kinase; TRAF, TNFR-associated factor; ODN, oligodeoxynucleotide; OME, otitis media with effusion.

Reagents

Nuclease-resistant phosphorylated oligonucleotide was purchased from Oligos Etc., Inc. The sequence was 5'-TCCATGACGTTCCCTGACGTT-3' (CpG-oligodeoxynucleotide (ODN)) (19). Mouse IL-1 α and TNF- α were purchased from R&D Systems. PMA and LPS were purchased from Sigma-Aldrich. The TLR ligands flagellin, zymosan, and poly(I:C) were purchased from InvivoGen. Cytokine assays were performed using assay kits purchased from R&D Systems. Heat-inactivated *S. pneumoniae* was the kind gift of Dr. T. DeMaria (Department of Otolaryngology, Ohio State University College of Medicine, Columbus, OH).

Cell lines and cultures

RAW264.7 (murine monocyte/macrophage) cells (American Type Culture Collection) were cultured at 37°C in a 5% CO₂ humidified incubator and grown in DMEM (Invitrogen Life Technologies) supplemented with 10% (v/v) heat-inactivated FCS, 1.5 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin.

Cytokine secretion

RAW264.7 cells were plated at 1.5×10^5 cells/well in 48-well plates. After 24 h, the cells were incubated with peptide at various concentrations at room temperature in triplicate either before, simultaneous with, or after activation with various bacterial or viral PAMPs for 18 h. Cell-free supernatants were analyzed for cytokines by ELISA, in quadruplicate. RAW264.7 cells were stimulated with either CpG-ODN (1 μ g/ml), LPS (1 ng/ml), poly(I:C) (10 μ g/ml), flagellin (5 ng/ml), or zymosan (10 μ g/ml). Dose-response curves were done with each PAMP to determine optimal stimulation concentration.

Flow cytometry

Cells were analyzed by flow cytometry (FACScan; BD Biosciences) using CellQuest software to quantify internalization of peptides. Gates were drawn to exclude dead cells based on 7-aminoactinomycin D staining. Fluorescence due to cell surface binding of FITC-labeled peptides was quenched using trypan blue. Data obtained were geometric mean fluorescent units (*F*) with background autofluorescence subtracted.

Immunoblotting

RAW264.7 cells (6×10^5) were plated in 12-well plates overnight. Cells were incubated for 15 min at room temperature with either peptide P13 or control scrambled peptide, and then stimulated with medium or LPS (1 ng/ml) for either 15 or 30 min. Cells were lysed, and proteins were fractionated by SDS/PAGE (12%). Immunoblotting was done using Phospho-I κ B- α (Ser³²) Ab (Cell Signaling), detected using HRP-conjugated secondary Ab, and visualized by chemiluminescence. Measurements of band intensity were made using the Nucleo Tech Gel Expert software linked to an Epson expression 636 scanner and expressed as intensity/area.

Induction of otitis media

BALB/c mice, 8–12 wk of age, were anesthetized with a s.c. injection of xylazine and ketamine (0.1 mg/30 gm body weight), and their ears were examined under the operating microscope to ensure that they were free of infection or perforation. One group of animals ($n = 5$) was injected with PBS in one ear and with 10 μ M peptide in the opposite ear, to determine the effect of peptide without added bacteria. A second group of animals ($n = 20$) received 5.0 μ l of PBS plus heat-inactivated *S. pneumoniae* (10^9 CFU/ml) in one ear and 5.0 μ l of peptide (10 μ M) plus heat-inactivated *S. pneumoniae* (10^9 CFU/ml) in the opposite ear. Injections were done through the tympanic membrane. Animals were killed 3 days after bacterial injection, and tissue was histologically processed to assess middle ear disease. Inflammation was quantified by measuring the following: 1) area of fluid present in the middle ear, 2) number of cells in middle ear fluid, and 3) thickness of the tympanic membrane taken at a point away from the injection site. From previous studies, data were obtained from mice ($n = 18$) injected with PBS alone for each of the histological parameters measured, to serve as a control group. Disease induction was defined as positive if the ear injected with *S. pneumoniae* without peptide demonstrated an increase of at least 2 SDs above the control PBS-treated mice in at least two of the three parameters assessed for middle ear inflammation: fluid area, cell number, and thickness of the tympanic membrane. Inflammation was successfully induced in 7 of 20 mice.

Tissue collection

At the end of the experimental treatment, mice were killed, and tissues were collected for histology. Mice were overdosed on anesthetic and per-

fused intracardially with 1.0 ml of saline, followed by 20 ml of fixative (1.5% paraformaldehyde-3% glutaraldehyde in 0.1 M phosphate buffer). The middle ears were left intact and connected to each other by the skull base so both ears were processed together for histology and sectioning. This enables all histologic embedding, sectioning, staining, and analysis to be done on the two sides simultaneously to reduce any impact of processing variables on the subsequent quantitative analyses. Middle ears were decalcified, embedded in glycol methacrylate plastic, sectioned at 5 μ m, mounted serially on glass slides, stained, and coverslipped.

Histopathologic analysis

Three consecutive sections at the level of the umbo and promontory were selected for measures of 1) area of fluid present in the middle ear, 2) number of cells in middle ear fluid, and 3) thickness of the tympanic membrane. Each measurement was taken on the three sequential sections per specimen. The value presented for each parameter represents the mean of the three sections.

Statistical analyses

To determine the effect of peptide without added bacteria, animals ($n = 5$) were injected in one ear with PBS alone, and the other ear with 10 μ M peptide. Paired *t* tests were done comparing the effect of PBS alone with the effect of peptide for each of the three histological parameters: 1) area of fluid present in the middle ear, 2) number of cells in middle ear fluid, and 3) thickness of the tympanic membrane. Of 20 animals injected with *S. pneumoniae*, 7 met the criteria for disease induction. Paired *t* tests were done using these seven animals, comparing the effect of peptide plus *S. pneumoniae* in one ear with *S. pneumoniae* alone in the opposite ear for each of the histological parameters described above.

Results

Peptide construction

The A52R protein from vaccinia virus has previously been shown to inhibit intracellular TIR signaling (15, 18). To investigate which amino acid sequence(s) of A52R was responsible for this inhibitory effect, we constructed 18 peptides whose design was based on the sequence of the vaccinia virus A52R protein. Each peptide contained a nine-arginine cell transducing sequence (20) positioned at the C terminus and an 11- to 18-aa sequence from the vaccinia virus A52R protein. Three peptides (P5, P6, and P14) were found to be insoluble and were eliminated from evaluation. The remaining 15 peptides were evaluated for their effect on cell viability by trypan blue exclusion staining over a range of concentrations, and then each peptide was tested for cytokine inhibition at the maximum concentration that had no effect on cell viability (data not shown). Using the FITC-labeled peptides, each of

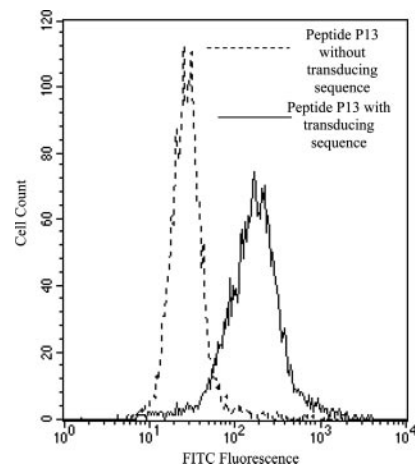


FIGURE 1. Internalization of peptide P13 requires the cell transducing sequence. RAW264.7 cells were incubated with 10 μ M peptide containing the transducing sequence (DIVKLTVYDCI-RRRRRRRRR; solid line) or with 10 μ M peptide lacking the transducing sequence (DIVKLTVYDCI; dashed line) for 15 min, and internalization of FITC-peptide was evaluated by FACS.

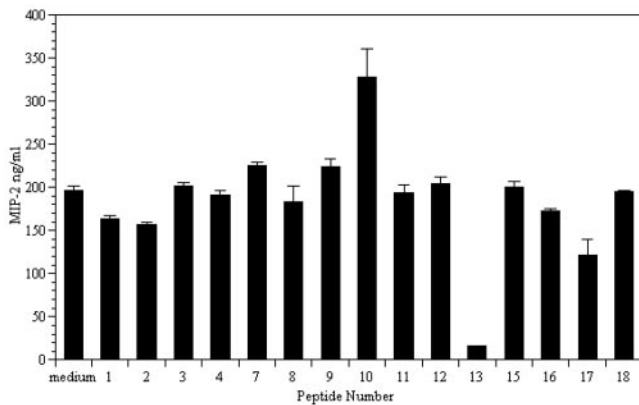


FIGURE 2. Effect of peptides on MIP-2 secretion. RAW264.7 cells were incubated 15 min with either medium (no peptide) or individual peptides at the maximal concentrations that did not affect cell viability. The cells were then stimulated with CpG-ODN (1 μ g/ml) for 18 h, cell-free supernatants were analyzed for MIP-2 by ELISA, and data were expressed as MIP-2 (nanograms per milliliter) \pm SD.

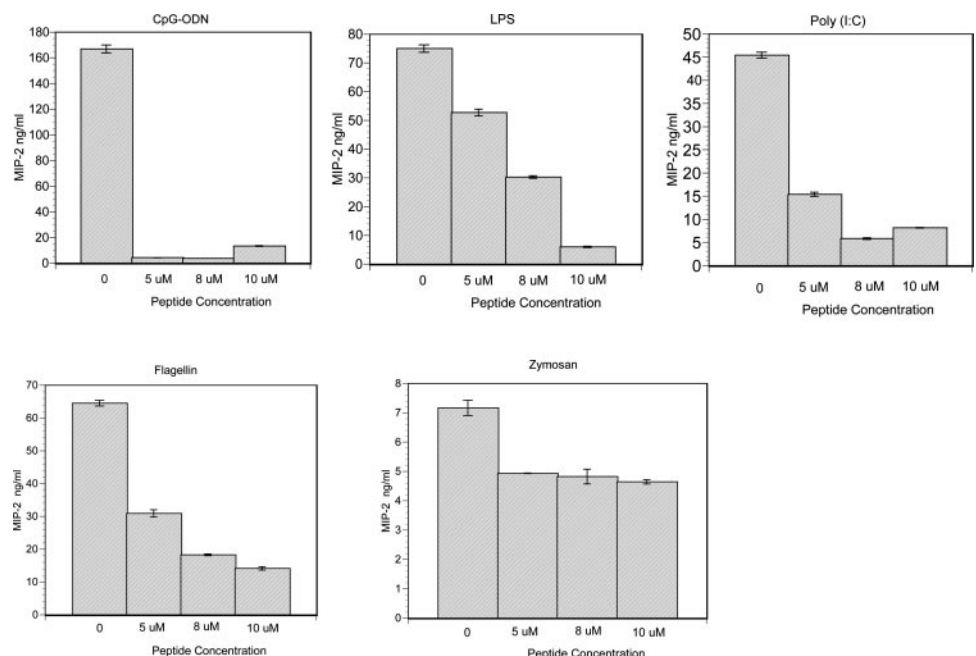
the peptides was shown to be internalized into RAW264.7 cells, a mouse monocyte/macrophage cell line, as assessed by FACS (data not shown). The necessity of the cell transducing sequence for cellular internalization was demonstrated when RAW264.7 cells were incubated with one of the FITC-labeled peptides (peptide P13), and internalization was assessed by FACS. The amount of FITC-peptide that was internalized into cells produced a geometric mean fluorescent unit (F) value of 151 (Fig. 1). As a control, peptide P13 was produced, which contained the 11-aa sequence from A52R but lacked the nine-arginine transduction sequence. This FITC-labeled control peptide showed significantly less internalization ($F = 17$) into RAW264.7 cells than the peptide containing the transduction sequence and was similar to the background level seen when cells were incubated with medium without peptide ($F = 8$). In initial experiments, individual peptides (lacking the FITC label) were examined for inhibition of MIP-2 secretion from RAW264.7 cells. MIP-2, a neutrophil chemoattractant factor, is important in development of inflammation. As a control, each pep-

ptide was tested for its effect on cytokine secretion without any added stimulants. These studies demonstrated that all peptides caused <4 ng/ml MIP-2 secretion in the absence of a stimulus. Individual peptides were then examined for inhibition of MIP-2 secretion from RAW264.7 cells activated by a variety of PAMPs (LPS, poly(I:C), CpG-ODN). Some peptides demonstrated moderate inhibition of MIP-2 secretion, whereas the majority of peptides examined had no significant effect on cytokine secretion, as demonstrated when cells were stimulated with CpG-ODN (Fig. 2). One peptide (P13), with the amino acid sequence DIVKLTVYDCI-RRRRRRRRRR, demonstrated significant inhibition of MIP-2 secretion for each of the TLR ligands examined and was used for further characterization. A scrambled peptide of P13 (ITCVDVLDLIYK-RRRRRRRRRR) was also produced as a negative control.

Inhibition of cytokine secretion

The initial studies with peptide P13 examined its effect on MIP-2 secretion at one concentration (10 μ M) in response to the stimulants LPS, CpG-ODN, and poly(I:C). Cell viability studies confirmed that a 10 μ M concentration of peptides P13 and the scrambled P13 control had no effect on cell viability (data not shown). Peptide P13 was then examined at various concentrations for inhibition of cytokine secretion in response to these and other TLR ligands. RAW264.7 cells were incubated for 15 min with 5, 8, or 10 μ M peptide P13 and then stimulated with either CpG-ODN, LPS, poly(I:C), flagellin, or zymosan for 18 h. Cell-free supernatants from treated cells were assessed for MIP-2 by ELISA. Treatment with peptide significantly inhibited MIP-2 secretion for each of the five TLR ligands examined (Fig. 3). Peptide inhibition of MIP-2 secretion was dose dependent for the TLR ligands LPS, poly(I:C), and flagellin. Inhibition was most dramatic when cells were stimulated with CpG-ODN, and ranged from ~ 90 to 35%, depending upon the TLR ligand used for cell activation. Testing of the control scrambled peptide at 10 μ M, under identical experimental conditions, showed no inhibition of MIP-2 secretion in response to the five PAMPs examined above (data not shown). To determine whether peptide P13 would be effective in inhibiting cytokine secretion induced by a combination of stimuli, RAW264.7 cells were incubated with both LPS (0.5 ng/ml) and

FIGURE 3. Inhibition of MIP-2 secretion by peptide P13. RAW264.7 cells were incubated 15 min with medium (no peptide) or 5, 8, or 10 μ M peptide P13, and then stimulated with CpG-ODN (1 μ g/ml), LPS (1 ng/ml), poly(I:C) (10 μ g/ml), flagellin (5 ng/ml), or zymosan (10 μ g/ml) for 18 h. Cell-free supernatants were analyzed for MIP-2 by ELISA, and data were expressed as MIP-2 (nanograms per milliliter) \pm SD.



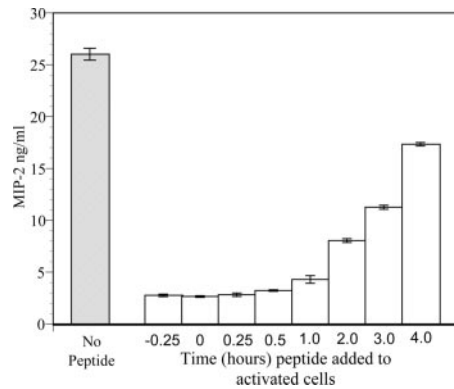


FIGURE 4. Peptide P13 inhibits MIP-2 secretion from activated cells. RAW264.7 cells were incubated with peptide P13 for various times; either before (15 min), simultaneous with (time 0), or after (0.25, 0.5, 1, 2, 3, or 4 h) stimulation for 18 h with CpG-ODN (1 μ g/ml). Positive control was cells stimulated with CpG-ODN (1 μ g/ml) without added peptide. Cell-free supernatants were analyzed for MIP-2 by ELISA, and data are expressed as MIP-2 (nanograms per milliliter) \pm SD.

CpG-ODN (0.5 μ g/ml). Each stimulus was used at half of its optimal stimulatory concentration. Incubation with 10 μ M peptide P13 reduced MIP-2 secretion 81% (data not shown). We next sought to establish the effect on MIP-2 secretion when peptide was added at various time points before, simultaneous with, or after stimulation with CpG-ODN. Inhibition seen after addition of peptide P13 up to 1 h after stimulation with CpG-ODN was similar to inhibition seen when peptide was added either before or simultaneous with CpG-ODN (>85% inhibition). Significant inhibition of MIP-2 secretion was demonstrated even when peptide was added as long as 4 h after stimulation of cells with CpG-ODN (Fig. 4). Peptide inhibition of cytokines other than MIP-2 was also examined. RAW264.7 cells were stimulated with CpG-ODN, and secretion of TNF- α , IL-6, and IL-10 was quantified by ELISA. Treatment of cells with peptide P13 significantly inhibited secretion of each of these cytokines (Fig. 5). In addition, peptide inhibited intracellular TNF- α production by 59% as assessed by FACS (data not shown). Inhibition of cytokine secretion by peptide P13 was also seen when human BJAB B cells were activated by TLR ligands (data not shown). In summary, the peptide demonstrated inhibition of cytokines stimulated by numerous TLR ligands, both alone and in combination. The inhibition was dose dependent and seen for a variety of cytokines produced by both macrophages and B cells. Peptide P13 was effective even when added after the stimulating PAMP, suggesting a potential application as an anti-inflammatory therapy.

Mechanism of peptide P13 inhibition of cytokine secretion

The A52R protein has been previously demonstrated to inhibit TIR signaling by interacting with both IRAK2 and TRAF6, intracellular signaling molecules involved in TIR signaling (18). We hy-

pothesize that P13, like the parent protein, inhibits cytokine secretion through interaction with IRAK2 and/or TRAF6. Data from the following experiments support this hypothesis.

Peptide P13 must be internalized to inhibit cytokine secretion.

To interact with IRAK2 and/or TRAF6, peptide P13 must be internalized. We compared treatment of cells with peptide P13 that either contained or lacked the nine-arginine cell transducing sequence. Peptide without the transducing sequence was not internalized into cells as previously demonstrated (Fig. 1). Treatment of RAW264.7 cells with peptide P13 lacking the transducing sequence had no effect on MIP-2 secretion in response to stimulation with either LPS or CpG-ODN (Table I). As previously demonstrated, peptide containing the cell transducing sequence significantly inhibited MIP-2 secretion (Fig. 3).

Peptide P13 does not inhibit cytokine secretion stimulated by PMA or TNF- α .

Both PMA and TNF- α activate RAW264.7 cells via signaling pathways independent of either IRAK2 or TRAF6, resulting in secretion of MIP-2. Treatment with peptide had no effect on MIP-2 secretion in response to stimulation with either PMA or TNF- α (Table II).

Peptide P13 inhibits phosphorylation of I κ B- α .

The intracellular signaling pathway triggered by the interaction of PAMPs with TLRs involves the IRAK family and TRAF6, resulting in translocation of NF- κ B to the nucleus, followed by secretion of proinflammatory cytokines. Activation of NF- κ B is dependent on the phosphorylation and proteolysis of the I κ B proteins. RAW264.7 cells were treated with either peptide P13 or control scrambled peptide and stimulated with LPS for either 15 or 30 min. Cells were lysed and analyzed by immunoblotting using phospho-I κ B- α Ab, which detects endogenous levels of I κ B- α only when phosphorylated at Ser³². Peptide P13 completely inhibited the phosphorylation of I κ B- α in LPS-activated cells compared with cells treated with control scrambled peptide, which demonstrated a 2-fold increase over background (Table III).

Peptide P13 inhibits cytokine secretion initiated by TLR3.

The TLR3 signaling pathway is different from the other TLR signaling pathways in that it requires TRAF6, but not the IRAK family or the upstream adaptor molecule MyD88, for the production of proinflammatory cytokines. Downstream of TRAF6, the pathways are similar, both resulting in the phosphorylation of I κ B and the translocation of NF- κ B to the nucleus. As demonstrated above, peptide P13 inhibits MIP-2 production from RAW264.7 cells stimulated with poly(I:C), a synthetic ligand for TLR3 (Fig. 3). Collectively, these data support the conclusion that peptide P13 inhibits cytokine secretion by interaction with an intracellular portion of the TIR signaling pathway upstream of I κ B. The inhibition of TLR3-mediated cytokine secretion, in combination with the other PAMP/TLR inhibitory data, suggests that the effect of peptide P13 is on TRAF6 or a downstream component of the TIR signaling pathway. The data are consistent with the hypothesis that peptide P13, like

FIGURE 5. Peptide P13 inhibition of TNF- α , IL-6, and IL-10 secretion. RAW264.7 cells were incubated for 15 min with 10 μ M peptide P13 and then stimulated with CpG-ODN (1 μ g/ml). Cell-free supernatants were collected after 18 h, cytokine secretion was quantified by ELISA, and data are expressed as nanograms per milliliter \pm SD.

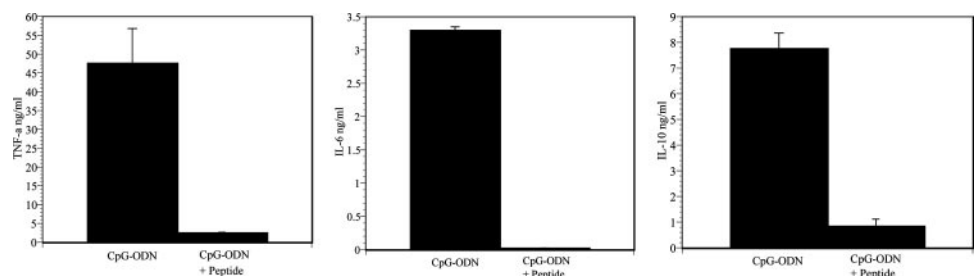


Table I. Peptide P13 lacking a cell transducing sequence fails to inhibit MIP-2 secretion

Treatment	MIP-2 (pg/ml \pm SD) ^a	% Inhibition
LPS	46,618 \pm 923	
LPS + peptide P13	12,435 \pm 269	73
LPS + peptide P13 (no transducing sequence)	46,931 \pm 1,335	0
CpG-ODN	31,194 \pm 743	
CpG-ODN + peptide P13	3,242 \pm 238	90
CpG-ODN + peptide P13 (no transducing sequence)	29,312 \pm 618	6

^a RAW264.7 cells were incubated for 15 min with either medium, peptide P13 containing the transducing sequence, or peptide P13 lacking the transducing sequence, and then stimulated with either LPS (1 ng/ml) or CpG ODN (1 μ g/ml). Cell-free supernatants were analyzed for MIP-2 by ELISA, and data are expressed as picograms per milliliter \pm SD.

the parent A52R protein, interacts in the TIR signaling pathway at TRAF6.

Inhibition of middle ear inflammation

The effect of peptide P13 on bacterial-induced inflammation *in vivo* was examined using a murine model of otitis media with effusion (OME). The inflammatory response in bacterial-induced OME is initiated by TLR activation and is characterized by infiltration of cells into the middle ear, fluid accumulation, and thickening of the mucosal epithelium and the tympanic membrane (21). To first examine any potential effects caused by peptide alone without added bacteria, five mice were injected in one ear with PBS and in the opposite ear with 10 μ M peptide P13. Three days later, the animals were killed, and middle ears were embedded, sectioned, stained, and evaluated for fluid area, infiltrating cell number, and thickness of the tympanic membrane. Paired *t* tests (two-tailed) were used to analyze each of the three parameters. In the absence of bacterial-induced inflammation, no differences were seen between the PBS-injected ear and peptide P13-injected ear in 1) fluid area ($p = 0.104$), 2) cell number ($p = 0.880$), or 3) tympanic membrane thickness ($p = 0.891$). To examine the effectiveness of the peptide to affect inflammation *in vivo*, 20 BALB/c mice were injected in the middle ear on one side with heat-inactivated *S. pneumoniae* plus PBS and in the middle ear on the opposite side with heat-inactivated *S. pneumoniae* plus 10 μ M peptide P13. Three days later, the animals were killed and evaluated for middle ear fluid area, infiltrating cell number, and thickness of the tympanic membrane. Disease development was defined as an increase over background controls (PBS-injected ears, $n = 18$) of at least 2 SDs in two of the three parameters quantified. A total of 7 of 20 mice met the criteria for disease induction. Analysis of middle ears by paired *t* tests from these 7 mice with disease

Table II. Peptide P13 does not inhibit non-TLR-induced MIP-2 secretion

Treatment ^a	MIP-2 (pg/ml \pm SD)
Medium	781 \pm 7
TNF- α	1,744 \pm 16
TNF- α + peptide P13	2,384 \pm 16
PMA	22,144 \pm 544
PMA + peptide P13	24,736 \pm 1,216

^a RAW264.7 cells were incubated for 15 min with either medium or peptide P13 and then stimulated with either medium, TNF- α (100 ng/ml), or PMA (100 ng/ml) for 18 h. Cell-free supernatants were analyzed for MIP-2 by ELISA, and data are expressed as picograms per milliliter \pm SD.

Table III. Peptide P13 inhibits phosphorylation of I κ B- α

Treatment	Phosphorylated I κ B- α Band Intensity/Area ^a	
	Scrambled peptide	Peptide P13
Medium	6.3	5.4
LPS (15 min)	13.1	4.9
LPS (30 min)	13.3	3.1

^a RAW264.7 cells were incubated for 15 min with either peptide P13 or control scrambled peptide, and then treated with either medium, or LPS (1 ng/ml) for either 15 or 30 min. Immunoblotting was performed using phospho-I κ B- α (Ser³²) Ab. Measurements of band intensity were made using the Nucleo Tech Gel Expert software linked to an Epson expression 636 scanner, and data are expressed as band intensity/area.

showed that peptide treatment significantly reduced the amount of fluid ($p = 0.004$), infiltrating cell number ($p = 0.02$), and thickness of the tympanic membrane ($p = 0.002$), all parameters of middle ear inflammation (Table IV). Examination of these three parameters of inflammation for each individual mouse with disease illustrates the dramatic effect seen with a single treatment of peptide P13 (Table V). Of interest, 6 of the 7 mice demonstrated reductions in all areas of inflammation, whereas 1 animal (no. 4-182) showed only modest reduction in fluid area and tympanic membrane thickness, and no reduction in cell number. Photographs from a normal, nondiseased animal and a representative animal with disease illustrate the effect of peptide on bacterial-induced inflammation *in vivo*. The middle ear of normal mice is free of fluid or cells (Fig. 6A), and the mucosal epithelium that lines the middle ear space is normally comprised of one to two low cuboidal cells (Fig. 7A). Injection of heat-killed bacteria resulted in a marked inflammatory response in the middle ear after 3 days. This was characterized by mucosal and tympanic membrane swelling, cellular infiltration, and significant fluid (effusion) secretion and accumulation that filled the middle ear space (Fig. 6, B and C). The inflammatory response led to significant mucosal cellular hypertrophy and active secretion of mucins and other fluids (Fig. 7B). When peptide P13 was injected with the bacteria, a significant reduction was seen in fluid accumulation into the middle ear space (Fig. 6D) and reduced mucosal hypertrophy (Fig. 7C).

Discussion

TLRs are conserved molecular receptors that recognize structures from bacteria, fungi, protozoa, and viruses. Activation of TLRs

Table IV. Peptide P13 inhibition of middle ear inflammation^a

Treatment	Fluid Area (μ m ² \pm SD)	Cell Number (\pm SD)	Tympanic Membrane Thickness (μ m \pm SD)
PBS ^b	1016 \pm 1397	31 \pm 41	44 \pm 20
<i>S. pneumoniae</i> ^c	5771 \pm 2077	252 \pm 140	105 \pm 33
<i>S. pneumoniae</i> + peptide P13 ^c	1486 \pm 1192	111 \pm 119	44 \pm 15
<i>p</i> value (two-tailed) ^d	$p = 0.004$	$p = 0.020$	$p = 0.002$

^a Middle ear inflammation was assessed by measuring three consecutive tissue sections for area of fluid in the middle ear, number of cells in the middle ear fluid, and thickness of the tympanic membrane measured at a point away from the injection site. Data represent the mean \pm SD of seven animals with middle ear inflammation. Statistical evaluation was done using a paired *t* test.

^b The PBS-treated animals ($n = 18$) received no bacteria or peptide P13.

^c Animals ($n = 7$) injected in one ear with *S. pneumoniae* plus PBS and in the opposite ear injected with *S. pneumoniae* plus peptide P13 (10 μ M).

^d Statistical evaluation using a paired *t* test was done using data collected from diseased animals ($n = 7$) injected with bacteria and comparing peptide vs no peptide P13 treatment.

Table V. Peptide P13 inhibits development of fluid, cell number, and tympanic membrane thickening in a murine model of OME^a

Animal No.	Fluid Area (% inhibition)	Cell Number (% inhibition)	Tympanic Membrane Thickness (% inhibition)
4-21	84	39	63
4-24	85	96	65
4-177	86	77	66
4-182	11	0	22
4-183	73	44	71
4-185	66	77	60
4-195	94	89	69

^a Middle ear inflammation was assessed as described in Table III. Percent inhibition is calculated by comparing fluid area, cell number, and tympanic membrane thickness seen in one ear injected with *S. pneumoniae* plus PBS with the same parameters of inflammation seen in the opposite ear injected with *S. pneumoniae* plus peptide P13 (10 μ M).

initiates a series of intracellular events, resulting in an innate immune response characterized by the production of proinflammatory cytokines (2–9). TLR signaling originates from the cytoplasmic TIR domain, conserved among all TLRs. The adapter molecule MyD88, containing both a TIR domain and a death do-

main, associates with the TIR domain of TLRs and IRAK proteins. Phosphorylation of IRAK leads to association with TRAF6 and subsequent activation of NF- κ B and secretion of proinflammatory cytokines (1, 14, 22–24). A52R, an immunoregulatory protein from vaccinia virus, has previously been shown to be an intracellular inhibitor of TIR-dependent signaling (15, 18). When expressed in HEK293 cells, A52R was shown to inhibit NF- κ B activation in response to stimulation by a variety of TLRs, including TLR4, TLR5, and the combination of TLR2 and -6, and TLR2 and -1. In addition, A52R inhibited NF- κ B activation in response to poly(I:C), a synthetic ligand for TLR3. TLR3 has been implicated in an antiviral innate immune response. The peptide P13 (sequence DIVKLTVYDCI) reported here was derived from the immunoregulatory protein A52R and demonstrates many of the same properties as the protein. The peptide inhibits cytokine secretion in response to a variety of TLR ligands, including LPS (TLR4), CpG-ODN (TLR9), poly(I:C) (TLR3), flagellin (TLR5), and zymosan (TLR2). Harte et al. (18) have demonstrated that the A52R protein inhibits TIR signaling by binding to both IRAK2 and TRAF6, key intracellular regulatory proteins. These authors further suggest that A52R binds independently to IRAK2 and TRAF6, suggesting the redundant targeting may indicate the importance of inhibiting TIR activation to enhance virulence. Consistent with this speculation,

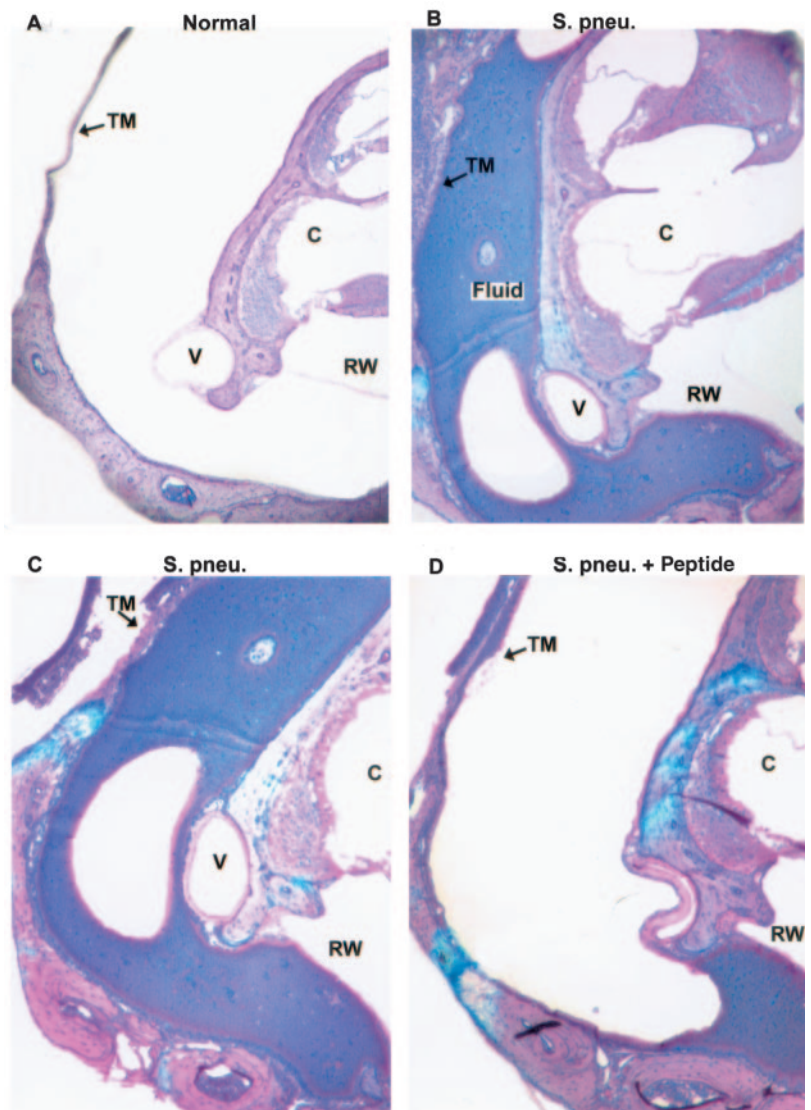


FIGURE 6. Photographs of the middle ear from after treatment with peptide P13. *A*, The normal middle ear of BALB/c mice is clear between the tympanic membrane (TM) and round window (RW). *B*, Injection of the middle ear with *S. pneumoniae* (*S. pneu.*) causes extensive inflammation. The middle ear space lateral to the cochlea (C) and around the stapedial artery (V) is filled with fluid, and the tympanic membrane is thickened. *C*, Higher magnification of the *S. pneu.*-injected mouse in *B* shows the extensive fluid accumulation in the middle ear space. *D*, The fluid and inflammation is significantly reduced and confined to the round window area when peptide P13 (*S. pneu.* + peptide) is injected with the bacteria.

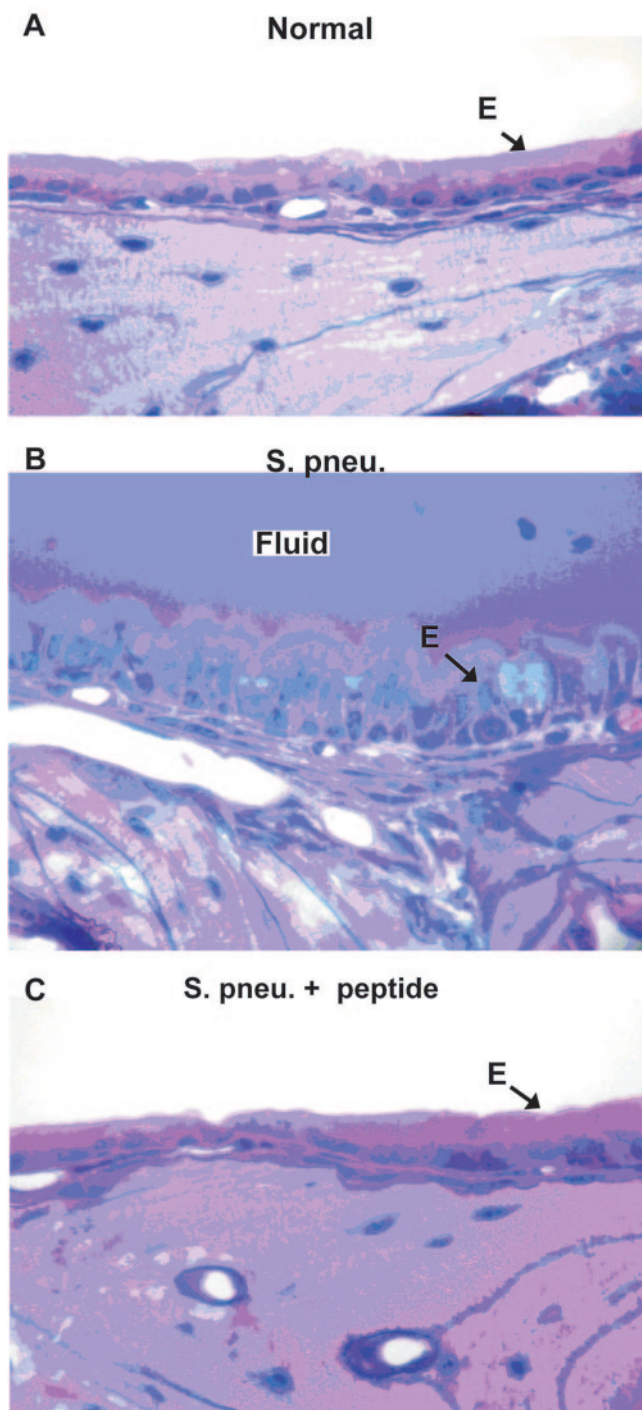


FIGURE 7. Photographs of the mucosal epithelium after treatment with peptide P13. *A*, The normal mucosal epithelium (E) of the middle ear lateral wall is typically comprised of only one to two layers of low cuboidal cells. *B*, *S. pneumoniae* (*S. pneu.*) injection causes hypertrophy of the epithelium, cells become secretory, and fluid accumulates. *C*, The epithelium retains a normal appearance and fluid disappears when peptide P13 is injected along with *S. pneumoniae* (*S. pneu.* + peptide).

deletion of the A52R protein from vaccinia virus resulted in reduced viral virulence. The mechanism by which peptide P13 inhibits TLR-dependent cytokine secretion remains to be defined. Our studies demonstrated that internalization of the peptide was required for inhibition, and that cytokine secretion, in response to non-TLR-dependent activation, was not affected. In addition, the

demonstration that peptide P13 inhibited phosphorylation of I κ B- α and inhibited TLR3 signaling, is consistent with the hypothesis that P13 acts on the TIR signaling pathway at some point between TRAF6 and I κ B. Whether peptide P13 associates with TRAF6, like the parent A52R protein, or another intracellular signaling protein further downstream, is currently under investigation.

The *in vivo* effectiveness of the peptide was demonstrated using a mouse model of OME. OME is an inflammatory disease of the middle ear accompanied by fluid accumulation. It is characterized by an infiltration of leukocytes, macrophages, and mast cells, and release of inflammatory mediators and enzymes (21). These mediators increase vascular permeability and secretory activity, and initiate a cascade of inflammatory events, resulting in fluid accumulation and mucin secretion (25, 26). The initiation of inflammation in OME has been attributed to a variety of factors, including bacterial or viral infections, eustachian tube dysfunction, and allergy. However, the evidence points to a bacterial etiology leading to cytokine activation in the majority of cases. Bacteria have been cultured from up to 40% of effusions, and studies have shown bacterial DNA by PCR in ~80% of effusions, often in the absence of viable organisms in culture (27). The most common bacteria invading the middle ear are *S. pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis*. These three bacteria account for 85% of acute middle ear infections (26), with *S. pneumoniae* being the most frequent cause. Initially, live bacteria trigger acute inflammation, which is designed to eliminate the pathogen. During acute infection, interference with the innate immune response would be potentially harmful to the host and may lead to further bacterial spread. Acute inflammation initiated by bacterial infections self-resolves or is treatable by antibiotics. Chronic inflammation involves continued activation of the immune system, often by nonviable bacterial products. OME is often prolonged or antibiotic resistant, suggesting TLR stimulation in absence of live bacteria. We would predict that agents that interfere with TLR-dependent signaling would be potential treatments for prolonged or antibiotic-resistant middle ear inflammation. In our studies, treatment of mice with peptide P13 resulted in a significant reduction in bacterial-induced inflammation in the middle ear. Fluid accumulation, infiltrating cells, and tympanic membrane thickness in the middle ear were all dramatically reduced with peptide treatment. Administration of heat-inactivated bacteria, which has a number of potential TLR ligands, induced an inflammatory response in the middle ear most likely resulting from activation of multiple TLRs. In our studies, the use of heat-inactivated bacteria allowed for an examination of peptide inhibition of inflammation without the potential for bacterial spread that may occur in an acute infection initiated with live bacteria. The ability of peptide P13 to significantly inhibit this response *in vivo* is consistent with the *in vitro* data showing inhibition of cytokine secretion in response to multiple TLR ligands used either individually or in combination. In these studies, a single dose of peptide was administered at the same time as heat-inactivated *S. pneumoniae* into the middle ears of normal BALB/c mice. Although these studies demonstrated a dramatic effect on inflammation, additional studies assessing the effect of peptide treatment on resolving an ongoing inflammatory response are needed. Of interest in this respect, our *in vitro* data showed inhibition of cytokine secretion even when peptide P13 was added several hours after initiation of TLR activation.

The initiation of an inflammatory response to pathogens is a critical component of the innate immune response and is designed to control infection. However, the sustained production of inflammatory mediators can lead to chronic inflammation, tissue damage, and disease development. The signaling cascade initiated by

PAMP/TLR interactions and culminating in cell activation has been associated with many disease states, including sepsis, autoimmune diseases, asthma, heart disease, and cancer (28). For example, it is hypothesized that sepsis occurs when bacteria and their products activate an uncontrolled network of host-derived mediators, such as proinflammatory cytokines, which can lead to multi-organ failure, cardiovascular collapse, and death. An abnormal TLR signaling response could lead to exaggerated cell activation responses contributing to sepsis (29, 30). Inflammation is also a key aspect of autoimmunity, and is hypothesized to play a role in tissue destruction in diseases such as multiple sclerosis, rheumatoid arthritis, and insulin-dependent diabetes mellitus (31). Cells of the innate immune system have an essential role in acquired/adaptive immunity. TLR proteins are involved in the maturation and activation of dendritic cells, the APC type considered most relevant to development of acquired immunity (32). Allergic asthma is an example of a chronic inflammatory disease with an adaptive immune response, and the TLR signaling pathway is hypothesized to play an important role in the induction phase of an allergic phenotype (29). Bacterial and viral infections, causing increased inflammatory cell activation, are the main cause of exacerbations in diseases such as asthma and chronic obstructive pulmonary disease (29). Understanding and manipulating the TLR cell activation pathway has the potential to provide therapeutic benefit for a variety of diseases with an inflammatory etiology. Treatments for inflammation have included the use of aspirin and glucocorticoids to block NF- κ B activation (28, 33, 34) and the targeting of specific inflammatory mediators such as TNF- α (35). Recent studies report blocking the interaction of TLRs and their ligands (36), or suppressing TLR expression (37–39) may provide new approaches for controlling inflammation. The identification of proteins involved in TIR signaling, and their molecular characterization, have led to development of agents to inhibit specific points within the TIR signaling cascade. Bartfai et al. (40) have recently reported the synthesis of a low-molecular-mass mimic of MyD88. The structure of the compound was based on the sequence of the TIR domain. The compound inhibited the interaction between MyD88 and the IL-1R1 TIR domain, thereby inhibiting IL-1-induced activation in vitro and was effective in vivo at blocking IL-1-induced fever in mice. The compound did not block the interaction of TLR4 and MyD88, and therefore LPS-induced activation was not inhibited. Inhibition of multiple TLR-dependent responses, by targeting a common signaling component, may prove to be a more effective approach to controlling an inflammatory response.

In this report, we have identified an 11-aa sequence from the vaccinia virus A52R protein that has many of the same immunoregulatory properties described for the whole protein. When linked with a cell transducing sequence, we showed this peptide inhibited in vitro TLR-induced cytokine secretion and in vivo significantly reduced bacterial-induced inflammation in a murine model of OME. The treatment and control of bacterial- and viral-induced inflammation represents a significant clinical challenge. The selective targeting of the TLR/TIR signaling cascade represents one approach to control inflammation, and the identification of this peptide from the A52R protein may have potential therapeutic application.

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Disclosures

Steven H. Hefeneider is the CEO of and has a significant financial interest in Targeted Gene Delivery, Inc. This potential conflict of interest has been reviewed and managed by the Oregon Health and Science University Conflict of Interest in Research Committee.

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