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Antimicrobial Peptides Inhibit Polyinosinic-Polycytidylic Acid-Induced Immune Responses

Maroof Hasan,* Catarina Ruksznis,* Yan Wang,† and Cynthia Anne Leifer* 

Viral proteins and nucleic acids stimulate TLRs to elicit production of cytokines, chemokines, and IFNs. Because of their immunostimulatory activity, several TLR agonists are being developed as vaccine adjuvants and cancer immunotherapeutics. However, TLR signaling is modified by disease state, which could enhance or impair therapeutic efficacy. For example, in the skin of psoriatic patients, the human cationic antimicrobial peptide LL37 is highly expressed and binds to host DNA. Association with LL37 enhances DNA uptake into intracellular compartments, where it stimulates TLR9-dependent overproduction of IFNs. Polyinosinic-polycytidylic acid (poly(I:C)), an analog of viral dsRNA, is recognized by TLR3 and is currently in preclinical trials as an inducer of type I IFN. If LL37 similarly enhanced IFN production, use of poly(I:C) might be contraindicated in certain conditions where LL37 is elevated. In this study, we show that TLR3 signaling was not enhanced, but was dramatically inhibited, by LL37 or mouse cathelicidin-related antimicrobial peptide in macrophages, microglial cells, and dendritic cells. Inhibition correlated with formation of a strong complex between antimicrobial peptides and poly(I:C), which partially inhibited poly(I:C) binding to TLR3. Therefore, after injury or during existing acute or chronic inflammation, when LL37 levels are elevated, the therapeutic activity of poly(I:C) will be compromised. Our findings highlight the importance of using caution when therapeutically delivering nucleic acids as immunomodulators. The Journal of Immunology, 2011, 187: 5653–5659.

The innate immune system recognizes molecular structures from infectious agents through pattern recognition receptors, including TLRs. Stimulation of the immune system through TLRs activates innate immune cells and facilitates inflammatory responses, which are necessary for host defense against invading pathogens (1–3). Because of their ability to activate the immune system, TLR agonists, especially those based on nucleic acids, are in current preclinical and clinical trials as immunomodulators.

Most TLR agonists are unique to the microbe and not present in the host; however, some detect shared nucleic acid structures such as DNA (TLR9), ssRNA (TLR7, TLR8), and dsRNA (TLR3). To limit response to host nucleic acids in the extracellular milieu, nucleic acid sensing TLRs are localized within the cell, traffic to endosomes, and are proteolytically processed (4–11). This permits detection of pathogen DNA released in phagosomes/endosomes. Host DNA is typically degraded extracellularly and does not enter the endocytic compartment. However, in certain disease states host DNA is chaperoned to TLR-containing endosomes where it elicits TLR-dependent inflammatory responses. High mobility group box proteins (12), the human antimicrobial peptide LL37 (13), and anti-DNA Abs (14) bind directly to host DNA and enhance uptake into the endosomal compartment. Enhanced uptake results in dramatically increased production of inflammatory cytokines and IFNs (13, 15–19). Given the potential of these proteins to modulate responses to TLR ligands, as well as the development of TLR ligands as therapeutics, it is important to understand the impact of preexisting or coexisting disease states on the therapeutic activity of TLR agonists.

Macrophages and dendritic cells are resident in tissues such as skin and are two of the cell types to first respond to topical therapeutic application of TLR ligands such as polyinosinic-polycytidylic acid ((poly(I:C)). The antimicrobial peptide LL37 is locally elevated in diseases such as psoriasis (micromolar concentrations), but it is not a significant product of either of these cell types (13). Therefore, it is important to test what effect elevated LL37 will have on macrophage and dendritic cell responses to poly(I:C). If LL37 enhances response to poly(I:C), it does not have therapeutic potential for CpG DNA, use of poly(I:C) as an immunotherapeutic would be potentially harmful for individuals with diseases such as psoriasis.

In this study, we show that TLR3-dependent responses were blocked by LL37 or the mouse equivalent peptide, cathelicidin-related antimicrobial peptide (mCRAMP). LL37 inhibited poly(I:C)-induced activation of macrophages, dendritic cells, and microglial cells. TLR3-dependent, poly(I:C)-induced NF-κB and IFN regulatory factor (IRF) 3 signaling pathways were inhibited by LL37. The inhibitory effect was due to the formation of a stable complex between antimicrobial peptide and poly(I:C), which partially blocked poly(I:C) binding to TLR3. Therefore, during infections, or in certain disease states, where LL37 is elevated, the therapeutic activity of poly(I:C) will be compromised. The interaction between immunomodulators and other immune components must be considered prior to widespread use of TLR ligands as therapeutic immunomodulators.

Materials and Methods

Reagents

The following reagents and Abs were used: poly(I:C) (EMD Biosciences, San Diego, CA), LL37 (a gift from Dr. Dawn Bowdish, McMaster University), scrambled peptide and mCRAMP (AnaSpec, Fremont, CA), CpG
oligonucleotide 10104 (5'-TTGTCGTTCGTTCGTTCGTTC-3') (Eurofins MWG Operon, Huntsville, AL). Pam3CSK4 (InvivoGen, San Diego, CA), and N-123,2-diacyloxypropyl)-3,5,5-trimethylammonium methylsulfate (DOTAP; Roche Diagnostics, Indianapolis, IN). The following Abs were used: phospho-IkBα (Ser72), phospho-JNK (Thr183/Tyr185), phospho-p38 (Thr180/Tyr182), phospho-IRF3 (Ser396) (Cell Signaling Technology), α-tubulin (eBioscience), and secondary Abs (SouthernBiotech).

Cell lines
RAW264.7 macrophages (American Type Culture Collection, Rockville, MD) were maintained in DMEM with 10% (v/v) heat-inactivated FCS, 2 mM l-glutamine, 10 mM HEPES, and 1 mM sodium pyruvate (complete DMEM) with the addition of 100 U/ml penicillin and 100 mg/ml streptomycin. Wild-type and TLR3-deficient microglial cell lines (BEI Resources) were cultured in complete DMEM and 10 μg/ml ciprofloxacin. All cell lines were cultured at 37°C with 5% CO2 and routinely tested negative for mycoplasma by PCR.

Electrophoresis of RNA/antimicrobial peptide complexes
Cationic peptides were incubated with poly(I:C) or 540-bp dsRNA for 1 h at room temperature in 10 mM HEPES buffer at pH 7.2. The mixtures were then resolved by electrophoresis on a 0.8% agarose gel (containing ethidium bromide) in 40 mM Tris-acetate/1 mM EDTA and visualized using UV-light. All samples were run in triplicate. Analysis was performed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

RNA isolation and RT-PCR analysis
Total RNA was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. RNA quantity and quality were confirmed using a NanoDrop ND-1000 spectrophotometer. cDNA was synthesized from 1 μg total RNA using SuperScript III first-strand synthesis (Invitrogen, Carlsbad, CA). The Power SYBR Green PCR Master mix (Applied Biosystems, Foster City, CA) was used. The GAPDH gene was used as a control for RNA normalization. All samples were run in triplicate. Analysis was performed on an Applied Biosystems 7500 (Applied Biosystems, Carlsbad, CA). The 2^{-ΔΔCt} method (20) was used to calculate relative changes in the gene expression determined from raw fluorescence data.

The following primers were used: IL-1β forward, 5'-CCGACGACAGCACATCAACAAGAGC-3'; reverse, 5'-TTGCCTCATCCTGAGAAGGTCTCAGC-3'; IL-6 forward, 5'-CACAAGTCCGGAGAGGAGAC-3'; reverse, 5'-AGGGGGCGAGATGATTACC-3'.

Cytokine detection assay
TNFα in the supernatants was measured by ELISA according to the manufacturer’s recommendations (BioLegend, San Diego, CA).

Nitrite assay
Accumulation of NO2⁻ was measured after 18 h stimulation using the Griess assay system as described previously (21).

TLR3 binding assay
TLR3 binding assay was performed as previously described (22). Briefly, microplate wells were coated with goat anti-mouse IgG followed sequentially by mouse anti-GFP mAb and cell lysate (lysis buffer: 1% Triton X-100, 10 mM Tris [pH 7.4], 150 mM NaCl, 5 mM EDTA, and protease inhibitors; Roche Applied Science) containing TLR3-yellow fluorescent protein. After blocking and washing, a mixture of peptides and biotinylated dsRNA was added to the microplate wells and incubated at room temperature for 2 h. Plates were then washed and incubated with streptavidin-HRP (Thermo Scientific, Rockford, IL) for 1 h at room temperature. Using HRP substrate reagent (R&D Systems) and a FLUOSstar OPTIMA plate reader (BMG Labtech), absorbance was measured at 450 nm.

Circular dichroism spectroscopy
Circular dichroism was performed with 10 μg poly(I:C) and 50 μM LL37 either alone or in combinations. Spectra were recorded with a model 400 Aviv circular dichroism spectrometer (Aviv Biomedical, Lakewood, NJ) in a 1-cm-path length cuvette at room temperature. Structural analysis was performed using the delta-epsion calculation method using K2D circular dichroism spectra deconvolution software.

Western blotting
Cells were stimulated as indicated, washed with ice-cold HBSS, and lysed with 300 μl 1× SDS-PAGE reduced sample buffer (62.5 mM Tris [pH 6.8], 12.5% glycerol, 1% SDS, 0.005% bromophenol blue, 1.7% 2-ME). Lysates were incubated at 95°C for 5 min prior to resolving by 10% SDS-PAGE. Proteins were transferred to nitrocellulose membranes and immunoblotted with the indicated Abs. Membranes were incubated with a Supersignal West Pico chemiluminescence Western blotting detection system (Thermo Scientific) and exposed to x-ray film. Films were scanned and images were assembled in Photoshop.

Preparation of DOTAP/poly(I:C) complexes
Poly(I:C) (5 μg) was prepared in HEPES-buffered saline at pH 7.4 in a sterile reaction tube (final volume, 50 μl). In a separate reaction tube 30 μl DOTAP was mixed with HEPES-buffered saline at pH 7.4 to a final volume of 100 μl. Then, poly(I:C)-containing solution was transferred to the tube containing DOTAP in HEPES-buffered saline. The two solutions were mixed by gentle pipetting several times and incubated at room temperature for 15 min prior to adding to cells.

Generation of bone marrow-derived macrophages and dendritic cells
Femurs and tibias were harvested from 8- to 10–wk-old C57BL/6 mice (The Jackson Laboratory). For macrophages, bone marrow was flushed from the bones with cold DMEM supplemented with 20% L-929 cell-conditioned medium, 10% (v/v) heat-inactivated FCS, 2 mM l-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 mg/ml streptomycin. Bone marrow cells were cultured in 10-cm petri dishes (10 ml vol) at 37°C, 5% CO2 for 7 d. At days 3 and 6, fresh medium was added to the cultured cells. For dendritic cells, bone marrow was flushed from the bones with cold RPMI 1640 containing 10 ng/ml mouse GM-CSF (PeproTech, Rocky Hill, NJ), 10% FBS, 50 μM 2-ME, 2 mM l-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 mg/ml streptomycin. Bone marrow cells were differentiated for 9 d in a 10-cm petri dish in 20 ml complete RPMI 1640.

Results
Cationic antimicrobial peptides inhibit poly(I:C)-induced signaling
Skin of psoriasis patients can have up to micromolar concentrations of LL37 that synergizes with DNA to induce inflammation (13). Therefore, we first asked whether high concentrations of LL37 that synergizes with DNA to induce inflammation (13). Therefore, we first asked whether high concentrations of LL37 enhanced poly(I:C)-mediated signaling. RAW264.7 macrophages produced TNF-α dose-dependently inhibited by mCRAMP or LL37 (Fig. 1A). Mouse, or human, antimicrobial peptide also inhibited nitrite production; a marker for NO (Fig. 1B). However, nitrite production induced by Pam3CSK4 was unaffected (Fig. 1B). Other proinflammatory cytokine messenger RNAs, such as IL-6 and IL-1β, were also induced by poly(I:C) (Fig. 1C, 1D). These responses were inhibited by LL37 and mCRAMP (Fig. 1C, 1D).

Early interaction of poly(I:C) and antimicrobial peptide is required for inhibitory signaling
To determine whether delayed addition of antimicrobial peptide has any effect on poly(I:C)-induced TNF-α production, we stimulated RAW cells either with premixed poly(I:C) and LL37 or LL37 was added at later time points as indicated. With a delayed addition of LL37 as short as 30 min, the inhibitory effect of LL37 was dramatically reduced (Fig. 2). With longer delays, up to 4 h, there was still some inhibitory effect on poly(I:C)-induced signaling (Fig. 2). Similar results were obtained using the mouse antimicrobial peptide mCRAMP. Therefore, these data provide the evidence that antimicrobial peptide is much more efficient as an inhibitor when it is preincubated with poly(I:C).
Antimicrobial peptides inhibit poly(I:C)-induced TNF-α signaling in primary macrophages and dendritic cells

We next examined whether LL37 and mCRAMP inhibited poly(I:C)-induced signaling in primary macrophages and dendritic cells. As expected, LL37 had no effect on Pam3CSK4-mediated, TLR2-dependent TNF-α production by bone marrow-derived macrophages (Fig. 3A). However, response to poly(I:C) was dramatically inhibited (Fig. 3A). LL37 had similar inhibitory activity on poly(I:C)-induced bone marrow-derived dendritic cell production of TNF-α (Fig. 3B). In bone marrow-derived dendritic cells, LL37 slightly increased CpG DNA-induced TNF-α; however, this effect was not as dramatic as that previously observed in plasmacytoid dendritic cells (13) (Fig. 3B). Importantly, LL37 alone did not have any stimulatory activity in either cell type (Fig. 3). Similar results were obtained using mouse antimicrobial peptide mCRAMP (M. Hasan and C.A. Leifer, unpublished observation). Therefore, cationic antimicrobial peptide significantly inhibited poly(I:C)-induced TNF-α production.

Antimicrobial peptide suppresses poly(I:C)-induced NF-κB and MAPK activation

The induction of proinflammatory cytokines depends on activation of MAPKs and the transcription factor NF-κB. Phosphorylation and subsequent degradation of the inhibitor protein IκB relieves repression of NF-κB, which translocates to the nucleus and initiates transcription of target genes (23, 24). In macrophages, treatment with poly(I:C), but not LL37 or mCRAMP, alone induced phosphorylation of IκB, as well as the MAPKs p38 and JNK (Fig. 4). Both mCRAMP and LL37 inhibited poly(I:C)-induced IκB, p38, and JNK phosphorylation (Fig. 4). However, Pam3CSK4-induced phosphorylation of these signaling mediators was not inhibited by mCRAMP or LL37 (Fig. 4). Therefore, antimicrobial peptides selectively inhibit poly(I:C) signaling pathways.

The inhibitory effect of cationic antimicrobial peptide is TLR3-dependent
dsRNA is detected by both TLR3 and by the cytoplasmic receptor melanoma differentiation-associated gene 5 (MDA5) (25). Whereas poly(I:C) alone stimulates TLR3, in complex with the lipid DOTAP, poly(I:C) also activates MDA5 (26, 27). Therefore, we compared the ability of LL37 to inhibit phosphorylation of IRF3 in macrophages treated with poly(I:C) alone or in complex with the lipid DOTAP. Poly(I:C) stimulation induced phosphorylation of IRF3, which was enhanced by lipid encapsulation with DOTAP, indicating that both the TLR3 and the MDA5 pathways were functional in macrophages (Fig. 5A). LL37 inhibited poly(I:C) alone-induced, but not poly(I:C) in complex with
DOTAP-induced, IRF3 phosphorylation (Fig. 5A). Neither LL37 alone nor DOTAP alone induced phosphorylation of IRF3 (Fig. 5A). These data support the conclusion that LL37 inhibited TLR3-dependent poly(I:C) signaling.

To confirm the TLR3 specificity of antimicrobial peptide inhibition, we compared poly(I:C)-induced signaling in wild-type and TLR3-deficient cells. Microglial cells respond to poly(I:C) through TLR3 (28). Whereas wild-type microglial cells secreted TNF-α in response to poly(I:C), TLR3-deficient microglial cells did not (Fig. 5B). These data confirm that in the microglial cells, poly(I:C)-induced TNF-α production was TLR3-dependent but not MDA5-dependent (Fig. 5B). Neither LL37 nor mCRAMP alone induced TNF-α (Fig. 5B). The poly(I:C)-induced TNF-α response was completely abolished when cells were treated with LL37 or mCRAMP (Fig. 5B). However, Pam3CSK4-mediated TNF-α production was unaffected by mCRAMP or lack of TLR3 expression (Fig. 5B). Therefore, antimicrobial peptides selectively inhibit TLR3-dependent poly(I:C)-induced responses.

Antimicrobial peptides bind directly to synthetic dsRNA and form a stable complex

We next asked whether the antimicrobial peptide and poly(I:C) directly associate to form a complex. Poly(I:C) alone migrated as a smear by agarose gel electrophoresis (Fig. 6A). Increasing concentrations of mCRAMP reduced the migration of poly(I:C), demonstrating that they form a stable complex (Fig. 6A). In fact, with the highest concentration of mCRAMP, most poly(I:C) never migrated into the gel but remained in the well (Fig. 6A). These data suggest that bulky, high m.w. complexes are formed, or that
LL37 neutralizes the charge of poly(I:C), preventing it from entering the gel. Circular dichroism analysis confirmed a strong interaction between LL37 and poly(I:C). The circular dichroism signal for poly(I:C) alone was between 2200 and 2250, but in the presence of LL37, the signal was dramatically reduced to less than 250 (Fig. 6B).

Because TLR3 signaling initiates in endosomes (29), we next asked whether the poly(I:C)/LL37 complex was stable at low pH. Complexes were formed in neutral pH buffer and transferred to reduced pH buffer (from pH 7.0 to 5.0). The complexes were then subjected to EMSA. LL37/poly(I:C) complexes were not dissociated, even at a pH as low as 5.0 (Fig. 6C). Therefore, the cationic peptide/poly(I:C) complexes are stable and pH resistant.

**Antimicrobial peptide partially inhibits dsRNA binding to TLR3**

We next asked whether antimicrobial peptides inhibited dsRNA binding to TLR3. Similar to poly(I:C), a synthetic 540-bp dsRNA bound to mCRAMP (Fig. 7A). Using our previously described assay, TLR3 was immobilized on a plate and incubated with a defined biotinylated dsRNA (22). Without any peptide, the biotinylated dsRNA bound to TLR3 (Fig. 7). This binding was strongly, and dose dependently, inhibited by increasing molar ratios of unlabeled dsRNA (Fig. 7). A 100-fold molar excess of mCRAMP, but not its scrambled peptide control, inhibited dsRNA binding to TLR3 (Fig. 7). However, inhibition by mCRAMP was much less efficient than that observed with unlabeled dsRNA. Therefore, although mCRAMP partially inhibited poly(I:C) binding to TLR3, other mechanisms likely account for the dramatic inhibition of signaling.

**Discussion**

Recognition of microbial products is critical for innate immunity, but increasing evidence suggests pathogenic roles for TLR sig-
naling in autoimmune and inflammatory disorders (13, 30, 31). The pathogenic role of TLRs in disease has been linked to facilitated uptake of self nucleic acids, which are normally poorly endocytosed and do not stimulate inflammation. The antimicrobial peptide LL37 is one protein that stabilizes and enhances uptake of self nucleic acids (13, 16). Given their therapeutic activity as immunomodulators (32), as well as their current evaluation in clinical trials (33, 34), it is important to understand how the immunomodulatory activity of TLR agonists is altered by host factors, such as LL37. In this study, we showed that LL37 inhibits the inflammatory activity of poly(I:C), a model dsRNA. The inhibitory effect was more pronounced when poly(I:C) was premixed with antimicrobial peptides. When the addition of LL37 was delayed, the inhibitory effect of LL37 was reduced significantly.

LL37 inhibited poly(I:C)-induced proinflammatory responses in mouse macrophages (Fig. 1), yet it enhanced CpG DNA-induced type I IFN responses in human plasmacytoid dendritic cells (13). Interestingly, in human myeloid dendritic cells and in HEK293 cells, both poly(I:C) and CpG DNA are endocytosed by a shared clathrin-dependent pathway (35). Recent studies have implicated CD14 and scavenger receptor in endocytosis of poly(I:C) (36, 37) and CpG DNA (38, 39). Therefore, it is unclear why LL37 has opposite effects on CpG and poly(I:C) signaling. Further studies are needed to determine whether uptake is dependent on as yet unidentified receptors in different cell types.

Although LL37 and mCRAMP dramatically inhibit poly(I:C)-induced responses (Figs. 1–3), they minimally inhibit binding of poly(I:C) to TLR3 (Fig. 7). Due to the presence of several positive charges, LL37 and mCRAMP can condense short, single-stranded CpG DNAs. In contrast, poly(I:C) length is variable (from 200 to 8 kb) and forms concatemer-like structures in solution. Therefore, unlike complete coverage that might be achieved by LL37 association with CpG DNA, only part of the poly(I:C) might be covered by LL37, or higher concentrations of LL37 would be required to saturate binding. This model would explain our data that show LL37 forms a complex with poly(I:C), but high concentrations are required to inhibit poly(I:C) binding to TLR3. Because LL37 does not efficiently block binding to TLR3, but TLR3 requires dimerization to signal (40), it is possible that LL37 inhibits signaling by preventing receptor dimerization. However, dsRNA does not stably bind to TLR3 without receptor dimerization (22) and if LL37 blocked dimerization, it should have inhibited poly(I:C) binding to TLR3. Because it did not inhibit binding (Fig. 6), it is unlikely that LL37 blocks ligand-induced receptor dimerization. Therefore, inhibition of poly(I:C) signaling by LL37 likely occurs by other mechanisms such as inhibition of poly(I:C) uptake into endosomes.

Taken together, our studies show that antimicrobial peptides form a stable complex with poly(I:C) and block TLR3 signaling. This is due, at least in part, to inhibition of poly(I:C) binding to TLR3. Because poly(I:C), and other nucleic acids are currently in clinical trials, it is important to understand complex interactions between these nucleic acids and host proteins that modify their activity. Our data suggest that during acute infection, or chronic inflammatory conditions, where LL37 is elevated, the therapeutic activity of poly(I:C) will be compromised. Therefore, more information is needed on how disease conditions and host proteins modify the therapeutic potential of dsRNA.

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Disclosures
The authors have no financial conflicts of interest.

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