The Human Antimicrobial Peptide LL-37, but Not the Mouse Ortholog, mCRAMP, Can Stimulate Signaling by Poly(I:C) through a FPRL1-dependent Pathway*

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Background: How the cathelicidin LL-37 enhances Toll-like receptor 3 signaling is poorly understood.

Results: We determined that LL-37-poly(I:C) complex can localize to early endosomes with TLR3 through the FPRL1 receptor, whereas poly(I:C) localizes to endosomes by a different mechanism.

Conclusion: Double-stranded RNAs can localize with TLR3 by multiple trafficking pathways.

Significance: This work establishes a mechanism for cross-talk between LL-37, double-stranded RNA, and TLR3.

LL-37 is an antimicrobial peptide produced by human cells that can down-regulate the lipopolysaccharide-induced innate immune responses and up-regulate double-stranded (ds) RNA-induced innate responses through Toll-like receptor 3 (TLR3). The murine LL-37 ortholog, mCRAMP, also inhibited lipopolysaccharide-induced responses, but unlike LL-37, it inhibited viral-induced responses in mouse cells. A fluorescence polarization assay showed that LL-37 was able to bind dsRNA better than mCRAMP. In the human lung epithelial cell line BEAS-2B, LL-37, but not mCRAMP, colocalized with TLR3, and the colocalization was increased in the presence of dsRNA. The presence of poly(I:C) increased the accumulation of LL-37 in Rab5 endosomes. Signaling by cells induced with both LL-37 and poly(I:C) was sensitive to inhibitors that affect clathrin-independent trafficking, whereas signaling by poly(I:C) alone was not, suggesting that the LL-37-poly(I:C) complex trafficked to signaling endosomes by a different mechanism than poly(I:C) alone. siRNA knockdown of known LL-37 receptors identified that FPRL1 was responsible for TLR3 signaling induced by LL-37-poly(I:C).

These results show that LL-37 and mCRAMP have different activities in TLR3 signaling and that LL-37 can redirect trafficking of poly(I:C) to effect signaling by TLR3 in early endosomes in a mechanism that involves FPRL1.

Cathelicidins are multifunctional peptides produced by many mammals to act as bactericides, to promote wound repair and angiogenesis, and to modulate innate immune responses (1, 2). LL-37 is a 37-residue cathelicidin that is released by proteolytic cleavage from the C-terminal fragment of the protein hCAP-18. Elevated levels of LL-37 in human tissues associated with autoimmune diseases such as psoriasis (2–5).

LL-37 can bind several ligands that are also recognized by innate immune receptors (6). LL-37 binding to lipopolysaccharides (LPS) can down-regulate signaling by Toll-like receptor 4 (TLR4),2 binding to ssRNA can up-regulate TLR7 and TLR8 signaling, and interaction with flagellin can activate TLR5 signaling (3, 7, 8). Most relevant to this work, LL-37 binds double-stranded (ds) RNA, including poly(I:C) to enhance TLR3 signaling above the level observed with poly(I:C) alone (7, 9, 10). How LL-37 functions in concert with dsRNA to affect TLR3 signaling remains to be better understood.

LL-37 consists of an N-terminal a-helical structure followed by more flexible sequence that may be intrinsically disordered until it binds to ligands (11). Multiple LL-37 molecules may also form higher order structures with TLR ligands. The mouse ortholog of LL-37, mCRAMP, is believed to have a similar structure. Both LL-37 and mCRAMP are secreted from neutrophil granules upon wounding (8, 12). Unlike LL-37, however, mCRAMP inhibited TLR3 signaling in mouse cells (13). It is unclear whether these effects are due to the species of the cell lines examined, the tissues from which the cell lines have been derived, and/or sequence/structural differences in the two peptides.

In this study we document that LL-37 can activate TLR3 signaling in three human cell lines derived from different tissues but inhibit signaling in three murine cell lines. In contrast, mCRAMP inhibited dsRNA-dependent signaling in both human and murine cells. The differences in the activities of LL-37 and mCRAMP are correlated with their abilities to bind poly(I:C) and their localization to early endosomes. We also observed that signaling in the presence of both LL-37 and poly(I:C) was sensitive to inhibitors of clathrin-independent endocytosis, whereas signaling by poly(I:C) alone was sensitive to the clathrin-dependent endocytosis inhibitor. siRNA knockdown of the LL-37 receptor, formyl peptide receptor-like 1 (FPRL1) (14), prevented enhancement of signaling by the poly(I:C)-LL-37 complex.

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Note added in proof: The abbreviations used are: TLR4, Toll-like receptor 4; FPRL1, formyl peptide receptor-like 1; RV, reovirus; Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; EGFR, epidermal growth factor receptor; IGF-1R, Insulin-like growth factor 1 receptor; RANTES, regulated on activation normal T cell expressed and secreted.
Materials and Methods

Reagents—The BEAS-2B, A549, RAW264.7, and NK-92MI cell lines were from ATCC. Mouse embryonic fibroblasts and L929 are the generous gifts of Pranav Danthi of Indiana University, Bloomington. Cell culture media were from Invitrogen. Endocytosis inhibitors methyl-$\beta$-cyclodextrin, genistein, chlorpromazine, and bafilomycin A1 were from Sigma and dissolved in either water or DMSO according to its solubility. Poly(I:C) and LPS were from Invivogen. Reovirus (RV) dsRNA S4 were prepared as described in Lai et al. (9). Anti-microbial peptides with or without covalently attached fluorophores were custom-synthesized to greater than 95% purity by AnaSpec Inc. Antibodies to detect the TLR3 ectodomain were from R&D Systems (catalog #AF1487). Antibodies to detect Rab5, Rab7, and Rab11 were from Cell Signaling Inc. (catalog #9385). The antibody to detect mouse anti-LAMP1 was from Santa Cruz Biotechnology (catalog #Sc-20011). The antibody to detect FPRL1 was from Novus Biologicals (catalog #NLS1878). Secondary antibodies conjugated to Alexa Fluor 488 or 594 were from Invitrogen. Alexa Fluor-conjugated cholera toxin B subunit (catalog #C-34777) and human transferrin (catalog #T-13343) were from Invitrogen. An HRP-conjugated secondary antibody that recognized rabbit IgGs was from Santa Cruz Biotechnologies.

Cell Culture—RAW264.7, L929, mouse embryonic fibroblasts, and A549 were cultured in DMEM, high glucose, GlutaMAX (Life Technology, Inc.) supplemented with 10% FBS at 37 °C with 5% CO$_2$. BEAS-2B cells were cultured in BEGM media with its supplements (Lonza Inc.) (9, 17). NK-92MI cells were propagated in α-minimum essential medium with final concentrations of 2 mm l-glutamine, 0.2 mm inositol, 0.1 mm 2-mercaptoethanol, 0.02 mm folic acid, 12.5% horse serum, and 12.5% FBS.

Quantification of Cytokine Production—A typical assay used $2 \times 10^4$ BEAS-2B cells/well grown for 24 h in flat-bottom 96-well plates. At that point, the media were removed and replaced with fresh media amended with TLR ligands and/or peptides. Human cell lines were treated with LPS at a final concentration of 1 μg/ml, poly(I:C) at 0.13 μg/ml, and the peptides at a final concentration of 3 μM. RAW264.7 cells were plated at $2 \times 10^4$ cells per ml and grown for 24 h. At that time, the media were removed and replaced with fresh media containing LPS at a final concentration of 1 μg/ml, poly(I:C) at 1 μg/ml, and/or the peptides at 3 μM. TLR ligand and peptides were not preincubated before their addition to the culture medium.

IL-6 was quantified by ELISA using the human or mouse BD OptEIA™ kit (BD Biosciences) from cell media clarified by centrifugation at 1000 × g for 2 min. All ELISA data in this work are represented as the mean ± S.D. of triplicates. The data were repeated twice after the initial experiment.

Message RNA for human and mouse IL-6, IL-1β, TNF-α, and CCL5/RANTES were quantified by real-time RT-PCR using a primer(s) whose sequences will be made available upon request. Briefly, RNA was isolated using the Qiagen RNeasy kit (Qiagen Inc.). Moloney murine leukemia virus reverse transcriptase (New England Biolabs) was used to synthesize the cDNA. Each experiment was performed in triplicate in 96-well plates using 1 × SYBR Green master mix (Bio-Rad) and 2 μg of the cDNA in a final volume of 25 μl. Amplifications were performed with each cycle consisting of 95 °C for
3 min followed by 50 cycles of 94 °C for 10 s and 60 °C for 30 s and 72 °C for 30 s. The data were analyzed as described in Qi et al. (15).

siRNA Knockdown—BEAS-2B cells were seeded at 2 × 10^6 cells per 6-well plate in BGM amended with supplements (Lonza). 6 h later the cells were transfected with 30 nM concentrations of a pool of three siRNAs from Santa Cruz Biotechnology specific to TLR3 (catalog #sc-36685), FPRL1 (sc-40123), IGF-IRa/β (sc-29358), epidermal growth factor receptor (EGFR; sc-29301), or a nonspecific control siRNA (catalog #sc-37007). Transfection of the siRNAs used Lipofectamine RNAiMax (Life Technology Inc.) according to the manufacturer’s protocol. The cells were transfected with siRNA then incubated for 48 h before transfection of plasmids to express proteins. The effects of the siRNAs on target mRNA levels were analyzed using real-time quantitative RT-PCR. Effects of the knockdown on IL-6 production were assessed in culture media collected 24 h after the siRNA transfection. The cells were also fixed at that time to allow localization of LL-37 and poly(I:C) using confocal microscopy.

Confocal Microscopy—Cells were grown on poly-l-lysine-coated coverslips to 60% confluency. 12 h after treatment with poly(I:C) or LPS in the absence or presence of antimicrobial peptide(s), the cells were fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized for 30 min on ice in T buffer (0.5% Triton X-100 in PBS with 1% normal goat serum). Nonspecific antibody binding sites were blocked with 2% BSA in TBS-T, the coverslips were mounted on glass slides with anti-fade mounting medium with DAPI and dried overnight in the dark. Micrographs were acquired with a Leica TCS SP5 confocal inverted-base microscope with a 63× oil objective. Images were analyzed by Leica LAS AF and Image J software. At least 20 cells were analyzed for each treatment, and each experiment was repeated at least twice. Colocalization of fluorophores was quantified using the ImageJ plug-in tool, JACoP (16).

Labeling of dsRNAs—Rhodamine-labeled poly(I:C) was from Invitrogen. RV 54 dsRNA was labeled with Alexa Fluor 594–conjugated Universal Linkage System (Invitrogen) according to the manufacturer’s instructions. 1 μg of RNA was heated at 95 °C for 5 min. Conjugated Alexa Fluor 594 was added to denatured RNA, and the reaction was incubated at 90 °C for 10 min. The labeling reaction was then cooled on ice, and unbound labels were removed by using a gel filtration–based spin column. RNA concentration was quantified by spectrophotometry.

Fluorescence Polarization Assay—LL-37 or mCRAMP binding to poly(I:C) was analyzed by a fluorescence polarization assay in a microplate reader (BioTek). Fluorescein-labeled LL-37 or mCRAMP (10 nM) was mixed with poly(I:C) in a 100-μl solution of buffer P (25 mM Tris and 50 mM NaCl, pH 7.4) before analysis. All binding assays were performed in triplicate, and the experiment was reproduced in two addi-
**TABLE 2**

**LL-37 and mCRAMP on IL-6 production in different human and mouse cell lines**

All means and S.E. were derived from a minimum of six independently processed samples.

| Ligands | Human IL-6 (mean ± S.D.) | Mouse IL-6 (mean ± S.D.) |
|---------|--------------------------|-------------------------|
|         | A549                     | NK-92MI                  |
|         | µg/ml                     | µg/ml                    |
| Mock    | 0.19 ± 0.09               | 0.08 ± 0.08              |
| LL-37   | 0.25 ± 0.043              | 0.06 ± 0.07              |
| mCRAMP  | 0.25 ± 0.021              | 0.05 ± 0.05              |
| Sc37    | 0.26 ± 0.051              | 0.05 ± 0.02              |
| LPS     | 1.40 ± 0.95               | 0.61 ± 0.59              |
| LPS + LL-37 | 0.44 ± 0.29 (0.03*) | 0.17 ± 0.19 (0.08*) |
| LPS + mCRAMP | 0.43 ± 0.17 (0.07*) | 0.09 ± 0.11 (0.07*) |
| LPS + Sc37    | 1.40 ± 0.55 | 0.62 ± 0.43 |
| poly(I:C)  | 0.96 ± 0.31               | 0.51 ± 0.40              |
| poly(I:C) + LL-37 | 1.98 ± 0.45 (0.01*) | 1.23 ± 0.90 (0.01*) |
| poly(I:C) + mCRAMP | 0.57 ± 0.17 (0.07*) | 0.25 ± 0.21 (0.00*) |
| poly(I:C) + Sc37 | 0.97 ± 0.39 | 0.51 ± 0.49 |
|         | 0.09 ± 0.07               | 0.08 ± 0.06              |
|         | 0.09 ± 0.06               | 0.09 ± 0.06              |
|         | 0.09 ± 0.07               | 0.09 ± 0.08              |
|         | 0.09 ± 0.07               | 0.08 ± 0.06              |
|         | 2.16 ± 1.25               | 1.42 ± 0.09              |
|         | 0.10 ± 0.08 (0.004*)      | 0.08 ± 0.07 (0.01*)     |
|         | 0.21 ± 0.06 (0.01*)       | 0.07 ± 0.06 (0.01*)     |
|         | 2.19 ± 1.19               | 1.12 ± 1.6               |
|         | 1.11 ± 0.58               | 0.98 ± 0.66              |
|         | 0.17 ± 0.19 (0.003)       | 0.14 ± 0.19 (0.01*)     |
|         | 0.18 ± 0.16 (0.002)       | 0.19 ± 0.25 (0.01*)     |
|         | 1.10 ± 0.58               | 1.03 ± 0.71              |

*The p values were determine between the sample that treated with LL-37 or mCRAMP to those treated with either only LPS or poly(I:C).*

**RESULTS**

**LL-37 and mCRAMP Have Different Effects on dsRNA-induced TLR3 Signaling**—Discrepant observations of the activities of LL-37 and mCRAMP on dsRNA-induced signaling in human and mouse cells prompted us to better document the activities of the peptides (9, 17). The sequences of the peptides LL-37, mCRAMP, and a scrambled version of LL-37 named Sc37 caused little effect (9, 17). The addition of either BEAS-2B or RAW264.7 cells inhibited LPS-induced IL-6 production in a concentration-dependent manner, whereas Sc37 had no effect (Table 1). The addition of mCRAMP along with poly(I:C) to BEAS-2B cells had either no effect or caused only a modest increase in the four cytokine messages (Table 1). The results are consistent with those from ELISA assays for IL-6 levels. All of the assays for cytokine and chemokine production show that LL-37 and mCRAMP have different effects on dsRNA-induced signaling in human BEAS-2B and mouse RAW264.7 cells.

Two additional human cell lines were tested to determine whether LL-37 and mCRAMP have different effects on signaling by TLR3. Human cell lines NK-93MI (natural killer origin) and A549 (alveolar adenocarcinoma origin) both showed increased IL-6 production when treated with LPS. The addition of LL-37 and mCRAMP inhibited the LPS-induced IL-6 production by more than 3-fold, whereas Sc37 had no effect (Table 2). When treated with poly(I:C), the two cell lines produced between 5- and 6-fold higher IL-6. The addition of LL-37 with RV S4 dsRNA (right panel, Fig. 1B). The addition of mCRAMP or Sc37 with either poly(I:C) or LPS resulted in little or no enhancement of IL-6 production in BEAS-2B cells (Fig. 1B).

**LL-37 and mCRAMP Modulation of TLR3 Signaling**—Approximately 5 × 10⁵ cells were solubilized in NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-Cl, pH 6.5, and 1% Nonidet P-40) amended with protease inhibitor mixture (Sigma). The lysate was clarified from insoluble materials by a brief centrifugation at 2000 × g for 5 min and denatured with an SDS-containing solution for 5 min at 70 °C. The proteins were separated by gel electrophoresis in a 4–12% Bis-Tris gel and processed for Western blot analysis as described by Qi et al. (15).

**Statistical Analysis**—All data shown are the means and ranges for one S.E. for a minimal of three independent samples. Data sets were compared using Student’s t test calculated with GraphPad Prism 5 software.
production in NK-93MI and A549 cells (Table 2). Thus, in three human cell lines derived from different tissues, LL-37 consistently increased TLR3 signaling by dsRNA, whereas mCRAMP inhibited signaling.

In mouse fibroblast cell lines L929 and mouse embryonic fibroblasts, LPS-induced IL-6 production was inhibited by LL-37 and mCRAMP, demonstrating that the peptides possess bioactivity with these two cell lines. Furthermore, poly(I:C)-induced IL-6 production increased by more than 10-fold in both cell lines. The addition of either LL-37 or mCRAMP along with poly(I:C) inhibited IL-6 production (Table 2). These results are consistent with those from RAW264.7 cells and demonstrate that multiple mouse cell lines have a consistently different response to LL-37 and mCRAMP when compared with human cell lines.

**LL-37 and mCRAMP Differ in Their Ability to Bind Poly(I:C)**—We seek to better understand the basis for the distinct effects of LL-37 and mCRAMP. We hypothesize that the difference in dsRNA-induced signaling could be due to LL-37 and mCRAMP having different abilities to bind dsRNAs. To test this, LL-37 and mCRAMP were synthesized with N-terminal fluorescein. Both labeled peptides modulated LPS- and poly(I:C)-induced IL-6 production in BEAS-2B cells, indicating that the addition of the fluorophore did not alter the ability of the peptides to affect LPS or poly(I:C) signaling (Fig. 2, B and C). However, when the peptides were assessed for poly(I:C) binding using a fluorescence polarization assay, the change in the LL-37 polarization was increased by poly(I:C) in a concentration-dependent manner, whereas that of mCRAMP was not (Fig. 2D). These results show that LL-37 is better at binding poly(I:C) than mCRAMP.

LL-37 and mCRAMP are predicted to form an α-helical structure that starts at the N terminus (region 1), whereas the C-terminal eight residues is more flexible (region 2) (Fig. 2A; Ref. 31). Region 1 of mCRAMP is shorter by three residues than that of LL-37 but possesses a central portion that is highly similar in sequence to that of LL-37. The respective region 2s of LL-37 and mCRAMP have four residues of eight that are identical (Fig. 2A). To better define the regions in LL-37 that contribute to dsRNA binding, we synthesized two chimeras with mixed and matched sequences from the two regions (Fig. 2A). Both chimeras inhibited LPS-dependent signaling by TLR4 (Fig. 2B) but failed to significantly induce IL-6 production in response to poly(I:C) (Fig. 2C). In the fluorescence polarization assay, Chimera A that contained region 1 from LL-37 could bind poly(I:C) at a reduced rate relative to that of LL-37 (Fig. 2D). Chimera B that contained region 1 from mCRAMP failed to bind RNA. These results show that region 1 of LL-37 retained some ability to interact with poly(I:C) but that this is insufficient to enhance TLR3 signaling. Furthermore, both regions 1 and 2 contribute to optimal dsRNA binding and enhancement of TLR3 signaling.

**LL-37, But Not mCRAMP, Preferentially Colocalizes with Poly(I:C) in Cells**—We examined whether fluorescently labeled poly(I:C) or S4 could colocalize with LL-37 and/or mCRAMP in cells. A schematic of the collage of the confocal micrographs is shown in Fig. 3A. In BEAS-2B cells, the dsRNAs and the peptides were localized to punctate structures that are likely to be endosomes (Fig. 3B). However, although 50% of LL-37 colocalized with poly(I:C), only 18% of mCRAMP did so (Fig. 3C). Similar results were observed with the colocalization of the peptides with S4 dsRNA (Fig. 3, B and C). These results demonstrate that in human cells, LL-37 and mCRAMP differ in their intracellular localization with respect to dsRNAs, likely as a consequence to their different abilities to associate with dsRNA.

**Colocalization of LL-37 and mCRAMP with TLR3**—We examined whether fluorescently labeled LL-37 and mCRAMP will colocalize with TLR3 in the absence or presence of poly(I:C) or RV S4 dsRNA. TLR3 was detected by immunostaining using a fluorescently labeled mAb. Approximately 33% of LL-37 colocalized with TLR3 in endosomes in the
absence of dsRNA (Fig. 4). When added to cells along with poly(I:C), 60% of LL-37 colocalized with TLR3 (Fig. 4). The presence of S4 also increased the colocalization of LL-37 with TLR3.

The situation was different with mCRAMP. In the absence of poly(I:C), only 10% of mCRAMP colocalized with TLR3, and colocalization was not affected by the addition of either poly(I:C) or S4 dsRNA (Fig. 4). These results suggest that LL-37 can colocalize to endosomes containing TLR3 even in the absence of exogenously provided dsRNA. However, the targeting of LL-37 in endosomes containing TLR3 will be increased.

**LL-37 and mCRAMP Localize to Different Subsets of Endosomes**—We next sought to determine whether LL-37 and mCRAMP were associated with specific subpopulations of endosomes. The localization of fluorescently labeled LL-37 and mCRAMP in endosomes marked with Rab5 or Rab7 was of interest as TLR3 signaling was associated with Rab5 endosomes (15). The early (Rab5), late (Rab7), and recycling endosomes (Rab11) as well as lysosomes (LAMP1) were detected with fluorescently labeled mAb specific to the endosomal markers (Fig. 5, A and B, and data not shown). Approximately half of the LL-37 and mCRAMP localized with LAMP1 in BEAS-2B cells, and the degree of this localization was not much changed by the presence of poly(I:C) or LPS (Fig. 5C). Less than 10% of LL-37 or mCRAMP localized to Rab11 endosomes, and this was also largely unaffected by the presence of poly(I:C) or LPS (Fig. 5C and data not shown).

Localization with the Rab5 and Rab7 endosomes showed more substantive changes. In the absence of poly(I:C), ~12–14% of LL-37 and mCRAMP localized with Rab5-positive endosomes and 45% localized to Rab7 endosomes (Fig. 5). In the presence of poly(I:C), LL-37 colocalization with Rab5 endosomes was increased by 2-fold or more in six independent experiments, and LL-37 colocalization with Rab7 endosomes was decreased by a comparable amount (Fig. 5C). In contrast, mCRAMP localization to Rab5 or Rab7 endosomes was unaffected by poly(I:C), consistent with the observation that mCRAMP does not interact with poly(I:C) (Fig. 2).

**Effect of Endocytosis Inhibitors on Activities of Antimicrobial Peptides**—We sought to determine whether a clathrin-dependent or clathrin-independent mechanism was responsible for LL-37 enhancement of TLR3 signaling. Given that numerous differences between LL-37 and mCRAMP have already been established, we will focus on LL-37 for the remainder of this work.

BEAS-2B cells were assessed for the effects of endocytosis inhibitors (21) on poly(I:C)-induced IL-6 production. Methyl-β-cyclodextrin, which removes cholesterols from cells to inhibit clathrin-independent endocytosis (21), caused only a modest change in both LPS and poly(I:C)-induced IL-6 production (Fig. 6A). However, methyl-β-cyclodextrin profoundly inhibited the LL-37-mediated enhancement of IL-6 production in cells treated with both poly(I:C) and LL-37 (Fig. 6A). The differential effects of methyl-β-cyclodextrin on cells treated with poly(I:C) and poly(I:C)-LL-37 suggest that LL-37 can direct poly(I:C) to enter cells by a different mechanism than used by poly(I:C) alone. To confirm this, we used genistein, another clathrin-independent endocytosis inhibitor (Fig. 6B). Again, genistein did not alter the uptake of LPS or poly(I:C) but...
did inhibit IL-6 production when the cells were induced by poly(I:C) and LL-37. These results suggest that a clathrin-independent mechanism for endocytosis is responsible for uptake of a complex of poly(I:C)-LL-37.

The involvement of a clathrin-independent trafficking mechanism for LL-37-poly(I:C) complex does not preclude the use of a clathrin-dependent trafficking mechanism (22). Therefore, IL-6 production was quantified in the presence of chlor-
promazine-HCl, an inhibitor of clathrin-dependent trafficking. Chlorpromazine-HCl inhibited the effects of poly(I:C) on IL-6 production in a concentration-dependent manner (Fig. 6C). It also inhibited the effects of LPS. Because LPS has been reported to signal from endosomes (9, 23), these results suggest that LPS and poly(I:C) are internalized by some clathrin-dependent mechanisms, consistent with previous observations (23, 24). Interestingly, chlorpromazine-HCl had only a mild effect on IL-6 production induced by poly(I:C)-LL-37, consistent with our notion that LL-37-poly(I:C) complex enters cells by a mechanism different than that used by poly(I:C).

Should LL-37-poly(I:C) enter cells through a clathrin-independent endocytosis, it is likely that the complex would colocalize with cholera toxin B, which also enters cells using a clathrin-independent mechanism (2, 25). As a control, we examined poly(I:C)-LL-37 colocalization with the human transferrin, which uses a clathrin-dependent mechanism (22). In BEAS-2B cells, 82% of poly(I:C)-LL-37 colocalized with cholera toxin B, whereas only 22% colocalized with transferrin. These results support that the mechanism for poly(I:C) uptake differs from that used by poly(I:C).

Receptor for Poly(I:C)-LL-37 Signaling and Endocytosis—LL-37 has been reported to traffic into cells using three receptors, EGFR, IGF-1R, and FPRL1 (12, 14, 26, 27). To determine whether one or more of these receptors was involved in trafficking poly(I:C)-LL-37, siRNAs specific to each receptor were used to knock down their expression. Real-time RT-PCR was used to monitor the efficiency of knockdown. All three sets of siRNAs achieved a >70% reduction in the mRNA levels for their respective receptors in BEAS-2B cells (Fig. 7A). The effects of EGFR, IGF-1R, and FPRL1 knockdowns on IL-6 production were determined in cells stimulated with LPS, LL-37-LPS, poly(I:C), and poly(I:C)-LL-37. Knockdowns of EGFR and IGR-1R decreased the response to LPS but did not affect the response to poly(I:C) (Fig. 7B, upper two panels). In the presence of LL-37, a reduction in IL-6 production was observed when LPS was added to cells with EGFR and IGR-1R knocked down. In contrast, knockdown of FPRL1 did not affect the response to LPS. When LPS was added to the culture media along with LL-37, FPRL1 knockdown actually increased IL-6 production, possibly due to some cross-talk between FPRL1 and other LPS-signaling pathways. Importantly, the knockdown of FPRL1 had only a minimal effect on the response to poly(I:C) but severely reduced the response induced by poly(I:C)-LL-37 (Fig. 7B). These results suggest that FPRL1 is responsible for the trafficking of poly(I:C)-LL-37 but that poly(I:C) entered cells by an independent mechanism.

**FIGURE 6. Effects of endocytosis inhibitors on IL-6 production by BEAS-2B cells induced by LPS or poly(I:C) in the absence of presence of LL-37.** A, shown are the effects of increasing concentration of clathrin-independent endocytosis inhibitor methyl-β-cyclodextrin. B, shown are the effects of increasing concentrations of clathrin-dependent endocytosis inhibitor chloropromazine-HCl. In the results for all three graphs, the inhibitor of endocytosis was added 6 h before the addition of the TLR ligand and the antimicrobial peptides. IL-6 production was quantified by ELISA, and the data are represented as percent changes in IL-6 production normalized to IL-6 production without inhibitor. D, colocalization of LL-37 and poly(I:C) with either cholera toxin-B (CTxB) or transferrin (TFN). LL-37 was labeled with fluorescein in this experiment, and cholera toxin-B and transferrin were labeled with Alexa Fluor 594. Poly(I:C) was added to a final concentration of 0.13 μg/ml but was not labeled. All the data are represented as mean and 1 S.D. of the percent colocalization of LL-37 with cholera toxin-B or transferrin from at least 20 cells.
LL-37 Modulation of TLR3 Signaling

**DISCUSSION**

Cathelicidins have been isolated from many mammalian species and are demonstrated to have a range of activities from modulating wound repair responses to regulation of innate immune responses. In this work we showed that LL-37 could bind dsRNA and up-regulate TLR3 signaling, likely by trafficking to endosomes where TLR3 is resident. In addition, although the mouse CRAMP peptide is an ortholog to the human LL-37, mCRAMP does not bind dsRNA as well as LL-37 and cannot activate TLR3 signaling. Localization of LL-37 in endosomes with TLR3 in BEAS-2B cells was increased by the presence of poly(I:C) or S4 dsRNA (Fig. 3, B and C). These observations suggest that LL-37 binds to poly(I:C) and that the complex is delivered to endosomes for TLR3 signaling. Perhaps the most important observation for TLR3 signaling in this work is that the poly(I:C)-LL-37 complex is trafficked into BEAS-2B cells by a different mechanism used by poly(I:C). Poly(I:C) trafficking primarily uses a clathrin-dependent mechanism, whereas poly(I:C)-LL-37 used a clathrin-independent mechanism. siRNA knockdown of FPRL1, a known LL-37 receptor (14), showed that FPRL1 was required to traffic the poly(I:C)-LL-37 complex into Rab5 endosomes of BEAS-2B cells.

Although both LL-37 and mCRAMP can inhibit signaling by LPS, we and others have observed that their activities do not overlap with regard to dsRNA-induced signaling. In three independently derived human cell lines, LL-37 enhanced TLR3 signaling, but mCRAMP did not (Fig. 1B, Table 1, Table 2). We note, however, that LL-37 may not always enhance TLR3 signaling, as it was shown to reduce IL-6 production in human gingival fibroblasts (28). A difference in the activities of LL-37 and mCRAMP could be due to mCRAMP having reduced ability to bind dsRNA when compared with LL-37 (Figs. 2D and 3B).

The activities of LL-37 and mCRAMP was also different between human and mouse cell lines. Both LL-37 and mCRAMP inhibited signaling by LPS and by dsRNA in murine cell lines (Fig. 1C, Tables 1 and 2). Although the presence of poly(I:C) or viral dsRNA increased the localization of LL-37 in Rab5 endosomes in human cells, this was not observed in the murine RAW264.7 (3). Thus, trafficking of either LL-37 and/or dsRNA is likely to be significantly different in mouse cells than in human cells. We note that all three murine cell lines we analyzed appear to express FPRL1 (Fig. 9), consistent with previous reports (29, 30). However, there may be differences in the post-translational modifications of the murine proteins that could affect the ability to interact with the antimicrobial peptides. There are also a number of differences in the sequences of the two proteins that could affect the functions of the human and mouse FPRL1 (supplemental Fig. 1). The differences in the

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3 D. Singh, unpublished observation.
murine and human FPRL1 molecules will require more thorough analysis. Nonetheless, it is worth noting that the innate immune systems of the mouse have a number of significant differences from those in human (31). Studies of LL-37 may thus require other animal models.

Several lines of evidence suggest that trafficking of the poly(I:C)-LL-37 complex and poly(I:C) in human cells function by different mechanisms. First, an inhibitor of clathrin-dependent endocytosis, chlorpromazine, inhibited poly(I:C)-mediated signaling but was much less effective in inhibiting signaling by the poly(I:C)-LL-37 complex (Fig. 6C). Second, poly(I:C)-LL-37 was sensitive to clathrin-independent inhibitors, whereas poly(I:C)-dependent signaling was not. Third, siRNA knockdown of the FPRL1 receptor known to traffic LL-37 was able to inhibit LL-37-poly(I:C)-induced signaling and localization to early endosomes (Figs. 7 and 8). These results further extend the previous observations of Limmon et al. (32) and Itoh et al. (24), who showed that poly(I:C) binds to Class A scavenger receptors and traffics to endosomes through clathrin-mediated endocytosis. The demonstration that poly(I:C)-LL-37 complexes enter cells using a different endocytic mechanism than poly(I:C) and the increased colocalization of the complex with TLR3 could provide a mechanism for LL-37 enhancing poly(I:C)-induced signaling by TLR3. Our results also suggest that in BEAS-2B cells, LPS and poly(I:C) trafficking could use distinguishable pathways in the presence of LL-37. Signaling by cells incubated with LL-37 and LPS is affected by siRNA knockdown of EGFR and IGF-1R but not by knockdown of FPRL1. In turn, signaling by cells incubated with LL-37 and poly(I:C) was largely unaffected by knockdowns of EGFR and IGF-1R but severely affected by knockdown of FPRL1. Interestingly, signaling in response to LPS by BEAS-2B cells was affected by knockdowns of EGFR and IGF-R1, demonstrating that there are distinct endocytic mechanisms for the ligands of TLR3 and TLR4.

These results suggest that recognition of LL-37 by the three receptors could be modulated by either LPS or dsRNA complexed to LL-37. Furthermore, because LL-37, mcRAMP, and the two chimeric peptides contained swaps within the two regions of the peptides could inhibit LPS-induced signaling, it is likely that binding to LPS by these cathelicidin peptides have less stringent requirements than the binding of double-stranded RNA.

Poly(I:C) is a potential adjuvant for vaccines (33). Poly(I:C) complexed with LL-37 could enhance the responses to increase the efficacy of vaccines. In addition, as high levels of LL-37 have been observed in the context of certain inflammatory diseases, such as psoriasis and rheumatoid arthritis (34), antagonizing
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