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Human Host Defense Peptide LL-37 Suppresses TNFα-Mediated Matrix Metalloproteinases MMP9 and MMP13 in Human Bronchial Epithelial Cells

Anthony Altieri[a, b] Courtney Lynn Marshall[a] Padmanie Ramotar[a]
Dylan Lloyd[c] Mahadevappa Hemshekhar[c] Victor Spicer[b]
Anne M. van der Does[d] Neeloffer Mookherjee[a, c]

[a]Department of Immunology, University of Manitoba, Winnipeg, MB, Canada; [b]Department of Immunology, University of Toronto, Toronto, ON, Canada; [c]Manitoba Centre for Proteomics and Systems Biology, Department of Internal Medicine, University of Manitoba, Winnipeg, MB, Canada; [d]PulmoScience Lab, Department of Pulmonology, Leiden University Medical Center, Leiden, The Netherlands

Keywords
Cathelicidin · LL-37 · TNFα · Host defense peptides · Inflammation · Matrix metalloproteinases · Airway remodeling

Abstract
Introduction: TNFα-inducible matrix metalloproteinases play a critical role in the process of airway remodeling in respiratory inflammatory disease including asthma. The cationic host defense peptide LL-37 is elevated in the lungs during airway inflammation. However, the impact of LL-37 on TNFα-driven processes is not well understood. Here, we examined the effect of LL-37 on TNFα-mediated responses in human bronchial epithelial cells (HBECs). ***Methods:*** We used a slow off-rate modified aptamer-based proteomics approach to define the HBEC proteome altered in response to TNFα. Abundance of selected protein candidates and signaling intermediates was examined using immunoassays, ELISA and Western blots, and mRNA abundance was examined by qRT-PCR. ***Results:*** Proteomics analysis revealed that 124 proteins were significantly altered, 12 proteins were enhanced by ≥2-fold compared to unstimulated cells, in response to TNFα. MMP9 was the topmost increased protein in response to TNFα, enhanced by ~10-fold, and MMP13 was increased by ~3-fold, compared to unstimulated cells. Furthermore, we demonstrated that LL-37 significantly suppressed TNFα-mediated MMP9 and MMP13 in HBEC. Mechanistic data revealed that TNFα-mediated MMP9 and MMP13 production is controlled by SRC kinase and that LL-37 enhances related upstream negative regulators, namely, phospho-AKT (T308) and TNFα-mediated TNFAIP3 or A20. ***Conclusions:*** The findings of this study suggest that LL-37 may play a role in intervening in the process of airway remodeling in chronic inflammatory respiratory disease such as asthma.

Introduction

Airway inflammation accompanied by airway remodeling leads to structural changes in the lungs such as narrow and thickened airways resulting in impairment of breathing, which is a characteristic feature of respiratory

karger@karger.com
www.karger.com/jin

Correspondence to:
Neeloffer Mookherjee, neeloffer.mookherjee@umanitoba.ca

inflammatory diseases such as asthma [1]. Although the immunophenotype of airway inflammation associated with airway remodeling in asthma is heterogeneous, the disease process results in increased accumulation of leukocytes such as eosinophils and neutrophils in the lungs, along with elevated levels of various pro-inflammatory cytokines, which includes TNFα [2, 3]. TNFα is elevated in both Th2-high eosinophilic and Th2-low neutrophilic airway inflammation; it can induce the expression of airway remodeling factor matrix metalloproteinase (MMP) 9 [4, 5] and thus is a critical mediator of both airway inflammation and remodeling.

Airway inflammation, especially the Th2-low neutrophilic immunophenotype characterized with neutrophil activation, is also associated with elevated levels of the cationic host defense peptide (CHDP) LL-37 in the lungs [6, 7]. LL-37 is released by neutrophils and NETosis in the lungs during neutrophilic airway inflammation [8]. LL-37, the only human cathelicidin CHDP, is an important effector molecule of innate immunity with a wide range of immunity-related functions including the ability to alter cytokine-mediated inflammation [9, 10]. LL-37 also contributes to wound healing and tissue remodeling [11, 12] and has been reported to decrease total MMP activity [13]. Although there is increased TNFα and LL-37 in the lungs, especially during neutrophilic airway inflammation [8, 14], the impact of LL-37 on TNFα-driven processes including effect on airway tissue remodeling is not well understood. As airway remodeling and the pathogenesis of chronic respiratory disease such as asthma is often driven by responses mediated by the bronchial epithelium [5], we examined the effect of LL-37 at a concentration range detected in the lungs [7, 15, 16], on TNFα-mediated responses in human bronchial epithelial cells (HBECs).

Elevated level of TNFα in the lungs is associated with increased citrullination of lung proteins, which is a post-translational modification wherein the arginine residues are converted to citrulline, mediated by peptidylarginine deiminase enzymes [17]. LL-37 can get citrullinated in the lungs under inflammatory conditions, and both unmodified LL-37 and citrullinated LL-37 (citLL-37) are found in the bronchoalveolar lavage fluid of the lungs [18]. Limited studies examining the effects of citrullination on the biological functions of LL-37 have shown that citrullination impairs antimicrobial and anti-endotoxin functions of the peptide [18–21]. Therefore, in this study we also compared the effect of citLL-37 on TNFα-induced responses, with that mediated by unmodified peptide LL-37, in HBEC.

Here, first we used a proteomics approach to define the HBEC proteome altered in response to TNFα. We showed that MMP9 and MMP13 are the dominant airway remodeling factors enhanced in response to TNFα, which is selectively suppressed by LL-37. Our findings demonstrated that LL-37 enhances the phosphorylation of AKT (T308), a signaling intermediate known to facilitate negative regulation of inflammation [22]. Furthermore, LL-37 and citLL-37 enhanced the expression of TNFα-mediated TNFAIP3 or A20, which is also a negative regulator of AKT signaling and inflammation. Taken together, the findings reported in this study suggest that LL-37 may play a role in intervening in the process of airway remodeling in chronic inflammatory respiratory diseases such as asthma.

Materials and Methods

Reagents

Peptides LL-37 and sLL-37 were manufactured by CPC Scientific (Sunnyvale, CA, USA), and citLL-37 was obtained from Innovagen AB (Lund, Sweden). Each of the peptides were reconstituted in endotoxin-free E-Toxate™ water, aliquoted in glass vials, and stored at −20°C until use. The reconstituted peptides were used within 3 months. The reconstituted peptides were thawed to room temperature, sonicated in a water bath sonicator for 30 s, and vortexed for 15 s before use. Recombinant human TNFα (carrier free) was obtained from R&D Systems (Oakville, ON, CA, USA).

Cell Culture of HBECs

HBEC cell line, HBEC-3KT (ATCC® CRL-4051™), was cultured in airway epithelial cell basal medium (ATCC® PCS-300-030™) supplemented with bronchial epithelial cell growth kit (ATCC® PCS-300-040™), as previously described by us [23–25]. The medium was changed to airway epithelial cell basal medium containing 6 mM L-glutamine without growth factors, 24 h prior to stimulation with TNFα and/or peptides. Human primary bronchial epithelial cells (PBEC) were isolated from macroscopically normal lung tissue obtained from anonymized patients undergoing resection surgery at the Leiden University Medical Center (LUMC), the Netherlands, and enrolled with a no-objection system for coded anonymous further use of such tissue (http://www.coreon.org/), as previously detailed by us [23–25]. Since September 1, 2022, patients are enrolled with written informed consent in accordance with local regulations from the LUMC biobank with approval by the institutional medical Ethical Committee (B20.042/Ab/ab and B20.042/Kb/kb). Briefly, PBECs were expanded and cultured in T75 flasks or tissue culture (TC) plates, respectively, which were pre-coated with coating media (containing 30 μg/mL PureCol [Advanced Biomatrix, CA, USA], 10 μg/mL fibronectin [Sigma], 10 μg/mL BSA [Sigma] in PBS [Gibco]), in supplemented keratinocyte serum-free medium (Gibco) containing 0.2 ng/mL epidermal growth factor (Life Technologies), 25 μg/mL bovine pituitary extract (Gibco), 1 μM isoproterenol (Sigma), and 1:100 dilution of antibiotics penicillin and streptomycin (Lonza), and maintained until ~80% confluent. PBECs were cultured with a 1:1 mixture of supplemented

Dulbecco's Modified Eagle's Medium (Gibco) with a 1:40 dilution of HEPES (Invitrogen) and basal bronchial epithelial cell medium (ScienCell) containing bronchial epithelial cell growth supplement (ScienCell), 1:100 dilution of Penicillin/streptomycin, and 1 nM of a light stable analog of retinoic acid, EC-23 (Tocris, UK). The culture medium was replaced every 48 h, and the medium was replaced with medium without EGF, BPE, BSA, and hydrocortisone (starvation media) 24 h prior to stimulation with cytokine and/or peptides. Air-liquid interface (ALI) cultures were established with PBEC as previously detailed by us [26]. Briefly, 0.4 μm pore size 12-well Transwell inserts (Corning Costar, Cambridge, USA) were coated with coating medium as described above. PBEC obtained after expansion to ~80% confluency in a T75 flask as detailed above was seeded onto the coated inserts at a concentration of 40,000 cells/insert. Cells were grown in PBEC culture medium (as described above on both basal and apical side) with medium changed every 48 h. When fully confluent (typically end of week 1), cells were exposed to air by removing the apical medium. Air-exposed cells were cultured for 4 weeks in ALI-PBEC culture medium with basal medium changed every 48 h to promote epithelial cell differentiation. When cells were fully differentiated, ALI-PBEC medium was replaced with medium without EGF, BPE, BSA, and hydrocortisone (starvation medium) 24 h prior to stimulation with cytokine and/or peptides.

Proteomics: Slow Off-Rate Modified Aptamer (SOMAmer)-Based Proteomics

Cell lysates were obtained from HBEC-3KT cells stimulated with TNFα (20 ng/mL) for 24 h, using M-PER™ lysis buffer (Thermo Fisher Scientific, Burlington, ON, Canada) with HALT protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Total protein concentration was determined in each cell lysate sample with microBCA assay (Thermo Fisher Scientific, MA, USA), according to the manufacturer's instructions. Samples containing 14 μg of total protein each, from five independent experiments, were probed in slow off-rate modified aptamer (SOMAmer®) v.2 protein array for 1,322 protein targets, as previously described by us [24, 25]. Protein abundance for each target on the array was determined as relative fluorescence unit using an Agilent hybridization array scanner, and the log2-transformed relative fluorescence unit values were used for pairwise analysis.

ELISA

HBEC-3KT cells were stimulated with TNFα (20 ng/mL) in the presence and absence of LL-37, citLL-37, or sLL-37 (2.5 μM each) for 24 h. TC supernatants were centrifuged (250 × *g* at room temperature for 5 min) to obtain cell-free supernatants, aliquoted, and stored at −20°C until use. The abundance of MMP9 and MMP13 was examined in the TC supernatants using ELISA kits obtained from R&D Systems, as per the manufacturer's instructions. HBEC-3KT cells were stimulated with LL-37, citLL-37, or sLL-37 (2.5 μM each) for 30 min; cell lysates were obtained using ×1 Cell Lysis Buffer (Cell Signaling Technology) containing protease inhibitor cocktail (New England Biolabs). Cell lysates containing 25 μg total protein per sample was used to examine phosphorylation of SRC kinase using the PathScan® Phospho-Src (Tyr416) Sandwich ELISA Kit (Cell Signaling Technology) according to the manufacturer's instructions.

Western Blots

HBEC-3KT cells were stimulated with LL-37, citLL-37, or sLL-37 (2.5 μM each) for 30 min; cell lysates were obtained using ×1 Cell Lysis Buffer (Cell Signaling Technology) containing protease inhibitor cocktail (New England Biolabs). Total protein concentration for each cell lysate sample was determined using a microBCA protein assay kit (Thermo Fisher Scientific, MA, USA). Cell lysates containing 25 μg total protein per sample were used resolved on 4–12% NuPageTM 10% Bis-Tris gels (Invitrogen) followed by transfer to nitrocellulose membranes (Millipore, MA, USA). Membranes were blocked with Tris-buffered saline (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) containing 3% bovine serum albumin, followed by probing with phospho-site-specific antibodies obtained from Cell Signaling Technologies for phospho-AKT (T308) and phospho-AKT (473), and anti-human β-actin antibody obtained from Millipore (Burlington, MA, USA) for loading control, in Tris-buffered saline containing 2.5% BSA. The membranes were developed using ECL Prime detection system (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions.

Quantitative Real-Time PCR

HBEC-3KT cells were stimulated with TNFα (20 ng/mL) in the presence and absence of LL-37, citLL-37, or sLL-37 (2.5 μM each) for 2 h. Total RNA was isolated using the Qiagen RNAeasy Kit according to the manufacturer's instructions and mRNA expression was analyzed using SuperScript III Platinum Two-Step Quantitative Real-Time PCR with SYBR Green (Invitrogen), according to the manufacturer's instructions using the ABI Prism 7,000 sequence detection system (Applied Biosystems, CA, USA). QuantiTect Primer Assays for TNFAIP3 (GeneGlobe ID QT00041853) and 18S (GeneGlobe ID QT00199367) were obtained from Qiagen. Fold changes were calculated using the comparative ΔΔCt method after normalization with 18S RNA as the reference gene.

Results

Profiling of TNFα-Mediated Alteration of the HBEC Proteome

To identify the proteins altered in response to TNFα in HBECs, HBEC-3KT cells were stimulated with TNFα (20 ng/mL) for 24 h, and cell lysates (14 μg total protein per sample) from five independent experiments ($n = 5$) were individually probed using an aptamer-based proteomic array. The concentration of TNFα and the time point selected were based on our previous studies [23–25]. Pairwise differential analysis performed on normalized log2 protein expression values identified 124 proteins that were significantly altered in response to TNFα compared to unstimulated cells (Fig. 1). The abundance of 62 proteins was significantly increased, of which 12 proteins were significantly enhanced by ≥2-fold compared to unstimulated cells (online suppl. Table 1; for all online suppl. material, see https://doi.

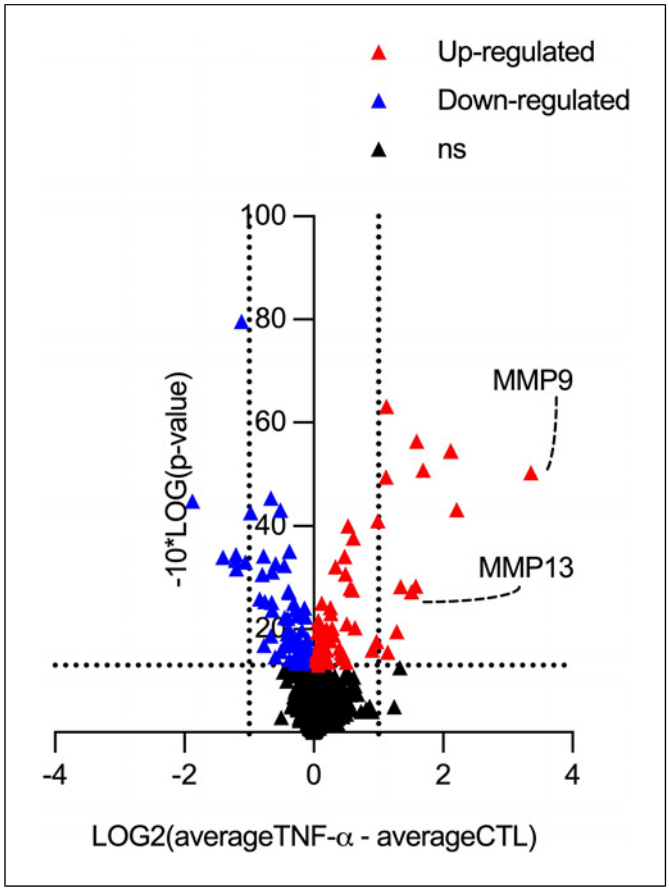

Fig. 1. Alteration of the bronchial epithelial cell proteome by TNFα. HBEC-3KT cells were stimulated with TNFα (20 ng/mL) for 24 h. Cell lysates (14 μg total protein per sample) obtained from five independent experiments were probed using the high-content aptamer-based proteomic array. Pairwise differential analysis was conducted on normalized log2 protein expression values, and Welch's *t* test with a cut-off of $p < 0.05$ was used to select protein abundance changes that were significantly altered in response to TNFα, compared to unstimulated cells. The volcano plot demonstrates differentially abundant proteins in response to TNFα compared to unstimulated cells.

org/10.1159/000537775). Five out of these 12 proteins were associated with extracellular matrix (ECM), MMP9 and MMP13, which are prominent enzymes that remodel the ECM, and three targets of MMPs, i.e., PLAU, PLAUR, and TNC [27]. The protein with the highest increase in abundance was MMP9, ~10-fold increase compared to unstimulated cells (Fig. 1). MMP9 is an airway remodeling factor associated with neutrophilic airway inflammation [28]. In addition, the airway remodeling factor MMP13, which is associated with pulmonary fibrosis [29], was also among the top 12 significantly increased proteins (online suppl. Table 1), with ~3-fold increase following TNFα stimulation compared to unstimulated cells (Fig. 1).

TNFα Significantly Increases the Abundance of Secreted MMP9 and MMP13 from HBECs Including in Differentiated ALI Cultures

As the proteomics study demonstrated significant increase in MMP9 and MMP13 levels in response to TNFα in cell lysates, subsequent independent studies were performed to examine the abundance of these MMPs secreted in cell culture supernatants, using both HBEC-3KT cell line and human PBECs. HBEC-3KT was stimulated with TNFα (20 ng/mL) or with IFNγ (30 ng/mL) as a paired negative control, and the abundance of MMP9 and MMP13 was monitored by ELISA in TC supernatants after 24 h. TNFα stimulation significantly enhanced the abundance of MMP9 and MMP13 in the TC supernatants, compared to unstimulated cells after 24 h (Fig. 2a). In contrast, stimulation with IFNγ did not increase the abundance of either MMP9 or MMP13 compared to unstimulated cells, thus serving as an appropriate negative control (Fig. 2a).

TNFα-mediated increase in secreted MMPs was also confirmed in primary cells using human primary PBEC, in both submerged and in physiologically representative, well-differentiated ALI cultures. Human PBECs in submerged cultures were stimulated with TNFα (20 ng/mL) or IFNγ (30 ng/mL), and TC supernatants were examined by ELISA for the abundance of MMP9 and MMP13, after 24 h. In submerged PBEC cultures, TNFα significantly enhanced MMP9 and MMP13 abundance in TC supernatant after 24 h (Fig. 2b). As the concentrations of cytokines used were optimized in submerged cultures as detailed above, differentiated ALI-PBEC in cultures was stimulated with a range of concentration of TNFα (10–40 ng/mL) or IFNγ (15–60 ng/mL). Cell culture medium from the basal side of the differentiated ALI-PBEC cultures was examined for the abundance of MMP9 and MMP13 by ELISA, after 24 h. Stimulation with TNFα (at 10, 20, and 40 ng/mL) significantly enhanced MMP9 and MMP13 abundance in ALI-PBEC cultures, but not with IFNγ, compared to unstimulated cells (Fig. 2c).

Taken together, these results demonstrate that TNFα significantly increases MMP9 and MMP13 secreted from HBECs, in submerged and ALI-PBEC cultures. As the results in HBEC-3KT cell line and human PBECs (submerged and ALI cultures) were similar (Fig. 2), the HBEC-3KT cell line was used for further experiments.

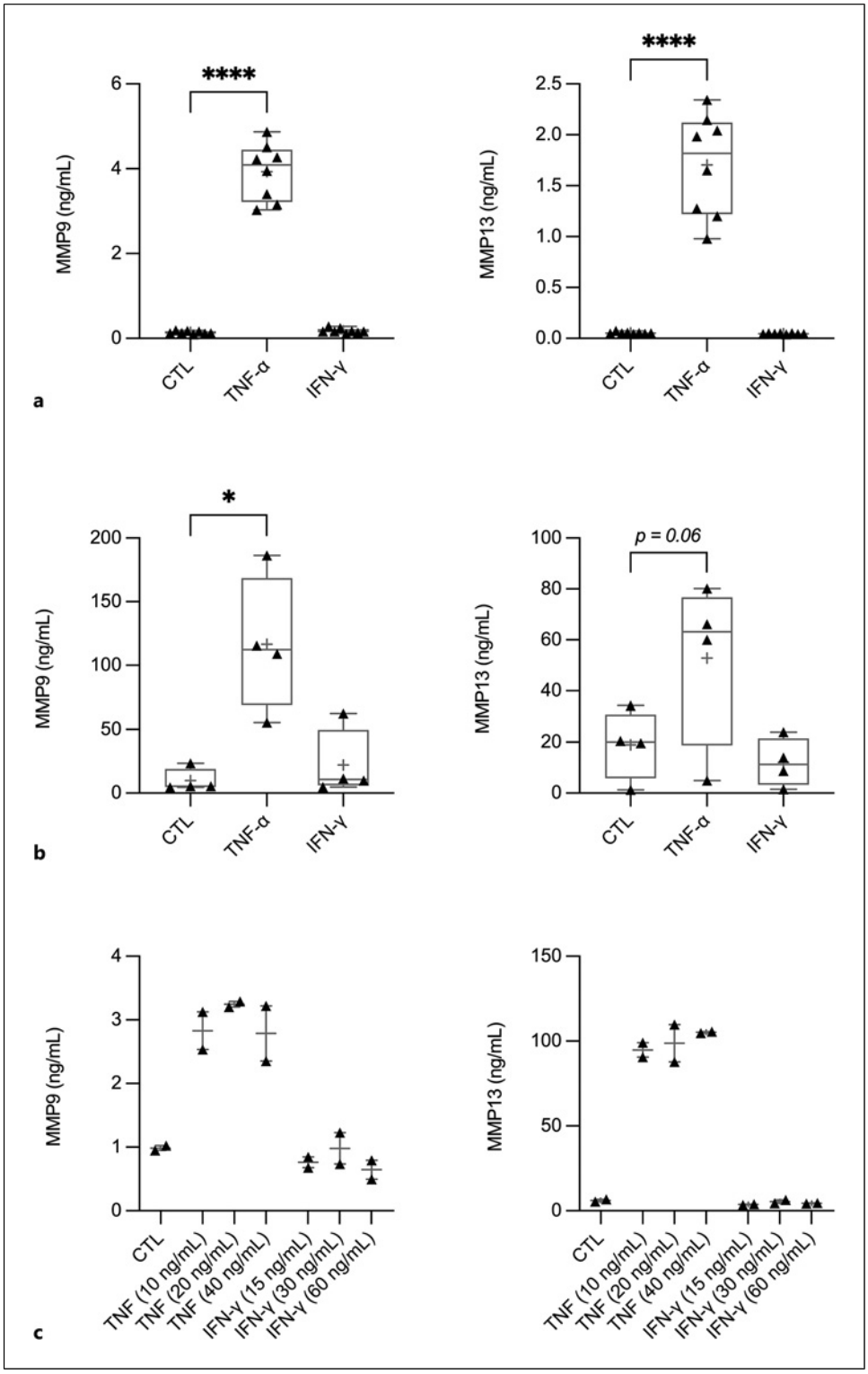

Fig. 2. TNFα induces secretion of MMP9 and MMP13 from HBEC cultures. HBEC-3KT cells (**a**) or submerged human primary bronchial epithelial cells (PBECs) (**b**) were stimulated with either TNFα (20 ng/mL) or IFNγ (30 ng/mL) for 24 h. Cell culture supernatants were examined for the abundance of MMP9 and MMP13 by ELISA. Each symbol represents an independent experiment, using cells from independent donors for PBEC, and bars show the median and min-max range. One-way ANOVA with Fisher's least significant difference test was used for statistical analysis (*p < 0.05, ****p < 0.0001). **c** PBEC was differentiated at the air-liquid interface (ALI) and stimulated with either TNFα (10–40 ng/mL) or IFNγ (15–60 ng/mL) for 24 h, after which cell culture medium from the basal side was examined for the abundance of MMP9 and MMP13 by ELISA. Each symbol represents an independent experiment using cells from an independent donor and bars show the median and min-max range.

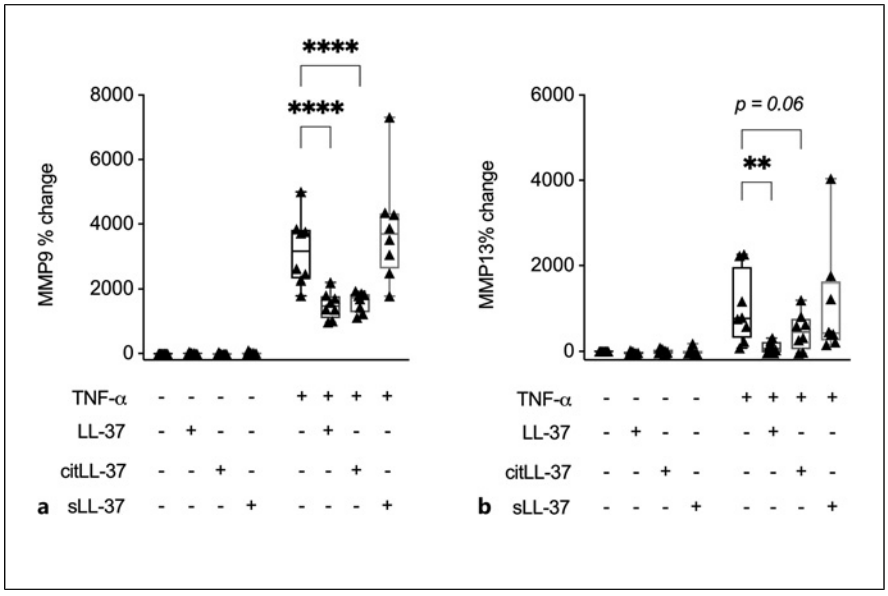

Fig. 3. LL-37 and citLL-37 suppress TNFα-mediated MMP9 and MMP13 production. HBEC were stimulated with LL-37, citLL-37, or sLL-37 (0.25 μm each) in the presence/absence of TNFα (20 ng/mL) for 24 h. TC supernatants were examined for the abundance of MMP9 and MMP13 by ELISA in HBEC-3KT (N = 5 independent experiments) (**a**) and human PBEC (independent experiments with N = 3 independent donors with n = 2 technical replicates each) (**b**). Y-axis represents % change compared to paired unstimulated cells within each independent experimental replicate. Each dot represents an independent experiment, and bars show the median and IQR, whereas whiskers show min-max range. Two-way analysis of variance with Fisher's least significant difference test was used for statistical analysis (**$p \leq 0.001$, ****$p \leq 0.0001$).

LL-37 and citLL-37 Suppress TNFα-Mediated Production of MMP9 and MMP13

We examined the effect of LL-37 and citLL-37 on TNFα-mediated MMP9 and MMP13 production. A scrambled peptide (sLL-37) was used as a paired control. The abundance of LL-37 detected in the lungs is between ~0.25 and 6 μM [7, 15, 16]. Therefore, HBEC-3KT cells were stimulated with LL-37, citLL-37, or sLL-37, using a concentration range of 0.25–5 μM of each peptide. Abundance of lactate dehydrogenase, a marker of cytotoxicity, was examined in the TC supernatants after 24 h. LL-37 at 0.25 or 0.5 μM concentrations was not significantly cytotoxic to the cells (online suppl. Fig. 1). Based on these results, HBEC-3KT cells were stimulated with LL-37, citLL-37, or sLL-37 (0.25 μM or 0.5 μM of each peptide), in the presence or absence of TNFα (20 ng/mL), to examine the effect of the peptides on MMP9 and MMP13 production. The abundance of MMP9 and MMP13 was measured by ELISA in TC supernatant. Both LL-37 and citLL-37 (0.25 μM) significantly suppressed TNFα-mediated MMP9 production by ~50%, 24 h post-stimulation (Fig. 3a). LL-37 significantly suppressed TNFα-mediated MMP13 production by >90%, while citLL-37 suppressed TNFα-mediated MMP13 by ~50% (p = 0.06), 24 h post-stimulation (Fig. 3b). Similarly, 0.5 μM of LL-37 suppressed TNFα-mediated MMP9 and MMP13 by ~50% and >90%, respectively, 24 h post-stimulation (online suppl. Fig. 2). Although 0.5 μM citLL-37 also reduced TNFα-mediated MMP9 and MMP13 (online suppl. Fig. 2), the effect was not as robust as that seen with 0.25 μM of the peptide (Fig. 3). Furthermore, the ability of these peptides to suppress TNFα-mediated MMP9 and MMP13 abundance was observed at earlier time points, 6 and 12 h post-stimulation (online suppl. Fig. 3). However, the abundance of the MMPs and ability of the peptides to suppress TNFα-mediated MMP production was more robust 24 h post-stimulation (Fig. 3). Overall, these results indicated that both LL-37 and citLL-37 can suppress TNFα-mediated MMP9, with no significant difference between the citrullinated and non-citrullinated forms of the peptide. However, there are quantitative differences in the effect of LL-37 and citLL-37 on suppression of TNFα-mediated MMP13, wherein LL-37 abrogates MMP13 abundance in TC supernatants, whereas citLL-37 decreases MMP13 by ~50%.

TNFα-Mediated Enhancement of MMP9 and MMP13 Involves SRC Family Kinase Activity in HBECs

Previous studies have demonstrated that SRC kinase signaling pathway regulates the production of MMP9 in airway epithelial cells and in alveolar epithelial cells [26, 27, 30, 31]. In addition, production of MMP13 was demonstrated to be controlled by SRC family kinase in breast cancer cells [32]. Therefore, we examined the involvement of SRC kinase signaling pathway in TNFα-mediated MMP9 and MMP13 production in HBECs, using specific pharmacological inhibitors. HBEC-3KT cells were pre-treated with pharmacological inhibitors specific to SRC (SRCi), SRC1i, and dasatinib, at various concentrations, for 1 h prior to treatment with TNFα (20 ng/mL). SRCi concentrations were selected based on previous studies [30]. TNFα-mediated abundance of both

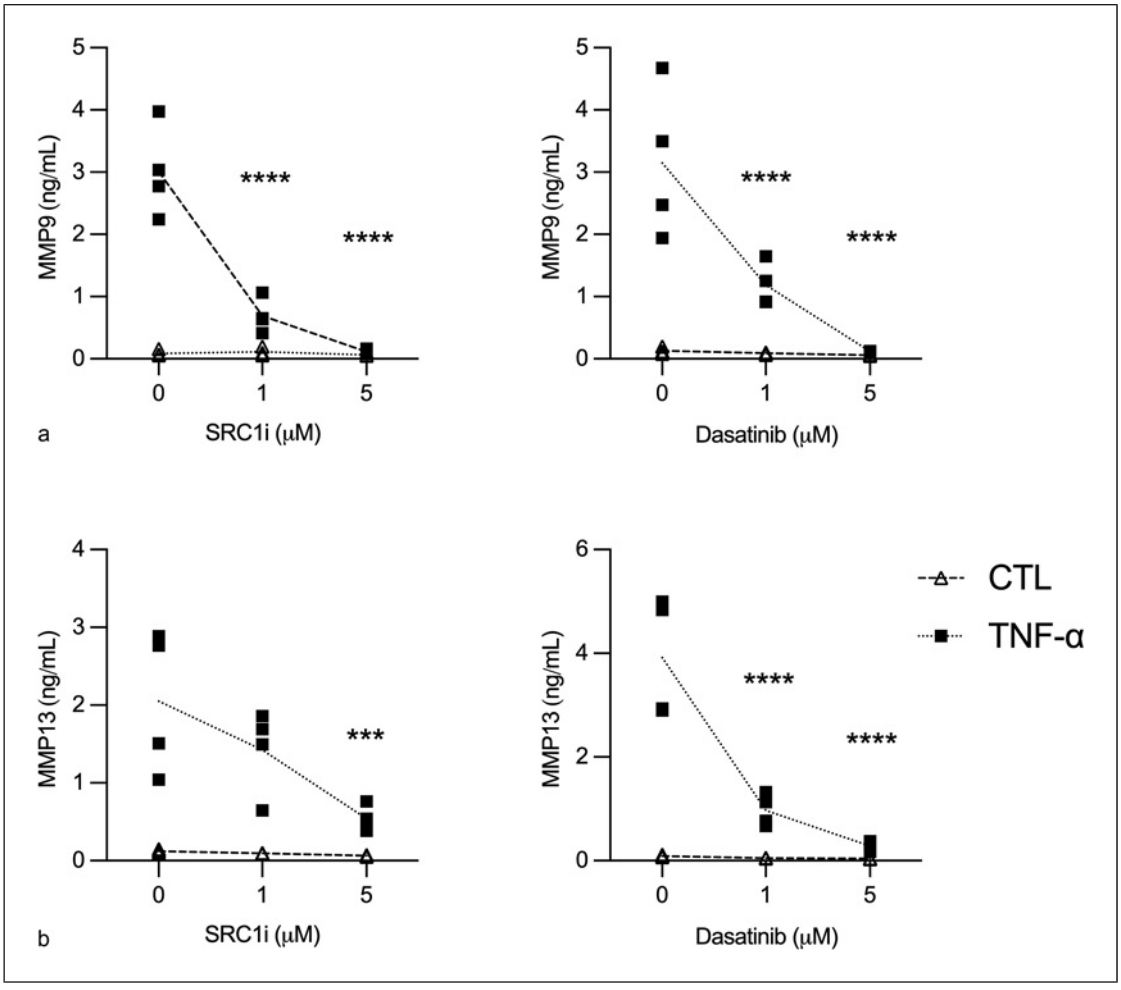

Fig. 4. SRC kinase inhibitors suppress TNFα-mediated MMP9 and MMP13 production. HBEC-3KT cells were pre-treated with pharmacological inhibitors SRC1 inhibitor (SRCi) and dasatinib for 1 h prior to stimulation with TNFα (20 ng/mL). TC supernatants were collected 24 h after TNFα stimulation and examined for the abundance of MMP9 (**a**) and MMP13 (**b**), by ELISA. Each data point represents an independent replicate ($N = 4$ independent experiments), and the line represents the average values. Two-way ANOVA with Dunnett's test for multiple comparisons was used to determine statistical significance (***$p < 0.001$, ****$p < 0.0001$).

MMP9 and MMP13 was suppressed by the inhibitors in a concentration-dependent manner, after 24 h. SRCi and dasatinib (1 μM each) significantly suppressed the production of TNFα-mediated MMP9 and MMP13 by ~50%, and 5 μM concentration of the inhibitors abrogated TNFα-mediated MMP9 and MMP13 production (Fig. 4a, b, respectively).

LL-37 and citLL-37 Modulate AKT Phosphorylation in Bronchial Epithelial Cells

We have previously shown that the immunomodulatory activity of LL-37 suppressing TNFα production involves suppression of Fyn (Y420) SRC kinase signaling, while increasing the phosphorylation of AKT (T308), in human blood-derived cells [33]. Maximal AKT signal transduction requires phosphorylation at both sites T308 and S473 simultaneously, resulting in promotion of inflammation. However, phosphorylation of AKT at site T308, but not S473, results in negative regulation of the AKT pathway with anti-inflammatory effects [22]. Phosphorylation of AKT (T308) is also controlled by SRC family kinases [34]. As we have shown that TNFα-mediated MMP9 and MMP13 production is dependent on SRC kinase signaling pathway (Fig. 4), we examined the effect of the peptides LL-37, citLL-37, and sLL-37 on phosphorylation of SRC, AKT (T308), and AKT (S473). HBEC-3KT cells were stimulated with 0.25 μM of either LL-37, citLL-37, or sLL-37, and phosphorylation of

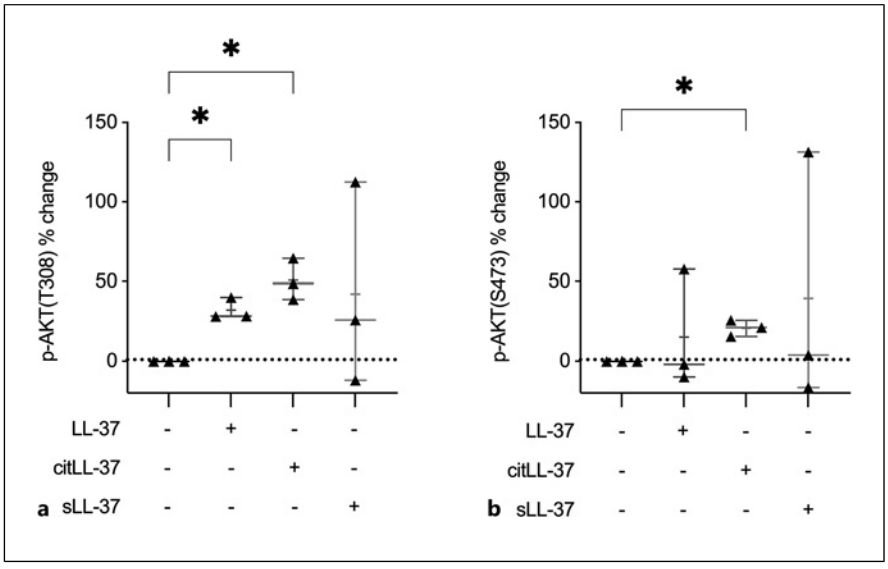

Fig. 5. LL-37 and citLL-37 differentially alter AKT phosphorylation in bronchial epithelial cells. HBEC-3KT (N = 3 independent experiments) was stimulated with LL-37, citLL-37, or sLL-37 (0.25 μM each). Total cell lysate (25 μg) obtained 30 min post-stimulation was used to examine the abundance of phospho-AKT (T308) (**a**) and phospho-AKT (S473) (**b**) by Western blots. Densitometry was performed after normalization with β-actin as a paired loading control for each sample. Y-axis represents % change compared to paired unstimulated controls. Each symbol represents densitometry assessment from an independent experiment, and bars show the median and min-max range. Repeated-measures one-way ANOVA with Fisher's least significant difference test was used for statistical analysis (*$p \leq 0.05$).

signaling intermediates SRC, AKT (T308), and AKT (S473) was examined using specific antibodies by Western blot, after 30 min. There was no significant change on phosphorylation of SRC by the peptides after 30 min (online suppl. Fig. 4). LL-37 significantly enhanced the abundance of phospho-AKT (T308) by ~32% and citLL-37 enhanced it by ~50%, compared to unstimulated cells (Fig. 5a). In contrast, LL-37 did not alter the phosphorylation of AKT (S473); however, there was modest increase of phospho-AKT (S473) by citLL-37, compared to unstimulated cells (Fig. 5b). These results indicated that LL-37 enhances the phosphorylation of signaling intermediate AKT (T308) but does not alter phospho-AKT (S473), which may be limiting the maximal activation of AKT, thus resulting in an overall anti-inflammatory effect. Although citLL-37 also enhances phospho-AKT (T308), there is a modest increase of ~20% in phospho-AKT (S473), which may be aligned with the quantitative difference in the ability of citLL-37 to limit TNFα-mediated MMP13 compared to LL-37.

LL-37 Enhances mRNA Expression of TNFα-Induced Protein 3 in HBECs

The protein A20 or TNFα-induced protein 3 (TNFAIP3) is a negative regulator of inflammation [35] and negatively regulates maximal AKT activation [36]. It was previously shown that LL-37 enhances the expression of TNFAIP3 to limit endotoxin-induced inflammation in blood-derived monocytic cells and fibroblasts [37, 38]. Therefore, we examined the mRNA abundance of TNFAIP3 in this study. HBEC-3KT cells were stimulated with 0.25 μM of either LL-37, citLL-37, or sLL-37, in the presence and absence of TNFα (20 ng/mL), and mRNA abundance of TNFAIP3 was examined by qRT-PCR. LL-37 significantly enhanced the mRNA abundance of TNFAIP3 in the presence of TNFα, compared to that with TNFα alone (Fig. 6).

Discussion

In this study, we demonstrated that airway remodeling factors, namely, metalloproteinases MMP9 and MMP13, and targets of MMPs associated with the ECM, were prominently enhanced in response to TNFα in HBEC proteome. This is corroborated by studies demonstrating that TNFα facilitates the biological process leading to airway remodeling in respiratory disease such as asthma, the pathophysiology of which is associated with increased activity of several MMPs [39–42]. Patients with severe asthma and COPD have increased levels and activity of MMP9 in their sputum environment [43, 44]. Similarly, levels of MMP13 are positively correlated with markers of neutrophilic airway inflammation, which is associated with the pathogenesis of severe asthma [42]. Both MMP9 and MMP13 lead to remodeling of ECM and are associated with the development of lung diseases characterized with airway remodeling [27–29, 45]. In this study, we showed that the human CHDP LL-37, at concentrations detected in the lungs, suppresses TNFα-mediated MMP9 and MMP13 production in HBEC. Limited studies have examined the effect of CHDPs on MMP production and/or activity in epithelial cells. The effect of LL-37 on MMP production remains confounding. Some studies demonstrate

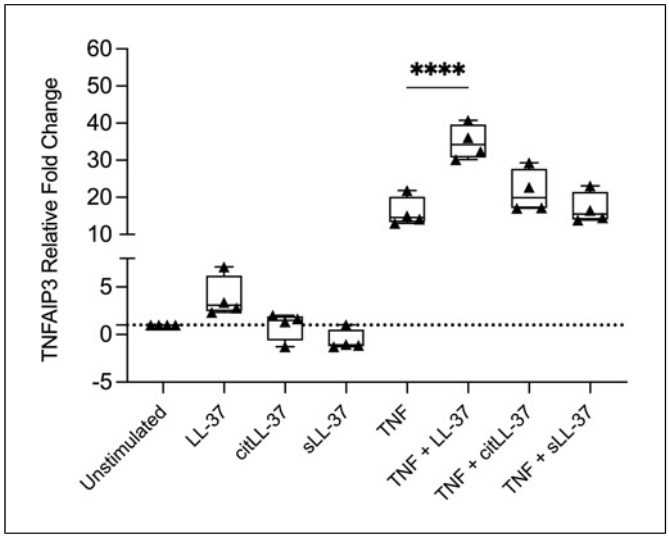

Fig. 6. LL-37 enhances TNFα-mediated negative regulator TNFAIP3 or A20 in bronchial epithelial cells. HBEC-3KT ($N = 4$ independent experiments) was stimulated with LL-37, citLL-37, or sLL-37 (0.25 μM each), in the presence and absence of TNFα (20 ng/mL). mRNA isolated 2 h post-stimulation was used to examine the expression of TNFAIP3 by qRT-PCR. Each symbol represents an independent experiment. Fold changes (*Y*-axis) for TNFAIP3 were normalized to 18S RNA and calculated compared to unstimulated cells normalized to 1, using the comparative ΔΔCt method. Results are shown as boxplots, wherein bars show median and IQR, and whiskers show minimum and maximum values. One-way ANOVA was used for statistical analysis (***$p \leq 0.001$). The dashed line represents normalized baseline value of 1 for unstimulated cells.

that LL-37 can increase MMP production [46]. In contrast, other studies show a negative correlation between LL-37 and MMP abundance. For example, a previous study showed an association of vitamin D-mediated increase in LL-37 along with decrease in TNFα and MMP9, in human corneal epithelial cells [47]. LL-37 was also demonstrated to decrease the combined enzymatic activity of MMPs and increase the negative regulator TIMP-1 in gingival fibroblasts [13] and suppress MMP1 secretion in chondrocytes [48]. To our knowledge, this is the first study to demonstrate that LL-37 can significantly suppress MMP production, namely MMP9 and MMP13, in HBEC.

Our results suggest that LL-37 may have the potential to limit TNFα-mediated airway remodeling and protect against detrimental airway structural changes, which is associated with chronic respiratory disease such as asthma or COPD. This is corroborated by a recent study demonstrating that LL-37 can counter the detrimental effect of cigarette smoke on epithelial barrier dysfunction [49]. However, it is possible that under certain inflammatory conditions regulation of MMP production by cathelicidin peptides such as LL-37 is compromised. We have previously shown in an allergen house dust mite-challenged murine model of acute airway inflammation that the abundance of mouse cathelicidin CRAMP (ortholog of LL-37) is decreased in the lungs [50], along with an increase in the abundance of TNFα and enhanced airway hyperresponsiveness [23, 51, 52]. Thus, it is likely that decrease in cathelicidin peptide abundance in the lungs may be one of the mechanisms for the loss of regulation of MMP production, subsequently leading to airway remodeling and hyperresponsiveness. This is corroborated by our previous study wherein exogenous administration of a cathelicidin-derived peptide significantly decreased airway inflammation and improved airway hyperresponsiveness in the house dust mite-challenged murine model of acute airway inflammation [23]. Taken together, these studies indicate that cathelicidin peptides may play a protective role in controlling biological processes that lead to airway remodeling and hyperresponsiveness. Therefore, examining the use of cathelicidin-derived peptides as interventions to control airway remodeling and its effect on lung function warrants further investigation.

A nuance to consider within the pulmonary inflammatory milieu is that arginine residues of peptides can get post-translationally modified to citrulline by peptidylarginine deiminase enzymes [19]. In vitro and in vivo studies have demonstrated that one to all five arginine residues in LL-37 can get modified to citrulline by PADI2 and PADI4 enzymes under inflammatory conditions [18–20]. Some studies have shown that citrullination of LL-37 decreases its antiendotoxin and antimicrobial functions [18, 19, 21]. These studies suggest that citrullination of LL-37 limits the binding capacity of the peptide to negatively charged bacterial components such as LPS, thus mitigating the peptide's antiendotoxin and direct antimicrobial functions [18]. Thus, certain LL-37-mediated immunity-related functions such as modulating cytokine-driven responses [10, 33], which is not dependent on charge-based interactions, may not be mitigated by citrullination of the peptide. This is corroborated by the results of this study demonstrating that citrullination of LL-37 does not mitigate the ability of the peptide to suppress TNFα-mediated MMP9 or MMP13 production in bronchial epithelial cells. Here, we show that citLL-37, where all five arginine residues are modified to citrulline, also suppresses TNFα-induced MMP9 production, like that mediated by the unmodified peptide. In addition, our results demonstrate both LL-37 and

citLL-37 suppressed TNFα-mediated MMP13 production, albeit with quantitative differences. These results indicate that citrullination does not mitigate all immunomodulatory functions of LL-37 and provide the rationale to further comprehensively examine how citrullination changes the immunomodulatory functions of LL-37, specifically in the context of inflammation and airway remodeling.

Mechanisms related to the anti-inflammatory functions of LL-37 engage several prominent innate immune signaling pathways such as NF-κB, SRC family kinases, PI3K and/or AKT pathways, in different cell types [33, 36, 37, 53]. SRC family kinases and PI3K/AKT signaling pathways are interconnected and are critical regulators of airway remodeling [30, 39, 54]. In this study, we showed that TNFα-mediated MMP9 and MMP13 production is dependent on SRC signaling in HBEC. It is known that SRC signaling is upstream of AKT phosphorylation, and phospho-AKT (T308) is regulated by SRC signaling [44]. Here, we demonstrated that LL-37 and citLL-37 selectively enhance phospho-AKT (T308), which is a negative regulator of inflammation. Maximal AKT signaling with phosphorylation of both AKT sites at T308 and S473 simultaneously promotes pro-inflammatory responses [22]. In contrast, phosphorylation of AKT only at site T308, but not S473, results in negative regulation of AKT signaling and control of inflammation [22]. Our results demonstrate that LL-37 significantly enhances the abundance of phospho-AKT (T308), but not phospho-AKT (S473), suggesting that LL-37 can negatively regulate AKT signaling. Abundance of phospho-AKT (T308) was also enhanced by citLL-37; however, the citrullinated peptide also modestly enhanced the abundance of phospho-AKT (S473). This disparate regulation of AKT phosphorylation by LL-37 and citLL-37 may explain the differences in the reduction of MMP13 production between the two peptides. Our results showed LL-37-mediated suppression of MMP13 was >30% compared to that mediated by citLL-37. Nonetheless, the results of this study suggest that a possible mechanism related to LL-37-mediated negative regulation of TNFα-mediated MMPs may be via the negative regulation of the AKT pathway. This is also corroborated by our second line of investigation, wherein we demonstrated that both LL-37 and citLL-37 significantly enhances TNFα-mediated expression of TNFAIP3 or A20 in HBEC. A recent study has shown that A20 inhibits AKT signaling in hepatic cells [36]. We have previously shown that mechanisms related to the antiendotoxin functions of LL-37 involve enhanced expression of TNFAIP3/A20 in monocytic cells [37]. Here, we show that LL-37 enhances mRNA abundance of TNFAIP3/A20 in the presence of TNFα compared to that with TNFα alone, in bronchial epithelial cells. This indicates that the peptide can enhance a negative regulator of TNFα signaling under inflammatory conditions. Overall, the findings in this study suggest that the mechanisms related to the ability of LL-37 and citLL-37 to limit TNFα-induced MMPs and airway remodeling may be mediated by the enhancement of A20 and the negative regulation of AKT signaling.

A limitation of this study is that the mechanistic data provided for the function of LL-37 in the control of TNFα-mediated MMP production are indirect. As the signaling intermediates detailed in this study also play a role in TNFα-mediated responses, it is challenging to use approaches such as knockdown studies to directly demonstrate mitigation of the beneficial function of LL-37 in epithelial cells. Nevertheless, we demonstrate that the peptides can enhance the abundance of two negative regulators of the AKT pathway, phospho-AKT (T308) and A20, which are known to control inflammatory responses. Furthermore, it remains to be resolved if the regulatory function of LL-37 in inhibiting TNFα-mediated MMP is at the transcriptional level or post-transcriptional level. However, we have previously demonstrated that LL-37 enhances TNFAIP3 (A20) mRNA expression in macrophages [37]. Thus, it is likely that the effect of the peptide in enhancing this negative regulator of TNFα in epithelial cells is also at the transcriptional level. Another limitation of this study is the number of time points examined. It is likely that the kinetics of phosphorylation within the AKT pathway and enhancement of the negative regulator A20 are differentially modulated by LL-37 and citLL-37, which warrants further investigation.

In summary, this is the first study to demonstrate that the human cathelicidin peptide LL-37 suppresses TNFα-mediated MMP9 or MMP13 production in bronchial epithelial cells and that citrullination of LL-37 does not mitigate all its immunomodulatory functions. Our results indicate that it is likely that LL-37 modulates TNFα-mediated AKT signaling by enhancing negative regulators such as phospho-AKT (T308) and TNFAIP3 (A20) to selectively suppress MMP9 and/or MMP13 to intervene in the biological process of airway remodeling. Overall, the findings of this study highlight the interplay of LL-37 and TNFα in the context of airway remodeling, indicating that LL-37 may have the potential to protect against structural changes of the lungs that is associated with prolonged airway inflammation. To that end, the signaling intermediates enhanced by LL-37 as detailed in this study, such as AKT (T308) or A20, may be useful to

develop interventional strategies to intervene in biological processes associated with TNFα-driven airway remodeling in the lung, which will be beneficial for chronic respiratory diseases such as severe asthma and COPD.

Statement of Ethics

Anonymized patients undergoing resection surgery at the Leiden University Medical Center (LUMC), the Netherlands, were enrolled with a no-objection system for coded anonymous further use of such tissue (www.coreon.org). Since September 1, 2022, patients are enrolled with written informed consent in accordance with local regulations from the LUMC biobank with approval by the institutional medical Ethical Committee (B20.042/Ab/ab and B20.042/Kb/kb).

Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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Author Contributions

N.M. and A.A. conceived and designed the study. A.A. performed majority of the experiments, analyzed the data, and wrote the manuscript. M.H. assisted with the human PBEC studies and provided significant intellectual input into study design. D.L. assisted with Western blot assays and C.M. with cell cultures, and P.R. performed the transcriptional analyses. V.S. performed the bioinformatics for the proteomics study. A.M.D. provided the human primary bronchial cells and intellectual input for the study and edited the manuscript. N.M. obtained funding and resources for the study, provided overall supervision, and extensively edited the manuscript. All authors reviewed the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its supplementary material files. Further inquiries can be directed to the corresponding author.

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