RESEARCH ARTICLE

Low concentrations of LL-37 alter IL-8 production by keratinocytes and bronchial epithelial cells in response to proinflammatory stimuli

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Abstract

The immunomodulatory cationic host defence peptide LL-37 plays an important role in epithelial innate immunity; at higher concentrations (20–50 μg mL^{-1}) associated with inflammation, LL-37 elicits the production of cytokines and chemokines. It was demonstrated here that lower, physiologically relevant LL-37 concentrations (2–3 μg mL^{-1}) altered epithelial cell responses to proinflammatory stimuli. In combination with interleukin-1β (IL-1β) and the Toll-like receptor-5 (TLR5) agonist flagellin, these low concentrations of LL-37 synergistically increased IL-8 production by both proliferating and differentiated keratinocytes and by bronchial epithelial cells. In combination with the TLR2/1 agonist PAM3CSK4, LL-37 synergistically induced transcription and the release of both IL-8 and IL-6 from primary bronchial epithelial cells; the IL-8 response was demonstrated to be regulated by epidermal growth factor receptor signalling. Treatment of bronchial epithelial cells with LL-37 and the TLR3 agonist polyI:C resulted in synergistic increases in IL-8 release and cytotoxicity. These data indicate that low concentrations of LL-37 may alter epithelial responses to infecting microorganisms *in vivo*.

Introduction

Host defence peptides play important roles in the defence of the epithelia against a pathogenic challenge. The host defence peptide LL-37 is produced by bronchial epithelial cells, and secreted into the airway surfactant (Bals *et al.*, 1998), and while keratinocytes apparently do not normally express LL-37, elevated levels of the peptide are detectable in both skin and bronchial epithelium as a result of inflammation (Frohm *et al.*, 1997; Conner *et al.*, 2002; Heilborn *et al.*, 2003; Chen *et al.*, 2004; Lopez-Garcia *et al.*, 2006; Schauber *et al.*, 2007). LL-37 has a number of activities in the epithelia: concentrations of 1–5 μg mL^{-1} stimulate the proliferation and migration of keratinocytes (Tokumaru *et al.*, 2005) and bronchial epithelial cells (Shaykhiev *et al.*, 2005), while higher concentrations of > 20 μg mL^{-1} have been shown *in vitro* to elicit the production of proinflammatory cytokines, notably interleukin-6 (IL-6) and IL-8 (Tjabringa *et al.*, 2003; Niyonsaba *et al.*, 2006; Pistolic *et al.*, 2009). These findings suggest a role for LL-37 in the regulation of innate immunity within the epithelial layer, a possibility supported by the observation that mice deficient in the murine LL-37 homologue CRAMP are impaired in their ability to resist cutaneous infection by Group A Streptococci (Nizet *et al.*, 2001).

In nonepithelial cell types, LL-37 treatment can alter cytokine responses to proinflammatory stimuli. For example, the production of tumor necrosis factor-α by peripheral blood mononuclear cells (PBMC) treated with the Toll-like receptor-4 (TLR4) agonist lipopolysaccharide can be significantly reduced by LL-37 treatment (Mookherjee *et al.*, 2006); in contrast, LL-37 synergistically increases IL-6 production by PBMC in response to treatment with IL-1β (Yu *et al.*, 2007). As LL-37 is present in the epithelia during inflammation, we sought to determine whether LL-37 might also alter the responses of epithelial cells to proinflammatory stimuli. In this study, we demonstrate that very low concentrations of LL-37 can significantly increase the production of IL-8 and IL-6 by epithelial cells in response to proinflammatory stimuli, suggesting that LL-37 may play an important role in the initiation and regulation of inflammation in the epithelia.

FEMS IMMUNOLOGY & MEDICAL MICROBIOLOGY

Materials and methods

Cell cultivation

Normal primary adult keratinocytes were obtained from Cascade Biologics (Portland, OR) and maintained in their proprietary Epilife medium with the addition of a human keratinocyte growth supplement that contained bovine pituitary extract, bovine insulin, hydrocortisone, bovine transferrin, and human epidermal growth factor (EGF). Unsupplemented Epilife contained 0.65 μM calcium. The medium was changed every 2 days and cells were passaged before confluence to avoid differentiation. Cultures were used for a maximum of six passages.

Normal primary adult bronchial epithelial cells were obtained from Cambrex BioScience Inc. (Walkersville, MD) and cultivated in their proprietary BEBM basal medium with the addition of 'Singlequot' growth supplements, comprising human EGF, triiodothyronine, bovine pituitary extract, epinephrine, transferrin, insulin, hydrocortisone, gentamicin/amphotericin, and retinoic acid. The cells were maintained for up to six passages. All cells were cultivated in a 37 °C incubator containing 5% CO_2.

Reagents

Human peptide LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIK DFLRNLVPRTES) was synthesized at the Brain Research Centre at the University of British Columbia, using F-Moc chemistry. The synthesized peptide was resuspended in endotoxin-free water (Sigma-Aldrich, Oakville, ON) and stored at − 20 °C until further use. *Salmonella typhimurium* flagellin, polyI:C, and PAM3CSK4 were obtained from InvivoGen (San Diego, CA), while recombinant IL-1β and granulocyte-macrophage colony-stimulating factor (GM-CSF) were purchased from Research Diagnostics (Flanders, NJ). EGF receptor (EGFR) inhibitor AG1478, MEK inhibitor PD98059, and metalloprotease inhibitor GM6001 were purchased from Calbiochem (San Diego, CA).

Cell stimulation

Keratinocytes were seeded into tissue-culture-treated 24-well plates (Corning Inc. Life Sciences, Acton, MA) at a density of 7000 cells cm^{-2} and cultivated in supplemented medium until they attained the desired level of confluence. Subconfluent keratinocytes were used at about 70% confluence, while calcium-differentiated keratinocytes were grown to confluence and cultivated for 2 days in an unsupplemented Epilife medium containing 1.35 mM calcium. Bronchial epithelial cells were seeded into either tissue-culture-treated 24- or 48-well plates at a density of 10 000 cells cm^{-2} and cultivated in supplemented medium until confluence was attained.

Once cells had reached the appropriate level of confluence or had been differentiated, the medium was replaced with fresh unsupplemented medium (1 mL per well). After a 2-h rest, the cells were treated with LL-37 and/or proinflammatory stimuli. Twenty-four hours later, the supernatants were collected. The supernatants were stored at 4 °C until assayed for lactate dehydrogenase (LDH) release, and then frozen until assayed for IL-8/IL-6 concentration. For EGFR inhibitor experiments, AG1478 was diluted in basal BEBM media at 1 μM, and was added to cells for 1 h before the addition of peptide and/or PAM3CSK4. Twenty-four hours later, the supernatants were collected and stored at − 20 °C for subsequent enzyme-linked immunosorbent assays (ELISAs).

RNA isolation and quantitative PCR (qPCR)

Confluent bronchial epithelial cells were stimulated as described previously, and the supernatants were collected at a 4-h time point. Total RNA was isolated using the RNeasy Mini kit (Qiagen, MD), as per the manufacturer's instructions, and was DNAse treated using the RNAse-Free DNAse kit (Qiagen). Once RNA integrity was confirmed by 1% agarose electrophoresis and spectrophotometry, 1 μg of total RNA was converted to cDNA as per the manufacturer's instructions using the SuperScript III Platinum CellsDirect Two-step quantitative real-time PCR (qRT-PCR) kit with SyberGreen (Invitrogen), with a nontemplate negative control for each of the treatments. qRT-PCR was performed using the ABI Prism 7000 system, including a DISSOCIATION CURVE program, at 50 °C for 2 min, 95 °C for 2 min, then 50 cycles at 95 °C for 15 s, and 60 °C for 30 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene control. The PCR was conducted in a 12.5-μL reaction volume containing 2.5 μL of 1/10 diluted cDNA template and 10 μL of a master mix (0.25 μL of Rox, 6.25 μL of UDG, 0.5 μL of 10 μM primer mix, and 3 μL of nuclease-free H_2O per reaction). A melting curve was performed to ensure that any product detected was specific to the desired amplicon. Fold changes were calculated after normalization to endogenous GAPDH using the comparative C_t method (Pfaffl, 2001). The PCR primers used in this study were IL-8 (forward) 5′-GACCACACTGCGCCAACAC-3′, IL-8 (reverse) 5′-CTTCTCCACAACCCTCTGCAC-3′, IL-6 (forward) 5′-AA TTCGGTACATCCTCGACGG-3′, IL-6 (reverse) 5′-GGTTGT TTTCTGCCAGTGCC-3′, GAPDH (forward) 5′-GAAACTGT GGCGTGATGG-3′, and GAPDH (reverse) 5′-TCTAGAGGC ATGCTGACTTC-3′.

Assays

Cytotoxicity was monitored using a Cytotoxicity Detection Kit (Roche, ON), which measures LDH activity in the collected supernatants. Results were normalized using a

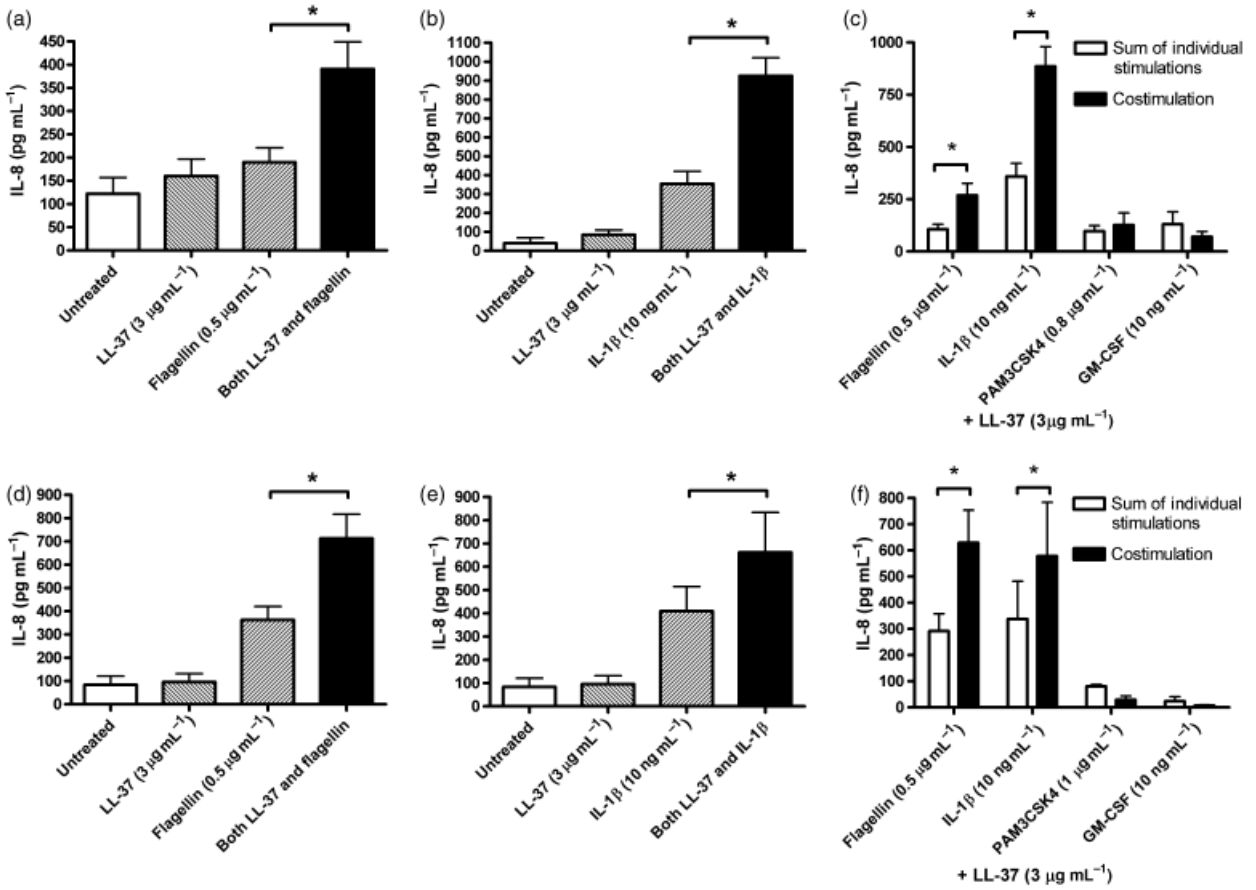

Fig. 1. Alteration of keratinocyte responses by LL-37. LL-37 altered IL-8 production by subconfluent keratinocytes in response to flagellin (a) and IL-1β (b). The increase in IL-8 production in response to flagellin and IL-1β was synergistic; in contrast, LL-37 did not alter IL-8 production in response to PAM3CSK4 or GM-CSF (c). Similarly, LL-37 altered IL-8 production by differentiated keratinocytes in response to flagellin (d) and IL-1β (e). The increase in IL-8 production in response to flagellin and IL-1β was synergistic, but LL-37 did not induce a synergistic increase in IL-8 release in response to PAM3CSK4 or GM-CSF (f). Supernatants were collected at 24 h. Error bars show SEM of at least three independent experiments. Statistical comparisons were performed using a two-tailed Student's *t*-test. A background subtraction was performed on data in (c) and (f). $^*P < 0.05$.

negative control (untreated cells) and a positive control (cells treated with unsupplemented media containing 2% Triton-X) according to the following formula: % Cytotoxicity = (sample − negative control)/(positive control − negative control) × 100.

IL-8 and IL-6 concentrations in the supernatants were assessed by ELISA, as per the manufacturers' instructions (IL-8 – Biosource International, Camarillo, CA; IL-6 – eBioscience, San Diego, CA).

Results and discussion

Low doses of LL-37 altered the responses of keratinocytes to proinflammatory stimuli

Keratinocytes were grown to about 70% confluence and were rested in an unsupplemented medium before treatment with synthetic TLR ligands or endogenous proinflammatory cytokines in the presence or absence of LL-37. Preliminary dose–response assessments were used to select the minimal level of LL-37 showing an effect based on the usually quoted physiological levels of LL-37 (*c.* 2–5 μg mL^{-1}).

Low concentrations of LL-37 (3 μg mL^{-1}) resulted in a statistically significant twofold increase in IL-8 production in response to the TLR5 ligand flagellin (Fig. 1a), and a 2.8-fold increase in IL-8 production in response to IL-1β (Fig. 1b). To ensure that the increased IL-8 production was not simply an additive effect, we compared the sum of the IL-8 response to the individual treatments with the IL-8 production observed when cells received both LL-37 and flagellin or IL-1β; in both cases, IL-8 production was synergistically increased (Fig. 1c). In contrast, LL-37 did not alter IL-8 production in response to the TLR2/TLR1 ligand PAM3CSK4 or to GM-CSF. Keratinocytes pretreated with the inhibitor of IκB phosphorylation Bay11-7085, which

blocks NFκB activation, demonstrated a reduction to baseline in IL-8 production after flagellin/LL-37 costimulation (data not shown).

To determine whether these effects were unique to proliferating cells, we repeated the experiments using confluent keratinocytes that had been terminally differentiated via cultivation in a high-calcium medium. Again, low concentrations of LL-37 altered IL-8 production in response to flagellin (Fig. 1d) and IL-1β (Fig. 1e) by synergistically increasing IL-8 production, but responses to PAM3CSK4 and GM-CSF were not altered (Fig. 1f).

Low-dose LL-37 altered the responses of bronchial epithelial cells to proinflammatory stimuli

We then investigated whether LL-37 could induce similar synergistic effects in bronchial epithelial cells, as these cells consistently encounter LL-37 in the airway surfactant. The presence of both flagellin and LL-37 together resulted in a threefold increase in IL-8 production when compared with the response seen to flagellin alone (Fig. 2a). Similarly, the presence of LL-37 resulted in a fourfold increase in IL-8 production in response to IL-1β (Fig. 2b) and a 2.2-fold increase in IL-8 release in response to PAM3CSK4 (Fig. 2c), stronger responses than those observed in keratinocytes. Costimulation of the cells with LL-37 and either flagellin or IL-1β resulted in a synergistic, rather than an additive, increase in IL-8 production (Fig. 2d).

Dose-dependent ability of LL-37 to synergistically increased IL-8 and IL-6 production by bronchial epithelial cells in response to PAM3CSK4

To test whether synergistic IL-8 induction could still be observed at peptide doses $< 3\,\mu g\,mL^{-1}$, we stimulated bronchial epithelial cells (in basal media) with concentrations of LL-37 ranging from 0 to $3\,\mu g\,mL^{-1}$ in the presence or absence of the TLR2/1 ligand PAM3CSK4 for 24 h. Peptide doses of 2 and $3\,\mu g\,mL^{-1}$ synergistically increased IL-8 release in response to PAM3CSK4 (Fig. 3a). In addition to IL-8, the presence of physiological doses of LL-37 at $3\,\mu g\,mL^{-1}$, together with PAM3CSK4, resulted in a synergistic increase in another important lung response mediator, IL-6 (Fig. 3b).

Transcriptional regulation of IL-8 and IL-6 levels in the presence of LL-37 and PAM3CSK4

To assess whether the synergistic chemokine induction was due to protein release or altered transcriptional regulation, bronchial epithelial cells were either left untreated or

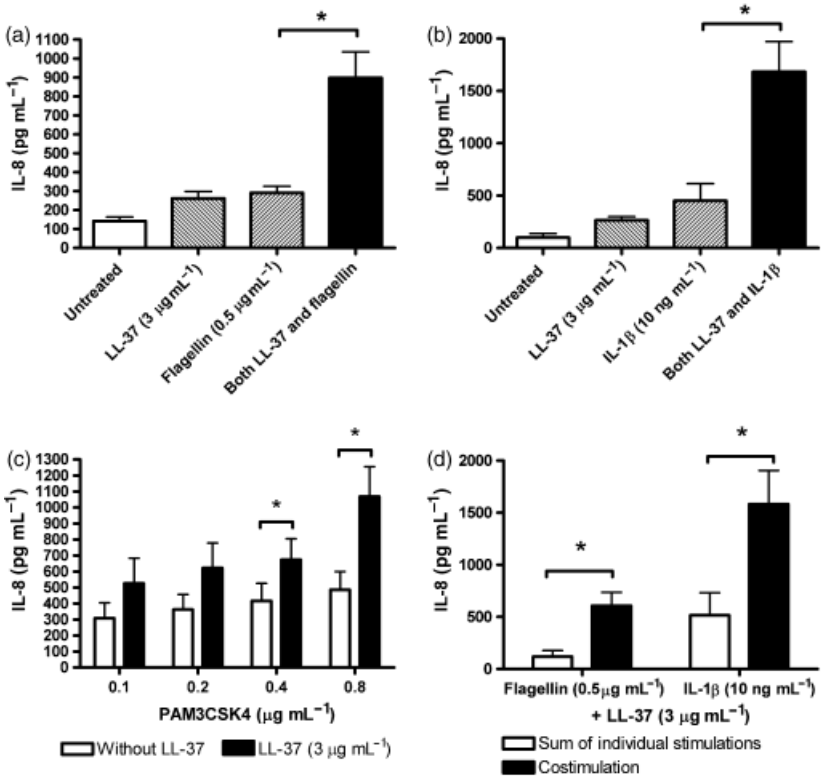

Fig. 2. Alteration of bronchial epithelial responses by LL-37. LL-37 altered IL-8 production by primary normal bronchial epithelial cells in response to flagellin (a), IL-1β (b), and PAM3CSK4 (c). The increase in IL-8 production in response to flagellin and IL-1β was synergistic (d). Supernatants were collected at 24 h and were assayed for IL-8 by ELISA. Error bars show SEM of at least three independent experiments. Statistical comparisons were performed using a two-tailed Student's *t*-test. A background qsubtraction was performed on data in (d). $^*P < 0.05$.

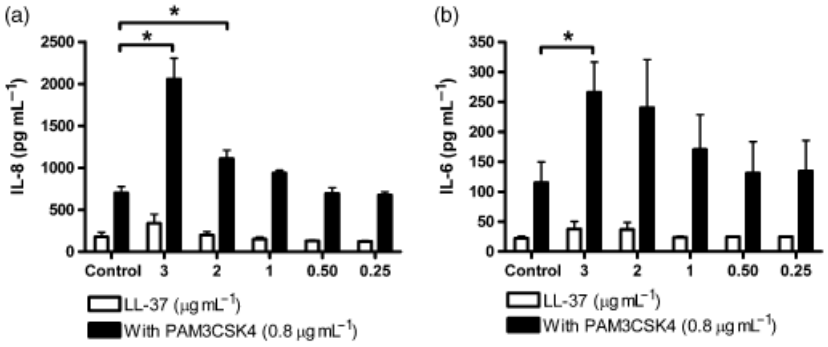

Fig. 3. Dose-dependent synergistic induction of IL-8 and IL-6 in bronchial epithelial cells by LL-37/PAM3CSK4 costimulation. Influence of LL-37 ± PAM3CSK4 on (a) IL-8 release and (b) IL-6 release from bronchial epithelial cells. Confluent bronchial epithelial cells were left untreated or treated with LL-37 at 3, 2, 1, 0.5, and 0.25 ± 0.8 μg mL^{-1} PAM3CSK4 for 24 h. Averages of three biological repeats ± SEM are shown. Statistical comparisons were performed using a two-tailed Student's *t*-test. *$P < 0.05$.

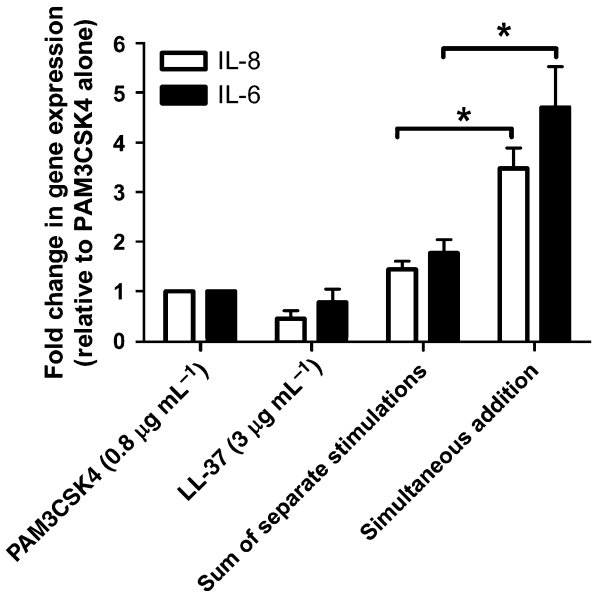

Fig. 4. Synergistic induction of transcription of the genes for IL-8 and IL-6 by LL-37/PAM3CSK4 costimulation. Costimulation of primary normal bronchial epithelial cells with LL-37 and PAM3CSK4 elicited synergistic increases in the transcription of IL-8 and IL-6 4-h poststimulation. Bronchial epithelial cells were either left untreated or treated for 4 h with LL-37 at 3 μg mL^{-1}, PAM3CSK4 at 0.8 μg mL^{-1}, or a combination of LL-37 and PAM3CSK4. Transcriptional effects were demonstrated by qPCR using cDNA generated from isolated total RNA. Average fold changes of three biological repeats relative to the PAM3CSK4 treatment alone ± SEM are shown. To demonstrate the synergistic nature of the response, the response to the combination was compared with the sum of the separate stimulations as generated by adding the fold change in gene expression observed after stimulation with LL-37 alone to that observed after stimulation with PAM3CSK4 alone. Statistical comparisons were performed using a two-tailed Student's *t*-test. *$P < 0.05$.

stimulated with 3 μg mL^{-1} of LL-37, PAM3CSK4 at 0.8 μg mL^{-1}, or a combination of LL-37 and PAM3CSK4; after 4 h, nucleic acid was collected and transcription assessed by qPCR of total RNA isolated from treated samples. As with IL-8 and IL-6 protein release, significant synergistic induction of IL-8 and IL-6 transcripts was observed 4 h after stimulation (Fig. 4).

Influence of inhibition of EGFR on the synergistically increased production of IL-8 by bronchial epithelial cells in response to LL-37 and PAM3CSK4

EGFR regulation in LL-37 stimulation of epithelial cell systems has been described in various processes such as cytokine induction, cell migration, and proliferation (Tjabringa *et al.*, 2003; Shaykhiev *et al.*, 2005; Tokumaru *et al.*, 2005; Pistolic *et al.*, 2009). Therefore, we investigated the effect of the EGFR inhibitor AG1478 on IL-8 production by bronchial epithelial cells in response to costimulation with LL-37 and PAM3CSK4. Confluent bronchial epithelial cells were pretreated with 1 μM AG1478 or dimethyl sulphoxide (vehicle control) for 1 h. Cells were either left untreated or were treated for 24 h with LL-37 at 3 μg mL^{-1}, PAM3CSK4 at 0.8 μg mL^{-1}, or a combination of LL-37 with PAM3CSK. As expected, the EGFR inhibitor significantly reduced IL-8 release from LL-37 and PAM3CSK4 treatments alone, but also significantly reduced the amount of IL-8 in the supernatant of costimulated cells by *c.* 60%, thus reaffirming the importance of EGFR signalling in LL-37-mediated responses in epithelial cells (Fig. 5). A connection between TLR signalling and EGFR activation in lung epithelial cells has been reported previously (Koff *et al.*, 2008), and so it is possible that the synergistic cytokine/chemokine induction occurred by enhancing the EGFR pathway. Furthermore, we studied the signalling regulation of synergistic IL-8 induction using the MEK-ERK inhibitor PD98059 and the metalloprotease inhibitor GM6001; however, significant inhibition of IL-8 transcription was not observed (data not shown). Using the 16HBE14o^{-} human bronchial epithelial cell line, we could demonstrate internalization of LL-37.

LL-37 increased IL-8 production and cell death in bronchial epithelial cells treated with polyI:C

Intriguingly, when we treated bronchial epithelial cells with the TLR3 agonist polyI:C in the presence of LL-37, we observed a dramatic and rapid induction of IL-8 (Fig. 6a), followed by extensive cytotoxicity detected through LDH

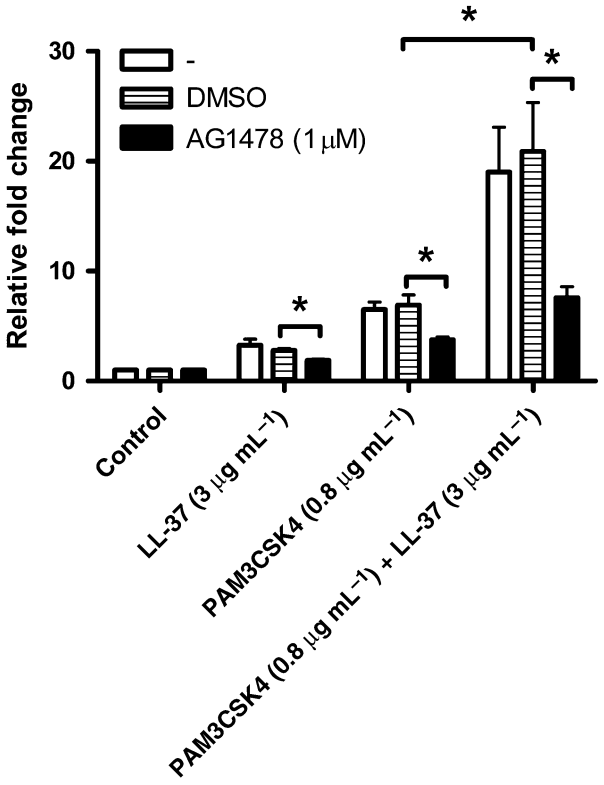

Fig. 5. Effect of the EGFR inhibitor AG1478 on LL-37/PAM3CSK4-stimulated IL-8 production by bronchial epithelial cells. Bronchial epithelial cells were pretreated with 1 μM AG1478 or dimethyl sulphoxide (DMSO) (vehicle control) for 1 h before leaving them untreated or treating cells for 24 h with either LL-37 alone (3 μg mL^{-1}), PAM3CSK4 alone (0.8 μg mL^{-1}), or the combination of LL-37 and PAM3CSK4. Supernatants were collected and assayed for IL-8 by ELISA. Average fold changes of three biological repeats relative to the respective untreated control ± SEM are shown. Statistical comparisons were performed using a two-tailed Student's *t*-test. The EGFR inhibitor AG1478 inhibited LL-37/PAM3CSK4-induced IL-8 release by *c.* 60%. $^*P < 0.05$.

release (Fig. 6b). Although polyI:C alone could elicit both IL-8 production and cytotoxicity, the combination of LL-37 and polyI:C dramatically increased the kinetics of the response, and synergistically increased both IL-8 production (Fig. 6c) and observed cytotoxicity (Fig. 6d). Although we failed to observe substantial cell staining using the TUNEL assay, an attempt was made to assess changes in cell morphology and permeability by a trypan blue exclusion time-course experiment (2, 4, 8, and 24 h). Throughout the time-course experiment, an increase was observed in the frequency of occurrence of morphological cell features such as membrane blebbing and cell fragmentation, which are known (Fink & Cookson, 2005; Labbe & Saleh, 2008) structural features of apoptotic cells, as well as some degree of trypan blue staining, which would indicate cell death by necrosis (data not shown). It appeared that the LL-37/polyI:C costimulation-mediated cell death was a complex phenomenon, which might include both apoptosis and necrosis.

These results indicate a novel role for LL-37 in the initiation and regulation of epithelial inflammation. The ability of such low concentrations of peptide to dramatically alter cytokine production suggests biological relevance when considered in the context of estimated LL-37 concentrations in the skin and lung. While LL-37 is not expressed by normal keratinocytes (Frohm *et al.*, 1997), it is present in sweat at concentrations of around 5 μg mL^{-1} (Murakami *et al.*, 2002); similarly, LL-37 is present in the lung surfactant of neonates at around 5 μg mL^{-1} (Schaller-Bals *et al.*, 2002). At these relatively low concentrations, LL-37 could act to enhance the inflammatory response of epithelial cells during the early stages of infection by increasing cytokine/chemokine production and a concomitant increase in immune cell recruitment in response to microbial antigens and other proinflammatory stimuli. As such, LL-37 could likely aid in a more rapid and efficient infection clearance from epithelial sites.

This increase of cytokines and chemokines in the early stages of infections would boost the innate immune defences. Conversely, a prolonged increase in cytokines can mediate the harmful inflammatory response often seen in diseases such as cystic fibrosis and psoriasis (Arlene *et al.*, 2001; Baker, 2003; Muir *et al.*, 2004; Greene *et al.*, 2005; Büchau & Gallo, 2007). Both keratinocytes and bronchial epithelial cells show increased expression of LL-37 as a consequence of wounding (Heilborn *et al.*, 2003; Schauber *et al.*, 2007) and inflammation (Frohm Nilsson *et al.*, 1999). An increase of the local LL-37 concentration could potentially lead to an even higher cytokine/chemokine release (Tjabringa *et al.*, 2003; Lau *et al.*, 2005; Niyonsaba *et al.*, 2006; Pistolic *et al.*, 2009), which might disrupt the carefully balanced immune response and lead to excessive inflammation during the pathogenesis of some diseases.

It seems possible that the increases in cell death observed after the costimulation of bronchial epithelial cells with LL-37 and the TLR3 ligand polyI:C, which mimics a viral nucleic acid, may represent a defence against viral infection. Accordingly, it would be of great interest to examine this phenomenon in more detail, especially in the context of real viral infections of lung epithelial cells. Such studies might decipher a potential role of LL-37 in the modulation of antiviral host responses.

High concentrations of LL-37 (*c.* 30 μg mL^{-1}) elicit the production of proinflammatory cytokines by epithelial cells (Tjabringa *et al.*, 2003; Niyonsaba *et al.*, 2006; Pistolic *et al.*, 2009). We have shown for the first time that low concentrations of LL-37 (3 μg mL^{-1}) can increase the production of IL-8 by both skin and bronchial cells, and IL-6 by bronchial epithelial cells in combination with exogenous and

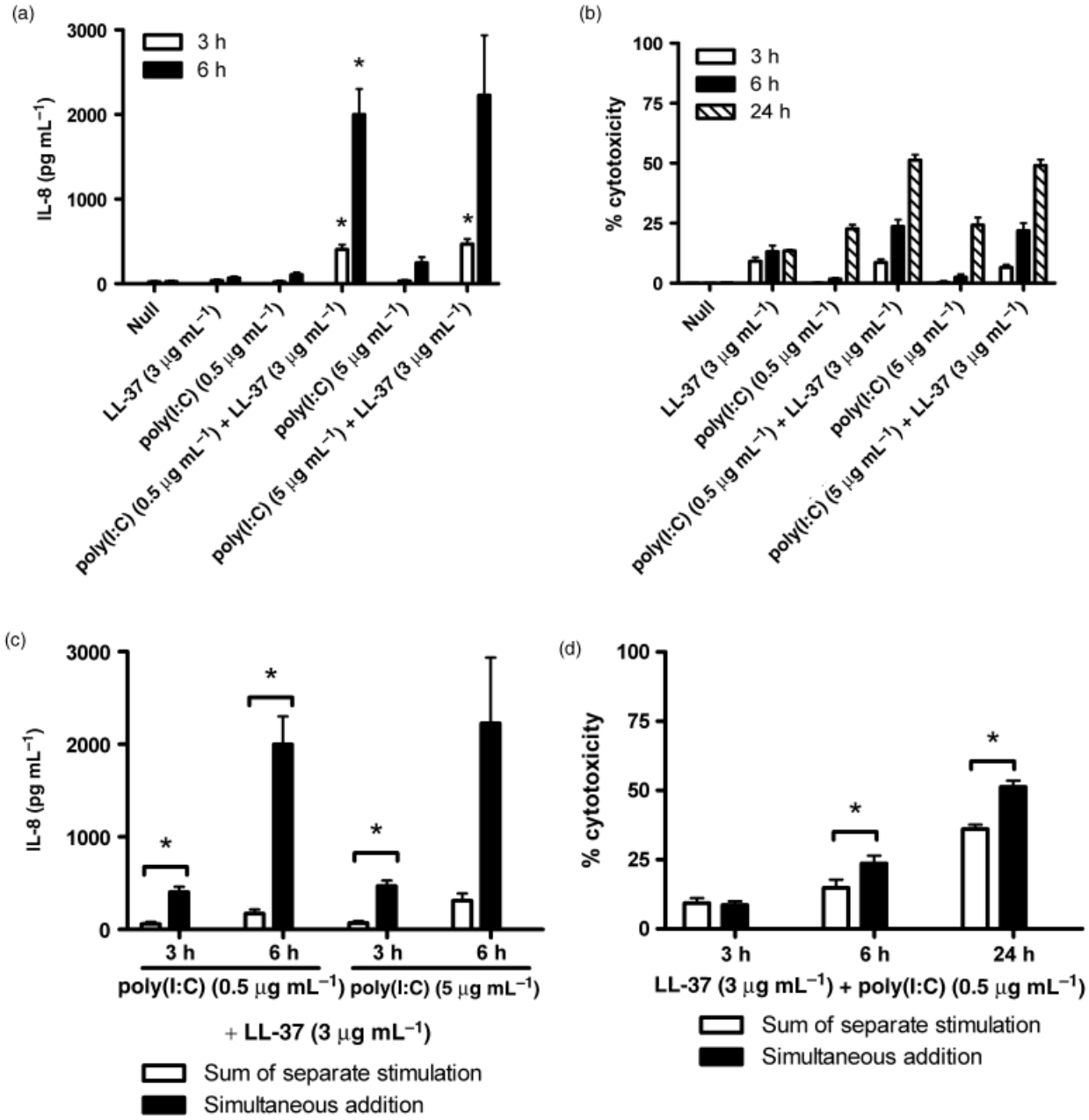

Fig. 6. Influence of LL-37 and polyI:C on IL-8 production and cell viability of bronchial epithelial cells. Costimulation of bronchial epithelial cells with LL-37 and polyI:C induced a rapid IL-8 response (a) and rapid and pronounced cytotoxicity (b). Low doses of LL-37 synergistically increased both IL-8 production (c) and cytotoxicity (d). Error bars show SEM of at least three independent experiments. Statistical comparisons were performed using a two-tailed Student's *t*-test; $^{*}P < 0.05$ when compared with null in (a) and as indicated in (c) and (d). A background subtraction was performed on data in (c) and (d).

endogenous proinflammatory molecules, indicating that LL-37 plays a broader role than previously suspected in the regulation of epithelial inflammation and immune cell recruitment. An improved understanding of the mechanisms underlying this response might facilitate the development of novel anti-infective therapeutics.

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