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The Role of Intermediary Domain of MyD88 in Cell Activation and Therapeutic Inhibition of TLRs

Monika Avbelj,*,† Simon Horvat,*‡ and Roman Jerala*†,§

Adaptor MyD88 has a pivotal role in TLR and IL-1R signaling and is involved in mediating excessive inflammation. MyD88 is composed of a death domain and a Toll/IL-1R domain connected by an intermediary domain (INT). The alternatively spliced form of MyD88 lacking the INT prevents signaling through MyD88-dependent TLRs. We designed a peptide from the INT and showed that it inhibits TLR4 activation by LPS when linked to a cell-penetrating peptide. As a new approach for the delivery of signaling-inhibitory peptides, INT peptide acylation also provided efficient cell translocation and inhibition of activation. We determined that INT peptide targets IL-1R–associated kinase 4. Furthermore, MyD88 mutant and molecular modeling refines the MyD88–IL-1R–inhibitory peptides, INT peptide acylation also provided efficient cell translocation and inhibition of activation. We determined that INT peptide inhibits TLR4 activation by LPS when linked to a cell-penetrating peptide. As a new approach for the delivery of signaling-inhibitory peptides, INT peptide acylation also provided efficient cell translocation and inhibition of activation. We determined that INT peptide targets IL-1R–associated kinase 4. Furthermore, MyD88 mutant and molecular modeling refines the MyD88–IL-1R–associated kinase 4 interaction model based on the Myddosome structure. In addition to TLR4, INT peptide also inhibited TLR5, TLR2, TLR9, and IL-1R signaling but not TLR3, which uses Toll/IL-1R domain-containing adapter inducing IFN-β signaling adaptor. Inhibition of signaling in murine and human cells was observed by decreased NF-κB activation, cytokine mRNA synthesis, and phosphorylation of downstream kinases. In the endotoxemic mouse model, INT peptide suppressed production of inflammatory cytokines and improved survival, supporting therapeutic application of INT peptides for the suppression of inflammatory conditions mediated by MyD88.

adaptors MyD88, Mal/TIRAP, TRIF, and TRAM, have been shown to inhibit TLR4 signaling (25–28). BB-loop peptides can bind, although with variable affinity, to proteins containing TIR domains. Another example of TLR4-inhibiting peptide is viral inhibitor peptide of TLR4, a peptide derived from vaccinia virus protein A46 that has been proposed to target Mal and TRAM (29).

To inhibit intracellular signaling, a peptide must enter into the cell. For peptide translocation, short sequences derived from segments of proteins capable of entering cells, called cell-penetrating peptides, such as the TAT protein of HIV-1 and sequence of homeodomain of transcription factor antennapedia (ANT) in Drosophila, have been used (30).

MyD88 as a master adapter involved in signaling through several TLRs represents an excellent potential target for development of inhibitory cell-penetrating peptides to attenuate ill symptoms due to misregulation of the innate immune system in the aforementioned medical conditions. That is why we designed inhibitory peptides derived from the INT of MyD88 linked to the motif for cell internalization. We showed that INT peptide inhibits signaling of MyD88-dependent TLR receptors as well as IL-1R. Binding of INT peptide to IRAK4 demonstrates that INT of MyD88 is important for MyD88 signaling and interaction with IRAK4. Furthermore, we showed that INT peptide has an in vivo inhibitory effect on inflammatory cytokine production and improves survival of LPS-sensitized mice, suggesting that it could be used for the therapy of MyD88-dependent inflammatory diseases.

Materials and Methods

Materials

Materials detailed below were obtained as kind gifts from investigators, purchased from companies, or produced in our laboratory. Plasmids used in luciferase and expression assays: plasmid coding for firefly luciferase under NF-κB promoter (pELAM-1 luciferase; from C. Kirschning, Institute for Medical Microbiology, University of Duisburg-Essen, Essen, Germany); plasmid coding for firefly luciferase under IFN-β promoter (IFN-β-luciferase; from J. Hiscott, Departments of Microbiology and Medicine, McGill University, Montreal, QC, Canada); plasmid with constitutive Renilla luciferase–phRL-TK (Promega); plasmids coding for different TLRs: hTLR4 (hTLR4 in pcDNA3; from T. Espevik, Institute of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway), hMD-2 (pEFBOSMD-2), hTLR2 (pFLAG TLR2 from C. Kirschning), hTLR5, hTLR3, hTLR8, and hTLR9 (pUNO-hTLR5, pUNO-hTLR3, pUNO-hTLR8-HA, pUNO-hTLR9-HA; Invivogen); pcDNA3 (Invitrogen); plasmid pcDNA3 MyD88-AU1 (from C. Kirschning); plasmid pcDNA3 MyD88-CFP (from T. Espevik); plasmid pECFP-N1 (Clontech); and plasmid pcDNA3 IRAK4-CFP was prepared with PCR ligation (specific primers used for IRAK4-CFP construction are available upon request).

FIGURE 1. INT peptides inhibit MyD88-dependent TLR4/MD-2 receptor signaling. (A) MyD88 INT sequence alignment. HEK293 cells were transfected with plasmids for TLR4, its coreceptor MD-2 (B), or plasmid for TLR3 (C) and reporter plasmids. Twenty-four hours after transfection, peptides were added, and 6 h later, HEK293 cells were stimulated with sLPS (final concentration of 25 ng/ml) (B) or poly (I:C) (final concentration of 10 μg/ml) (C). After 16 h, cells were lysed, and luciferase activity was measured. Data represented are means ± SD of triplicate samples and representative of at least three independent experiments. D, Peptide ANT-INT labeled with Alexa Fluor 555 (ii) was added to HeLa cells (final concentration of 40 μM) for 6 h (i). Nuclei were visualized using Hoechst dye (iii). Overlay (iv, v) indicates internalization of labeled ANT-INT. Data are representative confocal images of at least three independent experiments. Scale bars, 10 μm. ***p < 0.005.
request) from IRAK4 (Origene) and CFP (pECFP-N1; Clontech). Mutant pcDNA3 MyD88(110KKK) was prepared from pcDNA3 MyD88-AU1 with the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Specific primers were used to mutate aa E110, E111, and D112 into K110, K111, and K112 are available upon request. TLR ligands used in luciferase, quantitative PCR (qPCR), and Western blot (WB) assays: smooth LPS (sLPS) Salmo-
ella minnesota sp. Equi (from K. Brandenburg, Institute of Biophysics and Nanosystems Research, Austrian Academy of Sciences, Graz, Austria), polyinosinic-polycytidylic acid [poly (I:C)] (Invivogen), Salmonella typhimurium flagellin (Invivogen), lipoteichoic acid (LTA) from Staphylo-
coccus aureus (Invivogen), ODN10104 (Coley Pharmaceutical Group), ODN1826 (Alexis Biochemicals), R848 (Alexis Biochemicals), R848 (Alexis Biochemicals), recombi-
nant human TNF-α (Invitrogen), and human IL-1β (R&D Systems).

**Peptide synthesis**

Peptides were synthesized by Small Scale Peptide Synthesis (Yale University) and their quality analyzed by HPLC and mass spectrometry. Peptide ANT-INT was labeled with specific dye Alexa Fluor 555 according to the manufacturer’s instructions (Invitrogen).

**Cell cultures**

Human HEK293, murine RAW264.7, and HeLa cell lines were obtained from the European Collection of Cell Cultures. MyD88 knockout (KO) macrophages were a kind gift from K. Fitzgerald (Division of Infectious Diseases and Immunology, Department of Medicine, University of Massachusetts Medical School, Worcester, MA). MyD88 KO HEK293 cell line (HEK293-13A) was a kind gift from G. Stark (Department of Molecular Genetics, Lerner Research Institute, Cleveland Clinic, Cleveland, OH) and A. Weber (German Cancer Research Centre [DKFZ], Division Toll-like receptors and Cancer, Heidelberg, Germany). All cell lines were cultured in minimal DMEM (Invitrogen) supplemented with 10% heat-inactivated FBS (Life Technologies).

**Luciferase assay**

For dual luciferase assays, HEK293 or HEK293-13A cells were plated onto 96-well plates (Corning) and transiently transfected with plasmids coding for particular TLR receptor, MyD88-AU1, or mutant MyD88(110KKK) and reporter plasmids using jetPEI Transfection Reagent (Polyplus Transfection). Twenty-four hours after transfection, peptides were optionally added for 6 h and cells optionally stimulated for 16 h with appropriate TLR agonist, lysed in passive lysis buffer (Promega), and measured for luciferase assay.

**Isolation of mRNA, RT-PCR, and qPCR**

For mRNA expression assays, RAW264.7 were plated onto a 12-well plate (Techno Plastic Products). Twenty-four hours later, peptides were optionally added to cells for 2 h and then stimulated with appropriate TLR ligand. After 3 h, cells were lysed and RNA isolated using Pure Link RNA Mini Kit (Invitrogen). Reverse transcription was performed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems) with specific primers for desired cytokines. qPCR assay was performed using Power SYBGreen PCR Master Mix (Applied Biosystems).

**SDS-PAGE and Western analysis**

For phosphorylation assays, RAW264.7 were plated on 24-well plates (Techno Plastic Products). Twenty-four hours later, peptides were optionally added to cells for 2 h and then stimulated with sLPS Salmonella minnesota sp. Equi. Cells were stimulated for the indicated time and lysed in RIPA buffer with proteinase inhibitors (Sigma-Aldrich) and phosphatase inhibitors (Calbiochem). A total of 50 µg total cell protein was loaded on SDS-PAGE and transferred to nitrocellulose membrane (GE Healthcare).

### Table I. INT peptides

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Translocation Domain</th>
<th>Peptide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANT-INT</td>
<td>Antennapedia sequence</td>
<td>Sequence from INT of MyD88, 21 aa (EEDCQKYILKQQQEEAEKPLQ)</td>
</tr>
<tr>
<td>MYR-INT</td>
<td>Myristate</td>
<td>Sequence of cationic amino acid GRVRLS and sequence from INT of MyD88, 21 aa (EEDCQKYILKQQQEEAEKPLQ)</td>
</tr>
<tr>
<td>MYR-INT21</td>
<td>Myristate</td>
<td>Sequence from INT of MyD88, 21 aa (EEDCQKYILKQQQEEAEKPLQ)</td>
</tr>
<tr>
<td>MYR-INT17</td>
<td>Myristate</td>
<td>Sequence from INT of MyD88, 17 aa (EEDCQKYILKQQQEEAE)</td>
</tr>
<tr>
<td>MYR-INT13</td>
<td>Myristate</td>
<td>Sequence from INT of MyD88, 13 aa (LKQQQEEAEKPLQ)</td>
</tr>
<tr>
<td>MYR-INT9</td>
<td>Myristate</td>
<td>Sequence from INT of MyD88, 9 aa (LKQQQEEAE)</td>
</tr>
<tr>
<td>LAU-INT</td>
<td>Laurate</td>
<td>Sequence of cationic AA GRVRLS and sequence from INT of MyD88, 21 aa (EEDCQKYILKQQQEEAEKPLQ)</td>
</tr>
<tr>
<td>Control peptides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>INT-CTRL</td>
<td>–</td>
<td>Sequence of cationic AA GRVRLS and sequence from INT of MyD88, 21 aa (EEDCQKYILKQQQEEAEKPLQ)</td>
</tr>
<tr>
<td>MYR-CTRL</td>
<td>Myristate</td>
<td>Scrambled sequence of 21 aa from domain INT of MyD88</td>
</tr>
<tr>
<td>ANT-CTRL</td>
<td>Antennapedia sequence</td>
<td>Scrambled sequence of 21 aa from domain INT of MyD88</td>
</tr>
</tbody>
</table>

LAU, lauroyl.

### FIGURE 2. Identification of the minimal INT sequence and type of the internalization motif required for inhibition. HEK293 cells were transfected with plasmids for TLR4, its coreceptor MD-2, and reporter plasmids. Twenty-four hours after transfection, peptides were added, and 6 h later, HEK293 cells were stimulated with sLPS (final concentration of 25 ng/ml). After 16 h, cells were lysed and luciferase activity was measured (A, B). Data represented are means + SD of triplicate samples and representative of at least three independent experiments.
Detection of phosphorylation was done using specific primary Abs: phosphoplus(R) IκBα (Ser17/Ser18) Ab duet, phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) mouse mAb, phospho–IRF-3 (Ser396) rabbit mAb, α/β-tubulin Ab (Cell Signaling Technology), and appropriate secondary Abs: goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) and goat polyclonal Abs to rabbit IgG-HRP (Abcam).

For expression studies of MyD88 AU-1 and mutant MyD88(110KKK), HEK293 were seeded on six-well plates (Techno Plastic Products) and transiently transfected with 1 µg plasmid coding for wild-type or mutant MyD88. Twenty-four hours later, cells were lysed in RIPA buffer with protease inhibitors (Sigma-Aldrich), and 30 µg total cell protein was loaded on SDS-PAGE and transferred to nitrocellulose membrane (GE Healthcare). Detection of expression was done using specific primary polyclonal Ab to AU-1 tag (Abcam) and secondary goat polyclonal Abs to rabbit IgG-HRP (Abcam).

Peptide pulldown assay

HEK293 were seeded in six-well plates and transfected with plasmids coding for CFP, IRAK4-CFP, or MyD88-CFP (2 µg plasmid/well). Forty-eight hours later, supernatants were removed and cells washed with ice-cold PBS. Cells were lysed in modified 1% NETN lysis buffer (100 mM NaCl, 50 mM HEPES [pH 7.5], 1 mM EDTA, 1% Triton X-100, and 10% glycerol).

For peptide pulldown assay, Ni-agarose beads were first incubated at room temperature for 4 h with 100 µM peptide (target or control peptide). After that, beads were washed two times with PBS containing 50 mM imidazole. Cells lysates diluted in lysis buffer containing 50 mM imidazole and five times with lysis buffer. Bead samples were incubated at 50°C with SDS sample buffer for 10 min and resolved with SDS-PAGE. The resolved proteins were transferred to nitrocellulose membrane and immunoblotted for corresponding protein using primary anti-GFP Abs (Invitrogen) and secondary goat polyclonal Abs to rabbit IgG-HRP (Abcam).

For endogenous IRAK4 binding studies RAW264.7 cells were seeded in six-well plates. Twenty-four hours later, peptide ANT-INT-HIS or ANT-CTRL-HIS was added (final concentration of 150 µM) for indicated times (0, 30, and 120 min) and later stimulated with sLPS *Salmonella minnesota* sp. Equi for 5 min. Cells were then washed with ice-cold PBS and lysed in modified 1% NETN lysis buffer (100 mM NaCl, 50 mM HEPES [pH 7.5], 1 mM EDTA, 1% Triton X-100, and 10% glycerol). A total of 700 µg total protein was loaded on SDS-PAGE and transferred to nitrocellulose membrane. Immunoblots were performed with primary anti-GFP Abs (Invitrogen) and secondary goat polyclonal Abs to rabbit IgG-HRP (Abcam).

**FIGURE 3.** Comparative effect of INT peptides on different MyD88-dependent or -independent signaling pathways. HEK293 cells were transfected with plasmids for TLR5 (A), TLR9 (B), or TLR3 (D) and reporter plasmids. Twenty-four hours after transfection, peptides were added, and 6 h later, HEK293 cells were stimulated with flagellin (final concentration of 10 ng/ml) (A), ODN 10104 (final concentration of 3 µM) (B), human IL-1β (final concentration of 10 ng/ml) (C), poly (I:C) (final concentration of 10 µg/ml) (D), or human TNF-α (final concentration of 100 ng/ml) (E). After 16 h, cells were lysed, and luciferase activity was measured. Data represented are means + SD of triplicate samples and representative of at least three independent experiments. **p < 0.01, ***p < 0.005.
cell lysate was then incubated with Ni-agarose beads and incubated overnight at 4°C with gentle shaking. Later steps were similar to those described above. For IRAK4 detection, specific IRAK4 Ab (Cell Signaling Technology) and secondary goat polyclonal Abs to rabbit IgG-HRP (Abcam) were used.

Confocal microscopy
For microscopy assay, HeLa cells were plated on an eight-well plate (Ibidi). Later, Alexa Fluor 555-labeled peptide was optionally added to cells for 6 h (final concentration of 40 μM), and cells were then analyzed using Leica TCS SP6 confocal microscope (using software LAS AF, Leica Microsystems) under 63× oil immersion objective (numerical aperture 1.4). Observations were done at room temperature. Excitation and emission wavelengths used to visualize different fluorophores were: Alexa Fluor 555 (excitation 543 nm, emission 560–600 nm); nuclei dye Hoechst 33342 (Invitrogen) was used accordingly to the manufacturer’s instructions (excitation 405 nm, emission 420–460 nm). Sequential scan was used to detect different fluorophores.

Animal experiments
A murine model of LPS-induced inflammation and lethality was used (31) with some modifications. Female C57BL/6JoHsd (Harlan Laboratories) mice 10–12 wk of age (average weight 20 g) were injected i.p. with LPS (10 mg/kg) from *Escherichia coli* O55:B5 (Sigma-Aldrich). Peptides (15 μM/kg) myristate (MYR)-INT, ANT-INT, and MYR-CTRL were injected i.p. before (30 min) and after (30 min) LPS challenge. Blood was collected just prior to LPS challenge at 2 and 4 h after LPS injection. A tail tipping method was used under local anesthesia (ethyl chloride) followed by sealing the tail end by silver nitrate. Survival and weight changes in mice were monitored for several weeks; after 7 d, survival and weight figures stabilized. All of the procedures involving animals were performed according to local ethical and European Union regulatory guidelines under the Veterinary Administration of Republic of Slovenia permit number 34401:3012009/3.

Cytokine quantification in plasma samples
Blood samples from the tail vein were collected in heparinized tubes at 1 and 4 h after LPS challenge. As results for both time points were similar, only those for 2 h are reported in this study. Aliquots of plasma were kept at −80°C until assayed for cytokine levels using ELISA kits specific for mouse TNF-α and IL-6 (Bender MedSystems).

Statistical analysis
Statistical analysis was carried out using one-tailed unpaired Student t test. The p values were computed by comparing groups treated with a peptide and TLR agonist (ligand) versus TLR agonist (ligand) alone.

Results
Peptides comprising INT of MyD88 inhibit signaling of TLR4/myeloid differentiation factor 2 complex
INT of MyD88 is more conserved than would be expected from a simple unstructured spacer segment (Fig. 1A). Based on previous

**FIGURE 4.** Effect of INT peptides on the cytokine induction by different TLR agonists. RAW264.7 were preincubated with 40 μM peptides for 2 h before stimulation with sLPS (final concentration of 0.25 μg/ml) (A, B), LTA (final concentration of 0.2 μg/ml) (C), flagellin (final concentration of 50 ng/ml) (D), R848 (final concentration of 10 μg/ml) (E), or ODN1826 (final concentration of 5 μM) (F). Total mRNA was isolated 3 h after stimulation, and RT-PCR and qPCR were performed using primers for specific cytokine. Data represented are means ± SD of duplicate samples and representative of at least three independent experiments. **p < 0.01, ***p < 0.005.
reports (12) that MyD88 lacking INT is not able to transmit signal to downstream kinase IRAK4, we designed a set of peptides (Table I) from INT of MyD88 (hereafter referred to as INT peptides) to evaluate their ability to interfere with downstream signaling. To achieve intracellular localization of peptides, we added sequence of antennapedia homeodomain as a cell-translocation motif. As seen in Fig. 1B, INT peptides fused to the translocation motif of antennapedia homeodomain (ANT-INT) efficiently inhibit cell activation by LPS through TLR4/myeloid differentiation factor 2 (MD-2) complex, whereas control peptide with the same sequence but without translocation motive (INT-CTRL) did not show any effect. In contrast, peptide ANT-INT did not inhibit MyD88-independent TLR3 signaling, even at higher concentrations (Fig. 1C). Fig. 1D demonstrates that labeled ANT-

**FIGURE 5.** Effect of INT peptides on phosphorylation of different kinases. RAW264.7 or MyD88 KO macrophages were preincubated with 40 μM peptides for 2 h before stimulation with sLPS [final concentration of 0.2 μg/ml (A, C, E) or 0.1 μg/ml (G)]. Phosphorylation of p44/42 (A, upper panel, specific bands at 44 and 42 kDa, respectively), pIκBα (C, G, upper panel, specific band at 40 kDa), and pIRF3 (E, upper panel, specific band at 55 kDa) was assayed after 15, 5, or 120 min stimulation of RAW264.7 macrophages with sLPS, respectively, or after 30 min stimulation of MyD88 KO macrophages with sLPS. α/β-tubulin was used as a loading control (lower panels, specific band at 55 kDa). Densitometric analysis for p44/42 (B), pIκBα (D, H), and pIRF3 (F) was done using ImageJ (National Institutes of Health). Data represented are WB analyses of at least three independent experiments.
INT peptide indeed internalizes into cells (Fig. 1Dii). Because the antennapedia translocation sequence was reported to have an inhibitory effect on NF-κB signaling at higher concentrations (32), we decided to investigate an alternative cell-translocation motif. Peptides can be translocated into the cell by the addition of an acyl chain (33), although this approach has not yet been used for the inhibition of cellular signaling. Introduction of MYR-INT as well as lauroyl moiety to the INT peptide (LAU-INT) provided a very similar extent of inhibition of LPS-induced TLR4 activation (Fig. 2B) as the antennapedia translocation motif, but again, activation of TLR3 was not inhibited (Fig. 1C).

To further elucidate which segment of INT of MyD88 is responsible for the inhibitory effect, we designed serially truncated peptides comprising 9–21 aa residues of INT (Table I, MYR-INT9 to MYR-INT21, respectively). As shown in Fig. 2A, even the nine-residue peptide retains inhibitory activity, although it is slightly attenuated. Also, a control peptide consisting of a scrambled sequence of INT peptide and MYR group (MYR-CTRL) was used to assure that the MYR group did not affect signaling.

**INT peptides inhibit MyD88-dependent activation of membrane and endosomal TLRs**

As MyD88 is involved in signaling of almost all TLRs as well as in IL-1R signaling, we tested the specificity of inhibition of INT peptides on different receptors. Because TLR4/MD-2 is activated by LPS at a cell surface, we analyzed inhibition of another cell-surface TLR (TLR5), as well as endosomal TLR (TLR9), to determine if peptides discriminate between the surface and endosomal receptors. Inhibition of endosomal TLR9 is of particular interest because several autoimmune diseases are associated with its activation. Results in Fig. 3 demonstrate that INT peptides inhibit activation of TLR5 (Fig. 3A) as well as endosomal TLR9 (Fig. 3B). Additionally, we also observed inhibition of IL-1R by MYR-INT peptide, which also uses a MyD88-dependent pathway (Fig. 3C). Specificity of INT peptide inhibition for a MyD88-dependent pathway was confirmed by the lack of inhibition for MyD88-independent signaling of TLR3 and TNF-α receptor (Fig. 3D, 3E).

**INT peptides block proinflammatory gene expression in activated macrophages**

We next tested if INT peptides block TLR signaling pathways in mouse macrophages RAW264.7. As shown in Fig. 4, inhibitory peptides block LPS-induced MyD88-dependent expression of TNF-α (Fig. 4A) and IL-6 (Fig. 4B). Strong inhibition of signaling was also observed by stimulation of macrophages with LTA, a potent agonist of TLR2 (Fig. 4C), flagellin, an agonist of TLR5 (Fig. 4D), R848, an agonist of endosomal TLR7 (Fig. 4E), and ODN 1826, an agonist of endosomal TLR9 (Fig. 4F). Similar results were also obtained on human monocyte line MM6 (data not shown).

**INT peptides inhibit phosphorylation of downstream kinases**

To further evaluate the target(s) of inhibitory peptides in the signaling pathway, we next examined pathways upstream from cytokine expression. TLR4 signals both through the MyD88-dependent pathway to activate MAPKs and NF-κB and through the TRIF-dependent pathway to activate IRF3. MAPK kinase...
signaling diverges downstream from MyD88 at TRAF6 (34). We determined that INT peptides block phosphorylation of ERK kinases (p44/42) (Fig. 5A, 5B) that occurs 15 min after the stimulation of macrophages with LPS. INT peptides also inhibited even faster phosphorylation of IκBα (Fig. 5C, 5D). To determine whether INT peptides indeed inhibit MyD88-dependent signaling, phosphorylation of IRF3 was monitored, which showed that peptides have no effect on IRF3 phosphorylation (Fig. 5E, 5F). We further determined inhibition of MyD88-dependent signaling by using MyD88 KO macrophages. As seen in Fig. 5G and 5H, INT peptides block phosphorylation of inhibitor IκBα in RAW264.7 macrophages, but not in MyD88 KO macrophages. Together, these data indicate that INT peptides selectively inhibit receptor signaling through MyD88-dependent, but not through TRIF-dependent, signaling pathways.

**INT peptide targets kinase IRAK4**

In the crystal structure of the DDs of Myddosome, composed of DDs of MyD88, IRAK4, and IRAK2, the C-terminal segment of MyD88, containing the first eight residues of the INT, points away from the DD of IRAK4 and weakly interacts with the rest of its MyD88 DD but not with any other domain (16). To identify whether the inhibitory INT peptide binds to MyD88 or to the downstream kinase IRAK4, we examined the ability of peptide ANT-INT-HIS (ANT-INT peptide with added hexahistidine tag on C-terminal) to interact with MyD88 or IRAK4 by a pulldown assay. CFP, IRAK4-CFP, and MyD88-CFP were overexpressed in HEK293 cells and incubated with Ni-agarose beads with bound inhibitory (ANT-INT-HIS) or control (ANT-CTRL-HIS) peptide. Fig. 6A clearly demonstrates specific binding of target peptide to IRAK4 but not to MyD88. Furthermore, we wanted to demonstrate that INT peptides do not inhibit cells by binding to TLR4 agonist (sLPS) but indeed enter into cells and bind IRAK4. Therefore, we performed a similar pulldown experiment adding ANT-INT-HIS or control peptide ANT-CTRL-HIS to RAW264.7 cells 2 h and 30 min before or simultaneously to the stimulation with sLPS for 5 min, which is sufficient time to trigger phosphorylation of IκBα (Fig. 5C, 5D). As shown in Fig. 6B and 6C, ANT-INT-HIS entered into cells and bound to the endogenous IRAK4 after 30 min and even stronger after 2 h, whereas the control peptide ANT-CTRL-INT did not bind the endogenous IRAK4.

**Inhibitory peptides improve survival in LPS-challenged mice and attenuate acute cytokine response**

Inhibition of the excessive response in both acute and chronic inflammatory diseases mediated by MyD88 has large potential therapeutic importance. Therefore, we investigated the ability of peptides to inhibit proinflammatory cytokine production in vivo and their effect on survival in the LPS-challenged C57BL/6JolaHsd mice. Intraperitoneal injection of ANT-INT and MYR-INT peptides to mice challenged by LPS significantly suppressed production of IL-6 (Fig. 7A) and TNF-α (Fig. 7B). In contrast, the control scrambled peptide MYR-CTRL did not show this effect. Furthermore, we tested the protective effect of peptides on the lethal dose of LPS and monitored survival of mice several days after treatment. Both ANT-INT and MYR-INT improved survival of LPS-challenged mice (Fig. 7C) in comparison with LPS or LPS-control peptide-treated groups, demonstrating their therapeutic potential.

**Point mutations in MyD88 support the refined model of MyD88 and IRAK4 interaction**

To understand better which residues in the INT of MyD88 are important for interaction between MyD88 and IRAK4, we examined the crystal structure of the complex between the *Drosophila*-specific adaptor protein Tube and an IRAK4 orthologous kinase Pelle (Fig. 8B). The interaction between their DDs is mediated by a C-terminal extended segment of Tube DD (Fig. 8B).
interacting with the Pelle DD (35). Superposition of DD of MyD88 and IRAK4 to the Tube–Pelle complex suggests that the INT might mediate this interaction in the similar way (Fig. 8A).

Based on these observations, we identified that acidic residues in MyD88 around the residues 110 or 123 might be important for MyD88–IRAK4 interaction. MyD88 mutant MyD88(110KKK) with acidic residues within the INT mutated into the basic residues showed a strongly diminished activity when overexpressed in HEK293 cells (Fig. 8C). This mutant also could not reconstitute the IL-1R signaling in the MyD88-defective cell line HEK293-I3A in contrast to the wild-type MyD88 (Fig. 8D). Fig. 8E shows that both MyD88 and MyD88(110KKK) are equally expressed; therefore, the results are not a consequence of the lower expression of mutant.

Discussion

MyD88 is a central mediator of innate immunity signaling. Its alternative transcript MyD88s, which lacks the INT, does not support activation (12). However, it remained unclear whether the role of INT was important for the recruitment of downstream signaling kinase IRAK4 or its role was just to serve as a spacer between TIR and DD domains of MyD88. It has been proposed that INT might strengthen the interaction between MyD88 and IRAK4 (36). Therefore, we designed a set of peptides comprising up to 21 aa residues of the INT of protein adaptor MyD88. Our results demonstrate that these peptides specifically inhibit MyD88-dependent signaling when introduced into cell lines.

Recently, the crystal structure of the complex among DDs of MyD88, IRAK4, and IRAK2 has been determined (16). It shows a tower-like assembly with four DDs of each type in each layer. In this crystal structure, the N-terminal segment of the previously annotated INT forms a part of the folded domain, forming a long C-terminal extension of helix 6 of MyD88 DD (Fig. 8A). This helix 6 points away from the IRAK4 DD and does not interact with other protein domains. However, the last six residues of H6 in the crystallized DD have been introduced as a cloning artifact and purification tag (AAALEH) (Fig. 8A, black). Therefore, this helix is probably not as long in the native integral MyD88 as in the crystal structure. The residues of the assigned INT peptide do not interact in the crystal with DD of IRAK4, but because we determined that INT peptide interacts with IRAK4, we propose that this segment of MyD88 adapts a different conformation than in the crystal structure of DD complex. Additionally, bioinformatic analysis (37) predicts that INT forms a disordered segment. The anomalously elongated C-terminal helix of the MyD88 DD may be a result of the crystallization conditions. Interestingly, it has been reported that nonpolar cosolvent used for crystallization induced formation of an extended helix of DD of
Pelle that is absent in the solution (38). In support of this pro-
aposal, the crystal structure of the complex between DDs of the Drosophila-specific adaptor protein Tube and Pelle, an IRAK4 orthologous kinase (Fig. 8B), the interaction between DDs is mediated to a large extent by an extended segment C-terminal to the DD of Tube (Fig. 8B) interacting with the DD of Pelle (35). Superposition of DD of MyD88 and IRAK4 to the Tube–Pelle complex suggests that the INT might mediate this interaction in a similar way. We speculate that helix 6 from the crystal structure may be interrupted at the Gly106-Pro107 dipeptide (Fig. 8A), which has high propensity for the formation of β turn (39), and INT may therefore be extended and bind to the IRAK4 DD along the groove between its helix 2 and helix 5 (Fig. 8A). Favorable interactions mediated by the INT segment most likely include Tyr116, which is mutated in the inactive inkd mutant of MyD88 (40). Results (Fig. 8C–E) also suggest that a cluster of acidic residues of INT (Glu110, Glu111–Asp112) has a role in interaction with IRAK4. We conclude that the N-terminal segment of the INT peptide is important for inhibition, although truncation at its C terminus also decreases its potency. Previously, inhibition of TLR activation was demonstrated by cell-perpetrating peptides derived from the BB loop of the TIR domain of TLRs and adaptor proteins or their peptidomimetics (25–28). These peptides inhibit cell signaling with varying degrees of selectivity for different TLRs but have not yet demonstrated protection in animals. In addition to the anten-
apedapeptide-based cell penetration, we introduced cytoplasmic peptide delivery based on the N-terminal acylation and demonstra-
ated a significant attenuation of inflammation and lethality of endotoxin in mice. Peptides, either based on the peptide trans-
duction element or acylation, were nontoxic to animals and may find application for the therapy of wide range of inflammatory conditions caused by microbial infection or endogenous agonists such as sepsis, viral induction of inflammation (41), gout (19), rheumatoid arthritis (18), systemic lupus erythematosus (20), or other inflammatory diseases that are mediated by MyD88.

Acknowledgments

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Disclosures

A patent has been filed on INT peptides. The authors have no additional financial conflicts of interest.


