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Cathelicidin Peptide LL-37 Modulates TREM-1 Expression and Inflammatory Responses to Microbial Compounds

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Abstract—Inflammatory diseases remain an important cause of morbidity and mortality. Cathelicidins are immunomodulatory and antimicrobial peptides with potent anti-endotoxic properties. Although the effects of the human cathelicidin LL-37 on cellular responses to Toll-like receptor (TLR) ligands have been investigated, its effects on responses to other pro-inflammatory stimuli have not been well studied. Triggering receptor expressed on myeloid cells (TREM-1) acts to amplify inflammatory responses and plays important roles in the pathogenesis of endotoxemia. In this work, the effects of LL-37 on responses to TREM-1 stimulation, alone and in the presence of a range of microbial compounds, were analyzed. It was shown that in peripheral blood mononuclear cells LL-37 strongly suppressed synergistic responses to TREM-1 and TLR4 stimulation, partly through the inhibition of TREM-1 expression on monocytes; similar effects were observed using the TLR2 ligand lipoteichoic acid. In contrast, LL-37 stimulated TREM-1 upregulation by peptidoglycan (PGN, TLR2 ligand that is also recognized via nucleotide-binding oligomerization domain containing 2 after fragmentation and intracellular uptake), as well as the responses to combined TREM-1 and PGN stimulation, possibly via the p38 mitogen-activated protein kinase pathway. LL-37 did not affect TREM-1-induced neutrophil degranulation or the production of reactive oxygen species and interleukin-8 by neutrophils. These findings provide further insight into the roles of LL-37 during inflammation and may have implications for its *in vivo* immunomodulatory properties and for the design of synthetic cathelicidin derivatives as anti-inflammatory and anti-endotoxic molecules.

KEY WORDS: LL-37; cathelicidin; triggering receptor expressed on myeloid cells 1 (TREM-1); lipopolysaccharide; inflammation.

INTRODUCTION

LL-37 is a human cationic host defense peptide of the cathelicidin family [1–3]. It is produced from a precursor protein hCAP-18 by proteolysis [4, 5] and is found at mucosal surfaces and in various tissues at estimated concentrations of 2–5 μg/ml and at higher levels under inflammatory conditions [6, 7]. At physiological concentrations of divalent cations and serum, it is weakly or non-antimicrobial [8], but exhibits a broad range of immunomodulatory activities on the immune system of the host [1–3].

Importantly, the immunomodulatory properties of LL-37 include both immunostimulatory and anti-inflammatory activities. The peptide is anti-endotoxic and inhibits pro-inflammatory cytokine production and cell activation in response to lipopolysaccharide (LPS) in many cell types [9–12] and also shows protective

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ABBREVIATIONS: CRAMP, cathelicidin-related antimicrobial peptide; hCAP-18, human cationic antimicrobial protein 18 kDa; JNK, Jun N-terminal kinase; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MAPK, mitogen-activated protein kinase; MDP, muramyl dipeptide; NLR, NOD-like receptor; NOD2, nucleotide-binding oligomerization domain containing 2; PBMC, peripheral blood mononuclear cell; PGN, peptidoglycan; PI3K, phosphoinositide-3-kinase; ROS, reactive oxygen species; TREM-1, triggering receptor expressed on myeloid cells 1; TRIF, TIR-domain-containing adapter-inducing interferon-β

activity *in vivo* in murine models of endotoxemia [13]. However, microarray profiling of LPS-stimulated cells shows that LL-37 does not cause global suppression of all LPS-induced transcriptional responses, but instead results in a selective downregulation of, among others, a large group of pro-inflammatory genes, while maintaining the expression of many chemokines, adhesion molecules, and anti-inflammatory mediators [9]. The mouse orthologue of LL-37, cathelicidin-related antimicrobial peptide (CRAMP), also strongly inhibits pro-inflammatory responses to LPS in murine macrophages but does not suppress the production of anti-inflammatory cytokine interleukin (IL)-10 and chemokine MIP-2 [14]. CRAMP deficiency in mice leads to increased inflammatory responses in models of allergic contact dermatitis [15] although it does not result in increased susceptibility to endotoxemia [14].

Importantly, LL-37 and CRAMP also exhibit a range of immunostimulatory activities. These include chemokine induction [13, 16, 17], stimulation of mast cell antimicrobial and pro-inflammatory functions [18, 19], stimulation of dendritic cell activation in synergy with oligonucleotides [20, 21], modulation of neutrophil survival [22–24], and promotion of cytokine production in combination with IL-1β [25]. These activities of LL-37 result from stimulation of multiple cell-surface and intracellular receptor proteins, leading to modulation of signaling responses through the mitogen-activated protein kinase (MAPK), phosphoinositide-3-kinase (PI3K), protein kinase B (AKT/PKB), nuclear factor kappa B (NFκB), as well as other signaling pathways [3, 26]. The immunostimulatory effects of LL-37 and CRAMP have been further demonstrated by the *in vivo* adjuvant properties of these molecules [27, 28].

The unique properties of LL-37 in selectively stimulating anti-infective immunity while dampening pro-inflammatory responses to endotoxin [29, 30] raise considerable interest in the application of cathelicidin derivatives as anti-inflammatory and antisepsis therapeutics [12, 31, 32]. Indeed, short synthetic peptides with potent anti-infective activities have been designed to share the anti-inflammatory and immune-stimulating properties of natural cathelicidins ([12, 33, 34] and unpublished data) and are in phase I/II clinical trials (www.inimexpharma.com, www.octoplus.nl). Potential applications of cathelicidin-derived cationic peptides as anti-inflammatory therapeutics highlight the need for further in-depth understanding of the mechanisms of anti-endotoxic and anti-inflammatory properties of cathelicidins.

Triggering receptor expressed on myeloid cells 1 (TREM-1) is a type I transmembrane protein, expressed on monocytes, macrophages, neutrophils, and hepatic vascular endothelium [35–37], that functions as an amplifier of inflammatory responses [38]. The natural binding partners of TREM-1 are at present unknown but may include endogenous ligands expressed on granulocytes and platelets [39, 40] and present in the sera of some septic patients [41], as well as exogenous ligands for example on Marburg and Ebola viruses [42]. TREM-1 ligation with an agonist antibody induces cytokine production in monocytes and promotes antimicrobial functions of neutrophils [35, 43]. Further, TREM-1 ligation strongly enhances inflammatory responses to bacterial compounds, and TREM-1 expression is upregulated under inflammatory conditions *in vitro* and *in vivo* [44, 45]. Administration of decoy TREM-1-derived peptides, designed to inhibit the interactions between TREM-1 and its ligands, is protective in animal models of endotoxemia and sepsis [39, 44], colitis and inflammatory bowel disease [46], and hemorrhagic shock [47], suggesting a deleterious role of TREM-1 activity in inflammatory diseases; however, TREM-1 stimulation can also enhance bacterial clearance and promote resolution of infection [48, 49]. Thus, there is a large body of evidence suggesting that TREM-1 plays a prominent role in inflammatory responses, anti-infective immunity, and in the pathology of endotoxemia; however, the effects of cathelicidins on TREM-1 expression and activity have not been previously explored.

In the current work, the effects of LL-37 on cellular responses to TREM-1 stimulation, alone and in combination with a range of Toll-like receptor (TLR) and nucleotide-binding oligomerization domain containing 2 (NOD2) ligands, were analyzed. LL-37 strongly suppressed synergistic responses to TREM-1 and TLR4 stimulation in human peripheral blood mononuclear cells (PBMCs), at least in part through downregulation of LPS-induced TREM-1 expression on monocytes, but had distinct effects on responses to TREM-1 stimulation in combination with other TLR ligands. This provides further insight into the immunomodulatory properties of LL-37, with potential implications for its *in vivo* immunomodulatory activities and for the design of synthetic cathelicidin derivatives as anti-inflammatory and anti-endotoxic molecules.

MATERIALS AND METHODS

Cell Isolation and Culture

Venous blood was collected from healthy volunteers into heparin-containing Vacutainer tubes (BD Biosciences) in accordance with the ethical approval guidelines of the University of British Columbia Research Ethics Board. PBMCs were isolated as previously described [9]. Briefly, the blood was diluted with an equal volume of phosphate-buffered saline pH 7.4 (PBS, Invitrogen) and separated by density gradient centrifugation over Ficoll-Paque Plus (Amersham Biosciences). Mononuclear cell layers were collected and washed twice in RPMI-1640 (Invitrogen). In selected experiments, monocytes were isolated using either the Dynal Monocyte Negative Isolation kit (Invitrogen) or the Easy Sep Monocyte Negative Isolation kit (Stem Cell Technologies), according to the manufacturers' protocols.

Neutrophils were isolated from whole blood as previously described [23]. Blood was prediluted in 2% (*w*/*v*) Dextran T-500 (Amersham Pharmacia Biotech) in 0.9% saline and sedimented at room temperature for 30 min. The leukocyte-rich upper layer was centrifuged at 250×*g* for 7 min and erythrocytes hypotonically lysed with water for 30 s, followed by restoration of tonicity with 2.5% (*w*/*v*) saline. Neutrophils were separated by density gradient centrifugation over a Ficoll-Paque Plus (Amersham Pharmacia Biotech) at 4°C and washed in Krebs–Ringer phosphate buffer pH 7.3, containing 10 mM glucose and 1.5 mM Mg^{2+} at 4°C. All the cells were maintained at 1×10^6 cells/ml in RPMI-1640 with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, and 1 mM sodium pyruvate (all from Invitrogen), in a humidified incubator at 37°C and 5% CO_2.

Reagents and Cell Stimulation

Peptide LL-37 (LLGDFFRKSKEKIGKEFKRIVQ RIKDFLRNLVPRTES) was synthesized using F-moc chemistry at the Nucleic Acid/Protein Synthesis Unit of the University of British Columbia, confirmed by mass spectrometry, and HPLC-purified to >98% purity. LPS from *Pseudomonas aeruginosa* strain H103 was purified using the Darveau–Hancock method [50]. Other microbial compounds utilized were Pam3CSK4, *Staphylococcus aureus* lipoteichoic acid (LTA), *Staphylococcus aureus* peptidoglycan (PGN), poly(I:C), *Salmonella typhimurium* flagellin, imiquimod, and muramyl dipeptide (MDP) [all purchased from Invivogen], and phosphorothioate-stabilized CpG oligonucleotide 10101 [TCGTCGTTTTC GCGCGCGCCG, kindly provided by the Vaccine and Infectious Diseases Organization, U. Saskatchewan, Saskatoon, Canada].

For TREM-1 ligation, tissue culture dishes were precoated with an agonistic monoclonal anti-TREM-1 antibody (clone 193015, R&D Systems) or a mouse IgG_1 isotype control at 10 μg/ml for 1 h at 37°C and washed twice in PBS pH 7.4 (Invitrogen). The cells were plated into the precoated wells and allowed to settle naturally onto the surface to engage TREM-1.

Chemical inhibitors used were Bay117082 (NFκB inhibitor, 2 μM), LY294002 (PI3K inhibitor, 5 μM), PD98059 (MAP2K1/MEK-1 inhibitor, 5 μM), SB203580 (p38 MAPK inhibitor, 5 μM), and JNKII (Jun N-terminal kinase (JNK) inhibitor, 5 μM, all from Calbiochem). The inhibitors were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich); concentrations of DMSO in cell culture did not exceed 0.05% (*v*/*v*), and all experiments included DMSO vehicle controls. The inhibitors were checked against cytotoxic effects using lactate dehydrogenase (LDH) release colorimetric cytotoxicity detection kit (Roche Applied Science).

Flow Cytometry

Data were collected on FACSCalibur™ and analyzed using CellQuest™ software (Becton Dickinson). For the analysis of cell surface markers, the cells were suspended in Hanks buffered salt solution (HBSS) pH 7.4, with 2% FCS (Invitrogen), 0.2% (*w*/*v*) sodium azide, and 20 mM HEPES. The staining was at 4°C for 20 min, and the antibodies used were anti-human TREM-1-phycoerythrin (clone 193015, R&D Systems), CD11b FITC (clone ICRF44, Sigma), CD14 phycoerythrin (clone MEM-18, ImmunoTools), CD14 Alexa Fluor 488 (clone M5E2, BioLegend), and CD66b FITC (clone G10F5, BioLegend). After washing, the cells were resuspended in 0.5% (*w*/*v*) paraformaldehyde in PBS for analysis.

For the analysis of protein phosphorylation, stimulated cells were fixed in 2% (*w*/*v*) paraformaldehyde in PBS at room temperature for 15 min, permeabilized in Phosflow Perm Buffer III (BD Biosciences) at 4°C for 30 min, and washed in 0.5% (*w*/*v*) bovine serum albumin (Roche Applied Science) in PBS. The cells were stained for 1 h at room temperature for phospho-p38 (Thr180/Tyr182, clone 3D7, Cell Signaling), phos-

pho-AKT (S473, clone 193H12, Cell Signaling), or phospho-p65 NFκB (S529, clone K10-895.12.50, BD Biosciences), and following washing, with goat anti-rabbit IgG Alexa Fluor 647 (2 μg/ml, Invitrogen, Molecular Probes) or goat anti-mouse IgG Alexa Fluor 488 (0.8 μg/ml, Sigma-Aldrich) at room temperature for 30 min. The cells were counterstained for CD14 and resuspended in 0.5% (*w*/*v*) paraformaldehyde in PBS for analysis.

For the studies of reactive oxygen species (ROS) production, carboxy-H_2DCFDA (Invitrogen, Molecular Probes) was used according to the manufacturer's protocol. To load the cells with the carboxy-H_2DCFDA dye, the cells were resuspended in HBSS pH 7.4 with 1 mM Ca^{2+}, 1 mM Mg^{2+}, 1% (*v*/*v*) FCS, and 1 μM carboxy-H_2DCFDA at 10^7 cell/ml; incubated for 20 min at 37°C; and washed twice in fresh HBSS pH 7.4, prior to stimulation and analysis.

Enzyme-Linked Immunosorbent Assay

Samples were centrifuged at 1,000×*g* for 10 min to obtain cell-free supernatants and stored at −20°C. Cytokine levels were measured by enzyme-linked immunosorbent assay (ELISA), using anti-human tumor necrosis factor alpha (TNF-α) antibody clones unconjugated MAb1 and biotin-conjugated MAb11, followed by avidin horseradish peroxidase (all from eBioscience). The ELISAs were developed using TMB Liquid Substrate System (Sigma-Aldrich) and imaged with a PowerWave x340 plate reader (BioTek Instruments).

Quantitative Real-Time Polymerase Chain Reaction

RNA was isolated using the RNEasy mini kit (Qiagen) and treated with RNase-free DNase (Qiagen), and complementary DNA (cDNA) was prepared with the SuperScript™ III First-Strand Synthesis SuperMix kit (Invitrogen). cDNA levels were assessed by quantitative real-time polymerase chain reaction (qRT-PCR) with the SuperScript™ III Platinum® Two-Step qRT-PCR kit with SYBR® Green (Invitrogen) and the ABI PRISM® 7000 sequence detection system (Applied Biosystems). The data were analyzed using the comparative Ct method [51] and normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. The primers used were TREM-1-Fw ATGATCATGGTTTACTGCGCG, TREM-1-Rv CATGTGAGGCTCCTTGGGAG, GAPDH-Fw GTCGCTGTTGAAGTCAGAGG, and GAPDH-Rv GAAACTGTGGCGTGATGG.

Statistical Analyses

Prism 4.0 Software (GraphPad Inc.) was used for statistical data analyses, with a two-tailed Student's *t* test or non-parametric Mann–Whitney test used for comparisons of two datasets and analysis of variance (ANOVA) for multiple comparisons.

RESULTS

LL-37 Inhibited Synergistic Responses to TREM-1 Agonist and TLR4 Stimulation

The effects of LL-37 on cellular responses to TREM-1 stimulation and to combined stimulations through TLR4 and TREM-1 were tested in PBMCs. The cells were plated into wells precoated with an anti-TREM-1 agonist antibody or a mouse IgG_1 isotype control, as previously described [44], and stimulated with LPS (100 ng/ml) and LL-37 (20 μg/ml) for 4 or 24 h. As monocytes are the main TREM-1-expressing cell type within the PBMC population [44], monocytes are expected to be the main responders to TREM-1 stimulation in this study. As previously reported, TREM-1 stimulation strongly augmented LPS responses, especially at the 24-h time-point [44, 45], and LL-37 strongly inhibited LPS responses [9, 10, 15]. Furthermore, LL-37 strongly inhibited cellular responses to the combined stimulation with LPS and the TREM-1 agonist, although it did not have significant effects on responses to TREM-1 agonist by itself (Fig. 1a, b). To eliminate any direct interactions between LPS and LL-37, the cells were preincubated with LL-37 (20 μg/ml) for 3 h, washed twice, and then stimulated with LPS (10 ng/ml) and TREM-1 agonist for 18 h. LL-37 significantly inhibited response to LPS and TREM-1 stimulation in this study (Fig. 1c). This suggested that inhibition of TLR4/TREM-1 synergy may be an important component of the anti-endotoxic properties of the peptide.

LL-37 Did Not Affect TREM-1-Induced Neutrophil Degranulation or ROS Production

In addition to cytokine induction in monocytes, TREM-1 ligation is also known to promote the antimicrobial functions of neutrophils [35, 43]. Thus, the effects of LL-37 on TREM-1 activity were also investigated in neutrophils. LL-37 did not affect TREM-1-induced neutrophil degranulation (Fig. 2a), as measured by the increase in cell surface levels of CD11b and

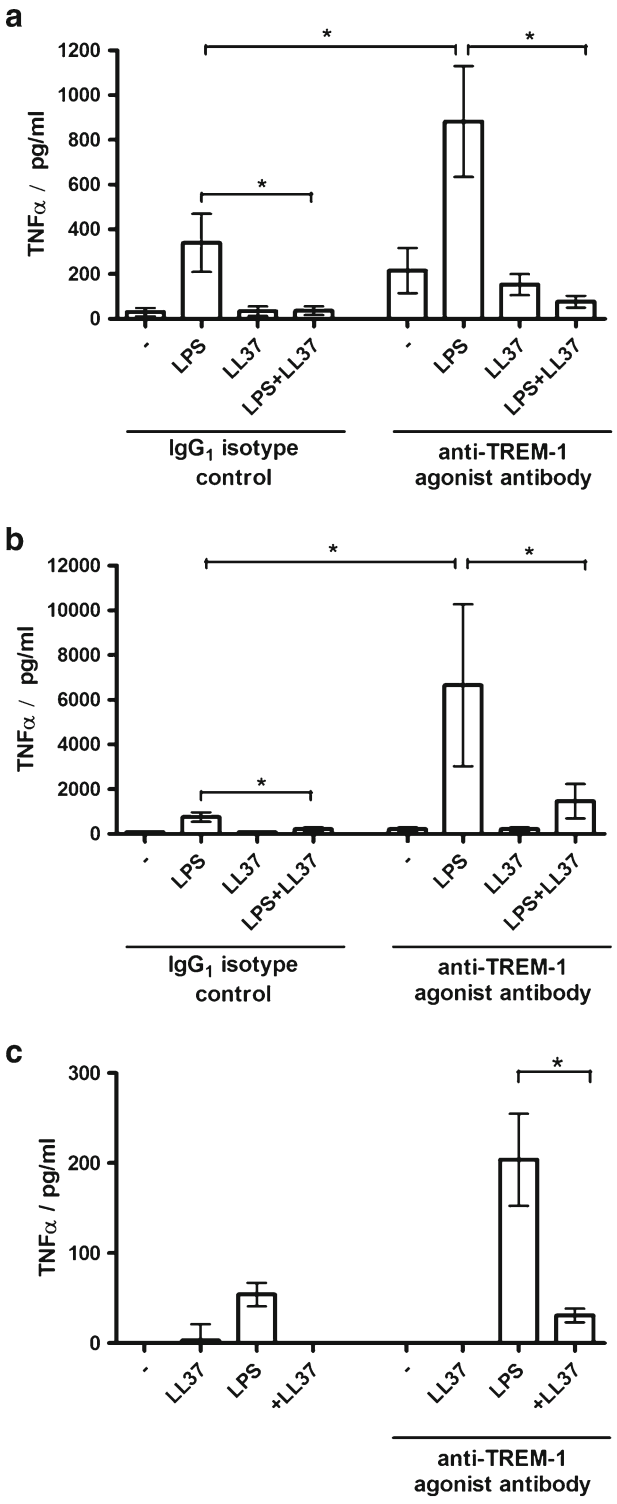

Fig. 1. LL-37 strongly inhibited TNF-α production by human PBMCs in response to combined stimulation with TLR4 and TREM-1 ligands. **a**, **b** The cells were stimulated with LPS (100 ng/ml), LL-37 (20 μg/ml), and an anti-TREM-1 agonist antibody or an IgG_1 isotype control for **a** 4 h and **b** 24 h and analyzed for TNF-α production by ELISA. **c** The cells were pretreated with LL-37 (20 μg/ml) for 3 h, washed twice, and then stimulated with LPS (10 ng/ml) and TREM-1 agonist antibody for further 18 h. *Bars* represent means ± standard errors, *$p<0.05$.

CD66b [43]. LL-37 also did not affect TREM-1-induced neutrophil oxidative burst, as shown by equivalent increases in the fluorescence of the ROS-sensitive dye carboxy-H_2DCFDA (Fig. 2b). The induction of IL-8 by TREM-1 stimulation was also unaffected by LL-37 (Fig. 2c).

LL-37 Inhibited LPS-Induced TREM-1 Expression in Monocytes

TREM-1 expression on monocytes is known to be upregulated in response to LPS and other inflammatory stimuli [35, 44, 45]. Thus, to investigate the mechanisms through which LL-37 inhibited TLR4/TREM-1 synergy, the effects of LL-37 on TREM-1 induction on monocytes were tested. PBMCs were treated with LPS (100 ng/ml) and LL-37 (20 μg/ml) for 24 h and analyzed for TREM-1 expression by flow cytometry, gating on monocytes ($CD14^+$). The levels of TREM-1 on monocytes were strongly upregulated by LPS stimulation, as previously reported [35, 44], and this effect was strongly suppressed by LL-37 (Fig. 3a). Further, LL-37 pretreatment was performed to rule out any direct interactions with LPS as the cause of the inhibitory activity. The cells were preincubated with LL-37 (20 μg/ml) for 3 h, washed twice, and then stimulated with LPS (10–100 ng/ml) for 18 h, and strong inhibition of TREM-1 expression by the peptide was observed (Fig. 3b). Neither LPS [52] nor LL-37 affected cell surface expression of TREM-1 on neutrophils (data not shown).

TREM-1 cell surface levels are known to be regulated both at the level of gene expression and post-transcriptionally [53]. Previous microarray studies showed that LL-37 selectively downregulates a subset of LPS-induced transcriptional responses; however, TREM-1 was not reported to be differentially expressed in these previous studies [9]. Thus, to differentiate between transcriptional and post-transcriptional effects, isolated monocytes were stimulated with LPS (100 ng/ml) and LL-37 (20 μg/ml) for 4 h and analyzed by qRT-PCR for TREM-1 messenger RNA (mRNA) levels. LPS upregulated TREM-1 mRNA levels in monocytes and this effect

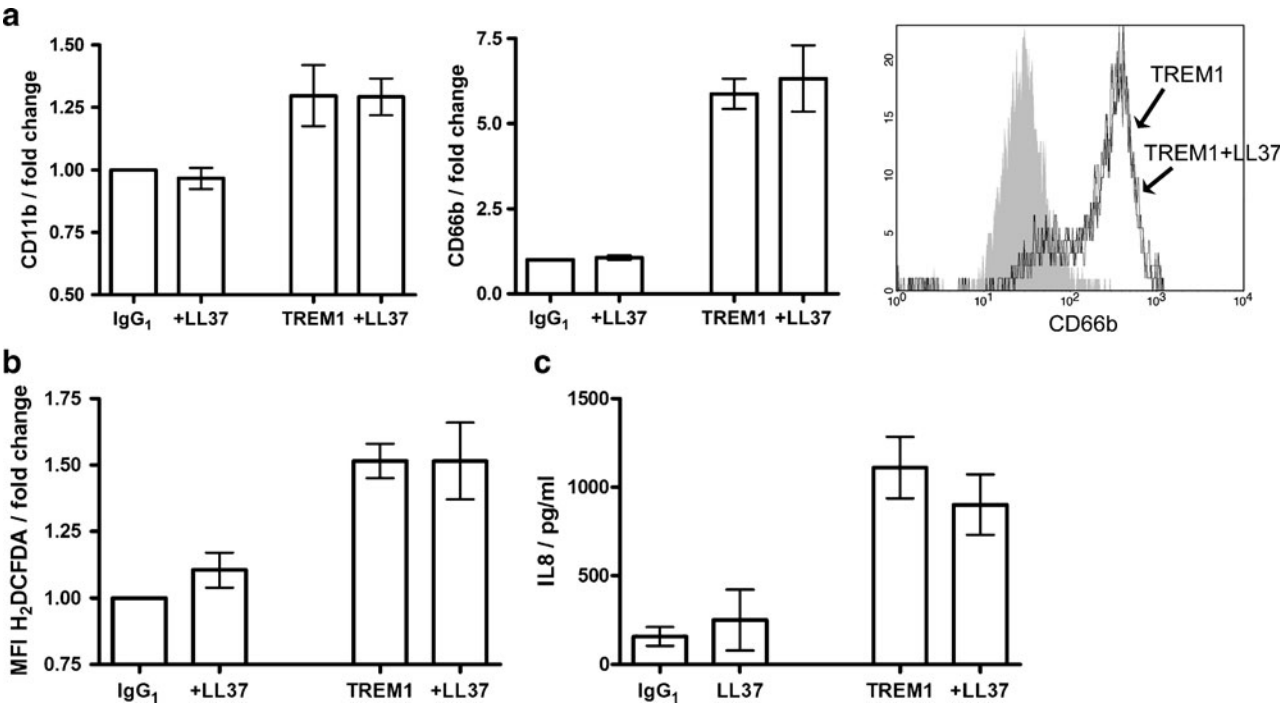

Fig. 2. LL-37 did not affect TREM-1-induced **a** neutrophil degranulation, **b** ROS production, or **c** IL-8 secretion. Human neutrophils were stimulated with the TREM-1 agonist antibody (TREM-1) or IgG_1 isotype control, with or without LL-37 (5 μg/ml), for 60 min (**a**, **b**) or 24 h (**c**). **a** CD11b and CD66b cell-surface levels analyzed by flow cytometry as a measure of degranulation; *filled area*—unstimulated cells, *black line*—TREM-1 stimulation, *gray line*—TREM-1 + LL-37, representative of three independent experiments. **b** Mean fluorescence intensity of carboxy-H_2DCFDA preloaded neutrophils; increased fluorescence as the result of carboxy-H_2DCFDA oxidation is a measure of ROS production. **c** IL-8 secretion at 24 h of stimulation analyzed by ELISA.

was suppressed by LL-37 (Fig. 3c), indicating that the inhibitory effects of LL-37 on TREM-1 induction were at least in part mediated at the transcriptional level.

Effects of LL-37 on TREM-1 Induction and Inflammatory Responses to Other TLR Ligands

LL-37 is known to have diverse effects on cellular responses to different TLR ligands, depending on the ligand, cell type, and response being studied [9–12, 14, 15, 20, 21], whereas its effects on NOD2 stimulation have not been extensively investigated, and the effects on TREM-1 induction have not been addressed. Thus, we investigated the effects of LL-37 on TREM-1 upregulation in response to a range of microbial compounds (Fig. 4). In monocytes, LL-37 significantly downregulated TREM-1 induction by LTA (TLR2 ligand, 2 μg/ml) but, conversely, enhanced TREM-1 induction by PGN (putative TLR2 ligand that can also be recognized via NOD2 after fragmentation and intracellular uptake, 10 μg/ml) [54–59]. LL-37 had no effects on TREM-1 induction in response to Pam3CSK4 (TLR1–TLR2 ligand, 1 μg/ml), flagellin (TLR5 ligand, 0.5 μg/ml), or muramyl dipeptide (fragment of PGN and a NOD2 ligand, 10 μg/ml). The distinct effects of LL-37 on TREM-1 induction by Pam3CSK4, LTA, and PGN are surprising and may be due to differences in the interactions of LL-37 with these compounds, differences in the recognition of these compounds by TLR1–TLR2 and TLR2–TLR6 heterodimers, and also due to recognition of PGN and its fragments by other non-TLR receptors [54–59].

TREM-1 levels on monocytes were not significantly altered by any of the ligands targeting endosomal TLRs: poly(I:C) (TLR3 ligand, 50 μg/ml), imiquimod (TLR7 ligand, 10 μg/ml), or CpG oligonucleotide (TLR9 ligand, 10 μg/ml), in agreement with previous reports [52], and there was also no significant change in TREM-1 expression following stimulation with these ligands in combination with LL-37 (data not shown). The effects of LL-37 on TREM-1 expression in LTA, Pam3CSK4, and PGN-stimulated neutrophils were also

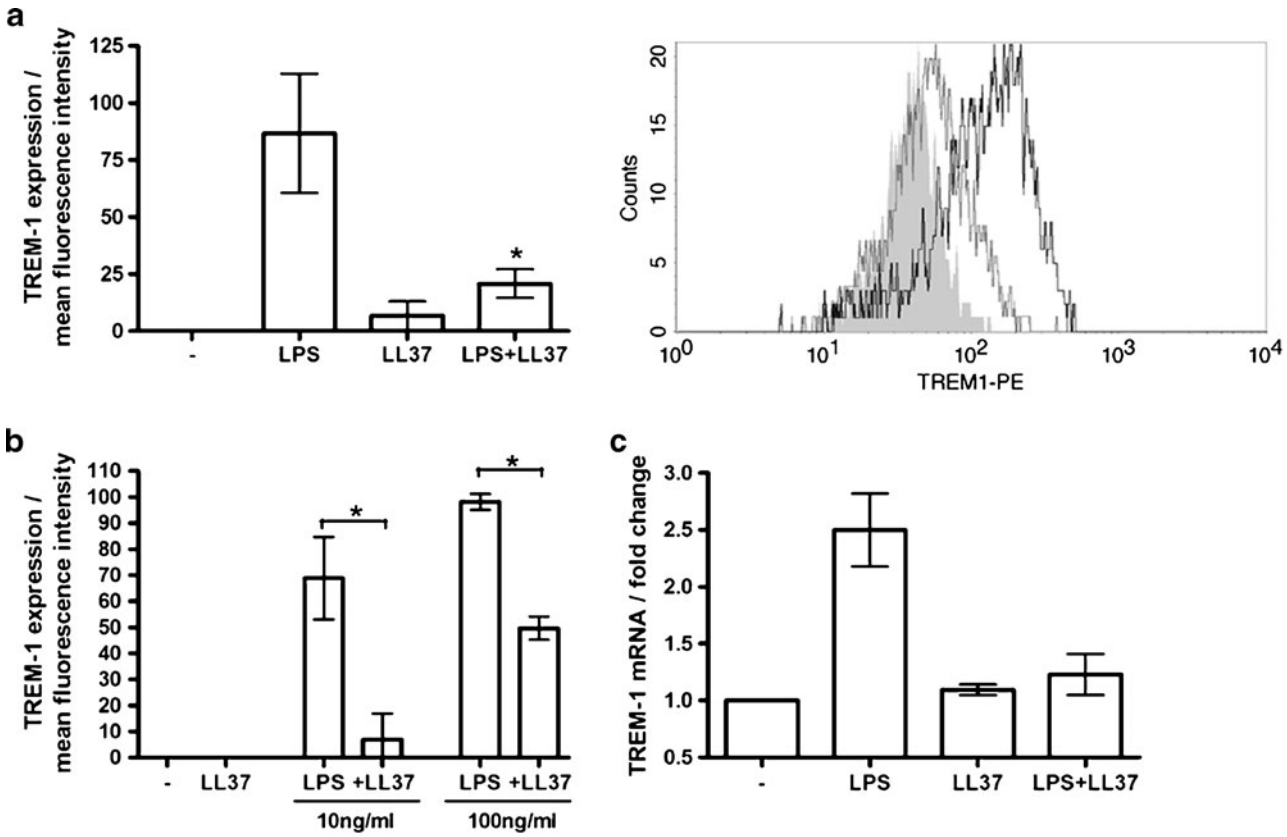

Fig. 3. LL-37 inhibited LPS-induced TREM-1 expression on monocytes. **a**, **b** TREM-1 induction measured by flow cytometry gating on $CD14^+$ monocytes, TREM-1 staining mean fluorescence intensity presented following background subtraction of TREM-1 levels on unstimulated cells. **a** The cells were stimulated with LPS (100 ng/ml) and LL-37 (20 μg/ml) for 24 h. **b** The cells were pretreated with LL-37 (20 μg/ml) for 3 h, washed twice, and then stimulated with LPS (10 and 100 ng/ml) for 18 h. Histogram representative of five independent experiments; *filled area*—untreated, *black line*—LPS-treated, *gray line*—LPS + LL37-treated cells. **c** Relative TREM-1 mRNA levels in monocytes stimulated with LPS (100 ng/ml) and LL-37 (20 μg/ml) for 4 h, measured by qRT-PCR. qRT-PCR data were analyzed using the comparative Ct method [51], normalized against GAPDH, and presented as fold change relative to unstimulated control. *Bars* represent means ± standard errors,* $p<0.05$.

investigated, but no significant changes in TREM-1 expression compared to unstimulated cells were seen (data not shown).

To test the functional significance of the distinct effects of LL-37 on TREM-1 induction by different microbial compounds, the effects of LL-37 on TNF-α responses to combined stimulations with these microbial compounds and TREM-1 agonist were investigated. There was a correlation between the effects of LL-37 on TREM-1 expression and its effects on TNF-α response. Thus, LL-37 significantly downregulated TNF-α responses to TREM-1 agonist in combination with LTA, but enhanced TNF-α responses to TREM-1 agonist in combination with PGN (Fig. 5a, b). LL-37 had no significant effects on responses to TREM-1 agonist in combination with Pam3CSK4 or MDP (Fig. 5c). These findings suggest that LL-37 may have distinct effects on cytokine responses to different classes of microbial stimuli through its differential effects on TREM-1 induction by these stimuli. This could potentially result in distinct effects of LL-37 on the progression of inflammatory reaction in response to different pathogens, depending on the combinations of TLR and NOD-like receptor (NLR) ligands presented by the pathogens.

Effects of LL-37 on Signaling Responses to Peptidoglycan in Monocytes

LL-37 enhanced PGN-mediated TREM-1 induction in monocytes (Fig. 4) and also TNF-α responses to stimulation with PGN and TREM-1 agonist (Fig. 5b). Previously, other studies also reported that the mouse

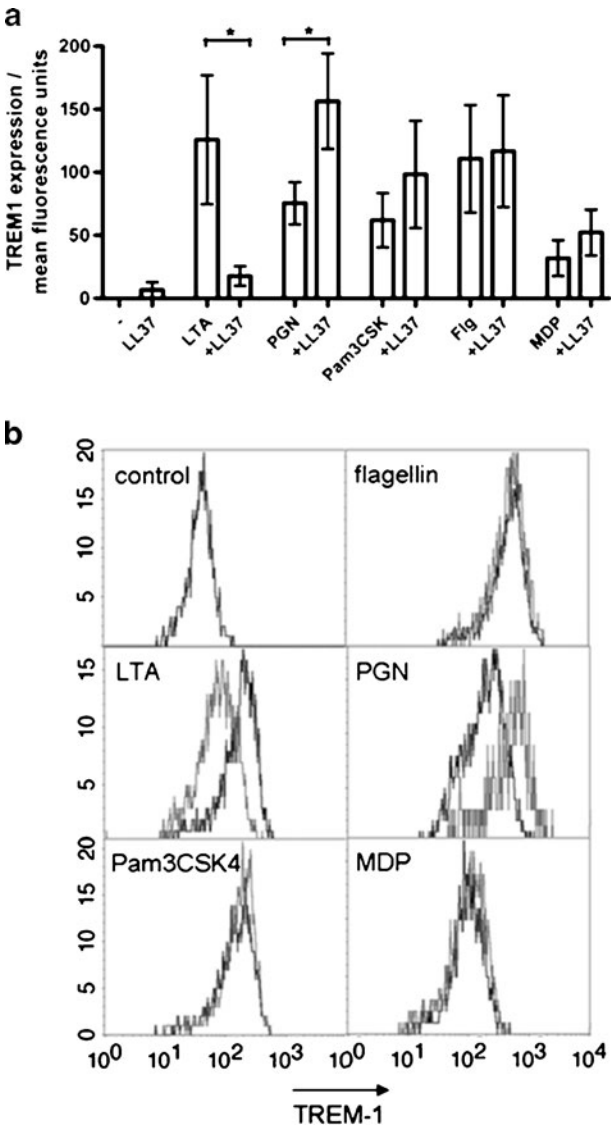

Fig. 4. a, **b** Effects of LL-37 on TREM-1 induction on monocytes in response to different classes of TLR ligands and microbial compounds. PBMCs were stimulated for 24 h with *S. aureus* LTA (lipoteichoic acid, TLR2 ligand, 2 μg/ml), *S. aureus* PGN (peptidoglycan, TLR2 and NOD2 ligand, 10 μg/ml), Pam3CSK4 (TLR1–TLR2 ligand, 1 μg/ml), flagellin (TLR5 ligand, 0.5 μg/ml), and muramyl dipeptide (MDP, NOD2 ligand, 10 μg/ml) and analyzed for TREM-1 expression, gating on $CD14^+$ monocytes. *Bars* represent means ± standard errors, $*p<0.05$. Histograms representative of ≥3 independent experiments; *black line*—without LL-37, *gray line*—with LL-37.

orthologue CRAMP augmented PGN-induced IL-6 production in dendritic cells [15], suggesting that cathelicidins may have wider effects on cellular responses to PGN. The signaling basis for these activities was investigated.

PBMCs were treated with specific inhibitors for signaling pathways known to be activated by PGN and LL-37 (MAP2K1/MEK-1 inhibitor: PD98059, p38 MAPK inhibitor: SB203580, JNK inhibitor: JNKII, NFκB pathway inhibitor: Bay117082, and PI3K inhibitor: LY294002) [3, 26, 60]. The cells were then stimulated with PGN (10 μg/ml) and LL-37 (20 μg/ml) for 24 h, and the effects on TNF-α production and TREM-1 expression were measured. All of the inhibitors tested strongly suppressed TNF-α secretion in response to combined PGN and LL-37 stimulation (Fig. 6a, all comparisons by ANOVA). In contrast, for TREM-1 induction, complete loss of the response was seen with the NFκB pathway inhibitor, partial but significant inhibition was observed with MAPK pathway inhibitors (including MEK-1, p38, JNK), while PI3K pathway inhibitor had no effects (Fig. 6b). This highlights the complexity of PGN and LL-37 signaling activities and shows that induction of TNF-α and TREM-1 in response to these stimuli is dependent on different but overlapping combinations of signaling pathways.

We further tested whether, in the presence of the specific inhibitors, LL-37 could enhance TREM-1 expression over and above the levels seen with PGN stimulation alone. p38 MAPK inhibitor SB203580 suppressed the stimulatory effects of LL-37 on TREM-1 induction (Fig. 6b), suggesting that augmentation of TREM-1 induction by LL-37 may be at least in part mediated through the p38 MAPK pathway. Further, NFκB inhibitor Bay117082 completely blocked TREM-1 induction by both PGN and PGN + LL-37. None of the inhibitors showed significant cytotoxicity at the concentrations used (LDH release assay, data not shown). The levels of DMSO, used as solvent for the inhibitors, did not exceed 0.05% in cell culture and no DMSO toxicity or effects on biological responses were seen at these levels (data not shown).

To explore the roles of p38 MAPK, NFκB, and other pathways in the signaling activities of LL-37 and PGN further, phosphorylation of specific signaling mediators was measured. PBMCs were stimulated with PGN (10 μg/ml) with or without LL-37 (20 μg/ml) for 30 min; stained intracellularly for phospho-p38 MAPK (T180/Y182), phospho-p65 NFκB (S529), or phospho-AKT (S473); and analyzed by flow cytometry gating on $CD14^+$ monocytes. LL-37 significantly enhanced the activation of p38 MAPK is response to PGN, and there was a trend towards enhanced activation of AKT although this did not reach statistical significance (Fig. 6c). These data further suggest that enhanced p38

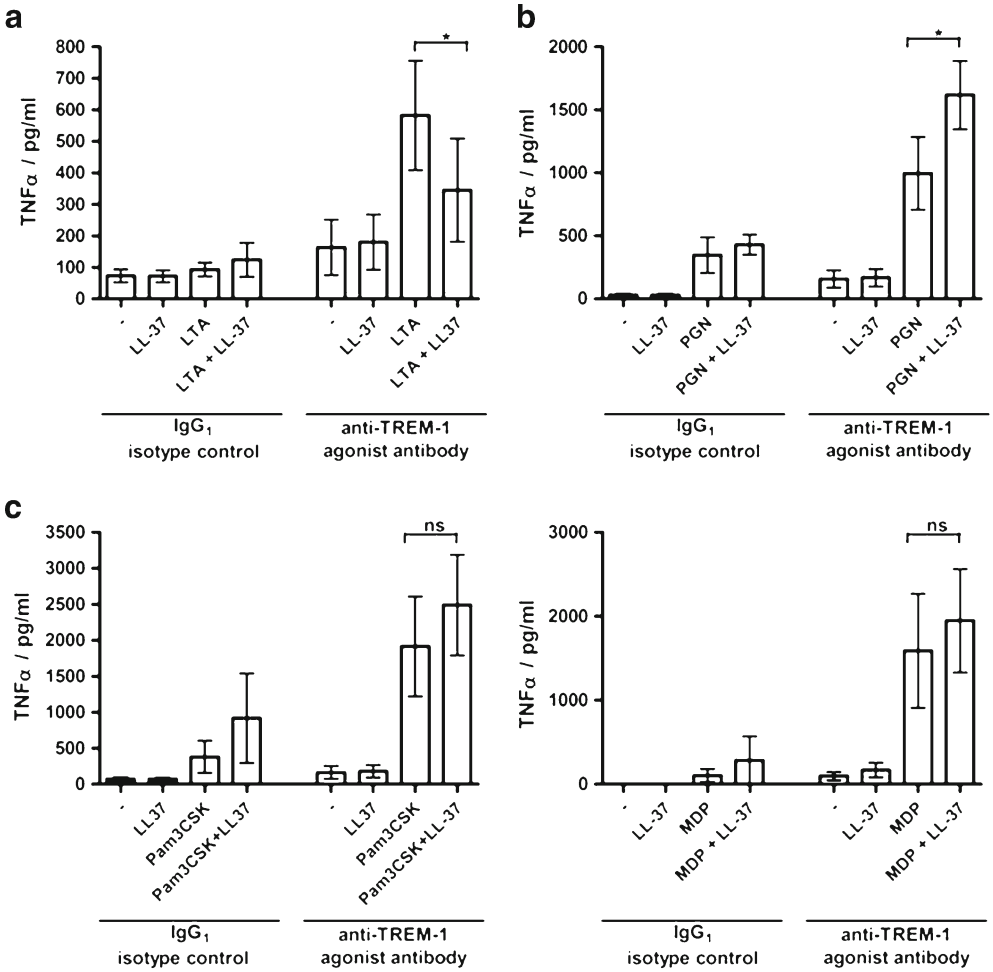

Fig. 5. a–c Effects of LL-37 on TNF-α responses to the TREM-1 agonist in combination with different microbial compounds. PBMCs were stimulated for 24 h with *S. aureus* LTA (lipoteichoic acid, TLR2 ligand, 2 μg/ml), *S. aureus* PGN (peptidoglycan, TLR2 and NOD2 ligand, 10 μg/ml), Pam3CSK4 (TLR1–TLR2 ligand, 1 μg/ml), or muramyl dipeptide (MDP, NOD2 ligand, 10 μg/ml), in combination with a TREM-1 agonist or an IgG_1 isotype control and analyzed for TNF-α responses by ELISA. *Bars* represent means ± standard errors from three to five independent experiments, * $p<0.05$.

MAPK activation may be in part responsible for the augmentation of TREM-1 induction in monocytes in response to PGN and LL-37.

DISCUSSION

Sepsis and inflammatory diseases are an important cause of morbidity and mortality worldwide [61, 62], and there is growing evidence that TREM-1 plays an important role in the pathology of these disorders [38]. In the current work, the effects of LL-37 on cellular responses to TREM-1 stimulation alone and in combination with a range of TLR ligands and other microbial compounds were investigated. LL-37 did not affect inflammatory cytokine production and antimicrobial activities in responses to TREM-1 stimulation alone. Importantly, LL-37 strongly suppressed the upregulation of TREM-1 on monocytes in response to LPS or LTA. This activity correlated with the strong suppression of inflammatory cytokine production in response to a combined stimulation with a TREM-1 agonist and these TLR ligands, and these effects might contribute to the anti-endotoxic and anti-inflammatory activities of LL-37.

Previous studies have shown that the anti-endotoxic activity of LL-37 does not result in indiscriminate suppression of all LPS-induced transcriptional responses, and some LPS activities, such as the induction of some

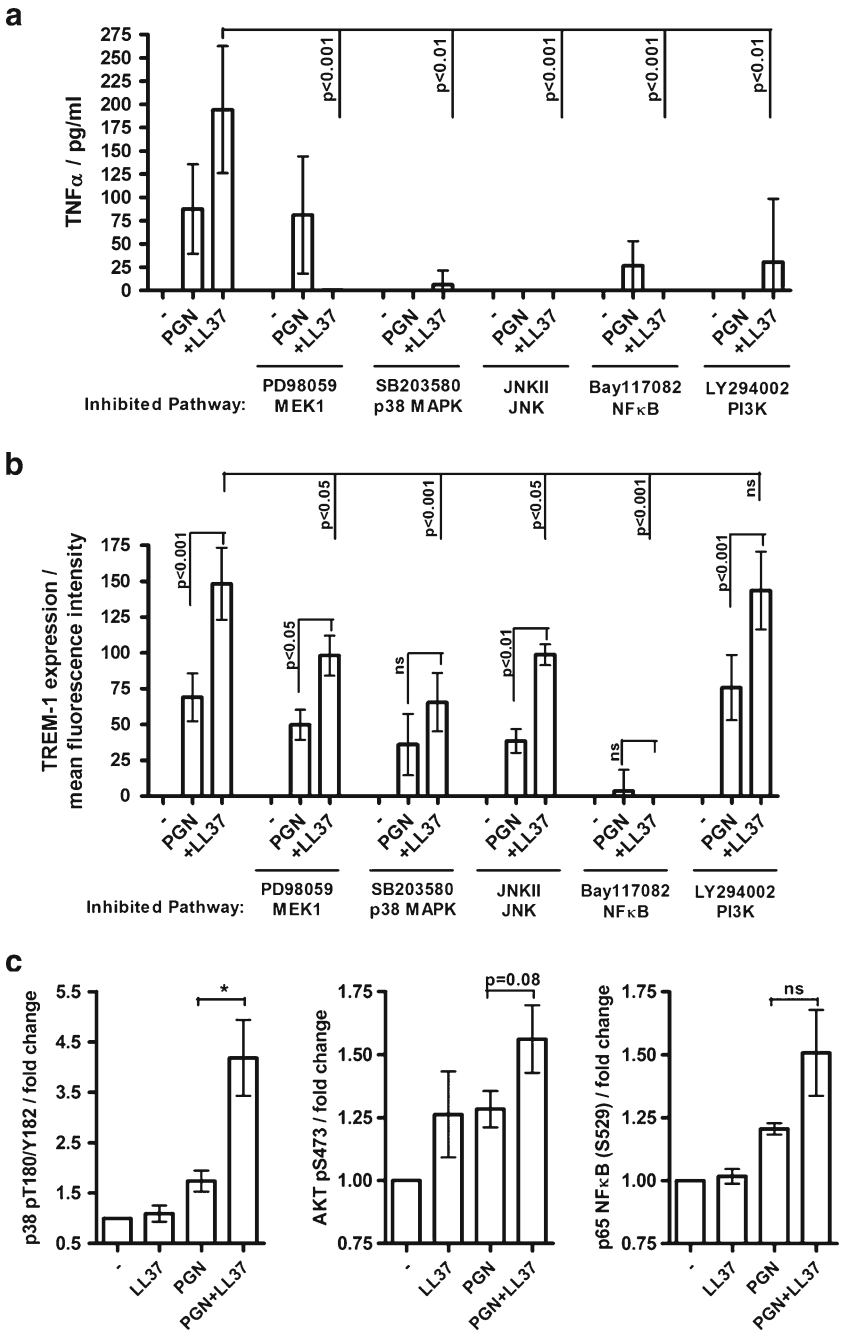

Fig. 6. Signaling activities of LL-37 and PGN. **a**, **b** Effects of the specific chemical inhibitors on biological activities of LL-37 and PGN. PBMCs were pretreated for 1 h with inhibitors of MEK-1—PD98059 5 μM, p38 MAPK—SB203580 5 μM, JNK MAPK—JNKII 5 μM, NFκB—Bay117082 2 μM, and PI3K—LY294002 5 μM; stimulated with PGN (10 μg/ml) with or without LL-37 (20 μg/ml) for 24 h; and analyzed for **a** TNF-α secretion by ELISA or **b** TREM-1 expression on monocytes by flow cytometry. Data presented after background subtraction of the measurements from unstimulated cells; monocytes were gated as $CD14^+$ cells. Statistical analysis employed ANOVA to control for multiple comparisons; *bars* represent means ± standard errors, *ns* nonsignificant. **c** Signaling responses to LL-37 and PGN in monocytes: PBMCs were stimulated with *S. aureus* PGN (10 μg/ml) with or without LL-37 (20 μg/ml) for 30 min; stained intracellularly for phospho-p38 MAPK (T180/Y182), phospho-p65 NFκB (S529), or phospho-AKT (S473); and analyzed by flow cytometry gating on $CD14^+$ monocytes. Increase in the mean fluorescence intensity of the staining is expressed as a fold change relative to the unstimulated sample. *Bars* represent means ± standard errors from three to four independent experiments; $*p<0.05$, *ns* nonsignificant.

chemokines and anti-inflammatory mediators, are maintained or enhanced by the peptide [9]; similar effects have been seen with the mouse orthologue peptide CRAMP [14]. The current study adds TREM-1, an important mediator of inflammatory responses, antibacterial immunity, and pathology of endotoxemia, to the list of genes strongly antagonized by the peptide in LPS and LTA-stimulated cells and suggests that the suppression of TREM-1 expression may be one of the factors contributing to the anti-endotoxic properties of LL-37 [13].

The signaling mechanisms responsible for the inhibitory effects of LL-37 on TREM-1 induction in response to LPS and LTA remain to be further investigated. Previous studies showed that LL-37 inhibits inflammatory cytokine production in response to LPS through the downregulation of p38 MAPK and NFκB signaling pathways [11, 14, 63, 64]; similar mechanisms may be responsible for the antagonistic effects of LL-37 on TREM-1 expression in response to LPS or LTA. It was recently reported that upregulation of TREM-1 by LPS is mediated through a signaling pathway dependent on the adapter protein TIR-domain-containing adapter-inducing interferon-β (TRIF) while its upregulation by LTA is mediated by MyD88 [65]. The ability of LL-37 to inhibit TREM-1 induction by both LPS and LTA therefore suggests that modulation of both MyD88 and TRIF-dependent TLR signaling responses is involved.

In contrast to the suppressive effects of LL-37 on TREM-1 expression in response to LPS and LTA, LL-37 augmented TREM-1 induction by PGN and also enhanced the production of TNF-α in response to the combined stimulation with PGN and TREM-1 agonist. This suggests that the effects of LL-37 on inflammatory processes will be highly dependent on the specific combinations of TLR ligands and other pattern recognition receptor ligands presented by a particular pathogen. Interestingly, induction of TREM-1 in response to LPS was previously shown to be PI3K-dependent [52], whereas this study showed that TREM-1 induction by PGN is dependent on the NFκB and to a lesser extent the MAPK signaling pathways, but is PI3K-independent. These differences in the signaling mechanisms mediating TREM-1 induction by LPS and PGN may be in part responsible for the distinct effects of LL-37 on TREM-1 induction by these compounds.

The opposite effects of LL-37 on cellular responses to LTA and PGN are surprising, as traditionally TLR2 was considered to be a receptor for both PGN and LTA [66–68]. However, recent studies suggested that PGN is not directly sensed by TLR2, attributing the original findings to lipopeptide contamination [57–59]. Other receptors have also been implicated in PGN recognition. For example, NOD2 binds PGN-fragment muramyl dipeptide [54–56] although it remains unclear whether extracellular PGN can be effectively recognized by cytosolic NLRs [69]. One can speculate that LL-37, which enters cells, affects membrane dynamics, and can assist passenger molecules in cell entry [15, 70–72], may facilitate membrane penetration by extracellular PGN fragments, thus enhancing recognition by intracellular receptors such as NOD2. However, we have shown here that LL-37 does not augment cellular responses to the NOD2 ligand MDP, indicating that at least for smaller ligands like MDP, LL-37 does not facilitate cell entry and access to intracellular receptors. Alternatively, the different effects of LL-37 on PGN and LTA responses may emerge from the complex interactions of TLR/NLR and LL-37 signaling networks, and their full understanding will require systems biology approaches, as recently applied to explore LL-37 signaling [26].

In summary, the work provides further insight into the immunomodulatory activities of LL-37, which include both direct effects on pro-inflammatory cytokine production in response to bacterial compounds and indirect effects mediated via modulation of TREM-1 expression. The lack of LL-37 inhibitory activity on direct responses to TREM-1 stimulation, especially in neutrophils, suggests that LL-37 does not interfere with the positive activities of TREM-1 in the clearance of bacterial infections [48, 49]; however, by blocking LPS and LTA-induced upregulation of TREM-1, LL-37 may disrupt the pro-inflammatory feedback loop responsible for the deleterious effects of TREM-1 in endotoxemia and sepsis [39, 44, 46]. Given the distinct effects of LL-37 on TREM-1 induction by different bacterial compounds, the overall influences of LL-37 on the activities of TREM-1 in the context of antimicrobial immunity will be dependent on the combination of signature molecules presented by a particular pathogen. The effects of LL-37 on the expression and activities of other TREM family receptors and the implication of these effects for the development of antiseptic and anti-infective cathelicidin-derived peptides for therapeutic applications remain to be further explored.

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