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Inhaled corticosteroid suppression of cathelicidin drives dysbiosis and bacterial infection in chronic obstructive pulmonary disease

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Abstract

Bacterial infection commonly complicates inflammatory airway diseases such as chronic obstructive pulmonary disease (COPD). The mechanisms of increased infection susceptibility and how use of the commonly prescribed therapy inhaled corticosteroids (ICS) accentuates pneumonia risk in COPD is poorly understood. Here, using analysis of samples from patients with COPD, we show that ICS use is associated with lung microbiota disruption leading to proliferation of Streptococcal genera, an effect that could be recapitulated in ICS-treated mice. To study mechanisms underlying this effect, we utilized human and mouse models of Streptococcal expansion with *Streptococcus pneumoniae*, an important pathogen in COPD, to demonstrate that

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Author contributions:
AS designed, conducted and interpreted all animal experiments, with input from NG, NWB and SLJ. AS performed the statistical analysis and prepared the manuscript. JF, MBT, MAC, PM and SLJ were instrumental in the design, recruitment and sample processing from the human COPD exacerbation studies. LJF, SVK, PF and JAW were instrumental in bronchoscopy studies to obtain primary airway epithelial cells. AS performed the in vitro experiments in collaboration with MRE, EB, LJF and PF. AS, in collaboration with LC, ET and PLJ, conducted quantitative PCR/16S rRNA sequencing work. TBC, MM, WOC provided key reagents and contributed discussions throughout the work.

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ICS impairs pulmonary clearance of bacteria through suppression of the anti-microbial peptide cathelicidin. ICS impairment of pulmonary immunity was dependent on suppression of cathelicidin as ICS had no effect on bacterial loads in mice lacking cathelicidin (*Camp*-/-) and exogenous cathelicidin prevented ICS-mediated expansion of Streptococci within the microbiota and improved bacterial clearance. Suppression of pulmonary immunity by ICS was mediated by augmentation of the protease cathepsin D. Collectively, these data suggest a central role for cathepsin D/cathelicidin in suppression of anti-bacterial host-defence by ICS in COPD. Therapeutic restoration of cathelicidin to boost antibacterial immunity and beneficially modulate the lung microbiota might be an effective strategy in COPD.

Introduction

Bacterial infection is a major complication of chronic obstructive pulmonary disease (COPD), contributing to airway colonisation, exacerbations and pneumonia(1–3). These sequelae of events are responsible for a large burden of morbidity and mortality associated with the disease(1, 4–7). Anti-bacterial immunity is likely to be impaired in COPD and recent studies have raised concern that commonly used therapies, inhaled corticosteroids (ICS), can further weaken host-defence increasing pneumonia risk.(8–10) Mechanisms of susceptibility to bacterial infection in COPD and how ICS use accentuates this risk remain poorly understood.

Historically, our insight into roles of bacteria in COPD was based on studies using classical microbial culture techniques(11, 12). Modern understanding of the importance of bacteria in COPD has been completely revised by culture-independent techniques that have shed light on the existence of a respiratory tract microbiota containing complex communities that are altered in disease states(13–15). Specific factors that lead to alterations in microbiota composition in COPD have not been elucidated and knowledge of how underlying disease, therapies and microbiota interact to promote infection is limited.

The critical immune effectors that that are compromized in COPD and impaired by ICS are poorly defined. Anti-microbial peptides (AMPs) and surfactant proteins are major sentinels of pulmonary innate immunity which have, in some studies, been shown to be reduced in the airways of COPD patients or smokers(16–20) and could thus represent a disease-specific impaired anti-bacterial mechanism. AMPs can act as critical regulators of the microbiota at other mucosal surfaces (21–23). ICS are broad anti-inflammatory agents and are capable of suppressing the production of host-defence proteins(24–27) and therefore could promote bacterial infection through lung microbiota disruption.

Here, we demonstrated that ICS alter the resident lower respiratory microbiota promoting proliferation of Streptococcal genera and impair pulmonary bacterial control in human and mouse models of *Streptococcus pneumoniae* infection, effects that occur through suppression of the AMP cathelicidin. We identified a mechanism for impairment of cathelicidin responses by ICS in COPD through augmentation of the protease cathepsin D. Deficient cathelicidin responses are a component of lung anti-bacterial host-defence that is impaired by ICS use and may contribute to the increased pneumonia risk associated with use of these agents in COPD.

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Results

Inhaled corticosteroids alter the resident lung microbiota promoting proliferation of Streptococcal genera

Clinical studies demonstrate that ICS use increases pneumonia risk in COPD(8, 9) but underlying mechanisms have not been elucidated. Because of its immunosuppressive effects, ICS could theoretically promote pneumonia by inducing bacterial proliferation within the existing lung microbiota. We therefore evaluated ICS effects on lung microbiota composition using 16S rRNA sequencing. We initially analyzed sputum samples from 23 patients with COPD during clinical stability, stratified according to non-use (n=13) or current use of ICS (n=10). Clinical characteristics of these patients are shown in Table 1. ICS-users showed a significant increase in relative abundance of Streptococcal genera compared to ICS non-users ($P<0.05$; Fig 1A and B). We confirmed these findings using quantitative (q)PCR specific for Streptococcus ($P<0.05$;Fig. 1C). Since ICS-users were significantly older than non-users in this cohort ($P=0.013$; Table 1), we also evaluated whether age might be a confounder for the increased Streptococcal load observed but we found no correlation between age and Streptococcus qPCR copies (fig. s1). We observed no difference in overall bacterial loads measured by 16S qPCR in ICS users versus non-users ($P=0.1$;Fig. 1D) and no difference in bacterial diversity (Fig. 1E). Given that cause and effect cannot be inferred from a cross-sectional human study, we evaluated whether experimental intranasal administration of the ICS fluticasone propionate (FP) in mice (Fig. 1F) at a dose previously shown to induce lung glucocorticoid receptor activation(27) caused a similar increase in Streptococci. 16S rRNA sequencing demonstrated that FP increased relative abundance of Streptococcal genera at 24 hours ($P<0.05$; Fig. 1G and H) and these findings were again confirmed using *Streptococcus* qPCR(Fig. 1I). FP also increased lung 16S bacterial loads ($P<0.01$; Fig. 1J) and bacterial diversity ($P<0.05$; Fig. 1K). These results indicate that ICS treatment promotes expansion of a genus that contains bacteria that are a major cause of infection in patients with COPD(1, 2, 28) and that these effects could be recapitulated in mice.

FP impairs bacterial control in models of Streptococcal expansion with *S. pneumoniae*

Having observed that ICS administration induces microbiota disruption in human and mouse models, we next sought to establish models of Streptococcal expansion with the COPD-relevant pathogen *S. pneumoniae* to understand how defects in pulmonary immunity associated with ICS use facilitate Streptococcal expansion. First, we demonstrated that intranasal *S. pneumoniae* infection in mice modelled Streptococcal lung expansion by confirming an increase in relative abundance of *Streptococcus* assessed by 16S rRNA sequencing compared to PBS-treated control mice ($P<0.05$; Fig. 2A and B). Then, we sought to investigate if FP promoted *S. pneumoniae* expansion. FP administration prior to *S. pneumoniae* challenge (Fig. 2C) increased lung bacterial loads assessed by quantitative culture ($P<0.01$; Fig. 2D). Despite increasing lung bacterial loads, FP had no effect on blood bacterial loads(Fig. 2E), suggesting that FP impairs local control of S*. pneumoniae* in the lungs without impacting upon systemic anti-bacterial defences.

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These findings confirmed that FP could suppress pulmonary immunity and impair bacterial control in the healthy lung. However, in clinical practice ICS treatment is given in the context of chronic lung inflammation. We therefore next investigated whether FP treatment promoted *S. pneumoniae* expansion in this context and thus established a mouse model of elastase-induced emphysema combined with *S. pneumoniae* infection (Fig. 2F). We have previously reported that a similar model of rhinovirus-exacerbated elastase-induced emphysema recapitulated many features of human virus-associated exacerbation(29). Elastase-treated mice infected with *S. pneumoniae* had increased lung bacterial loads compared to mice given PBS prior to *S. pneumoniae* infection with further augmentation of bacterial loads observed with FP administration ($P<0.01$ and $P<0.05$ respectively; Fig. 2G). Consistent with effects of ICS administration in mouse models, we also observed that FP augmented bacterial loads in *S.pneumoniae*-infected human airway epithelial cell cultures ($P<0.05$; Fig. 2H). These observations confirmed that ICS administration can impair pulmonary clearance of *S. pneumoniae*, a species member of the Streptococcus genus and a frequent cause of pneumonia in COPD(3).

Airway cathelicidin concentrations are reduced in COPD and suppressed by ICS during bacterial infection

We next sought to investigate mechanisms whereby ICS promote pneumonia in COPD. We focused on AMPs because studies have shown reduced expression in the airways of COPD patients and smokers(16–19). Additionally, our data indicated rapid effects of FP in murine models, suggesting that the drug interferes with an innate component of the immune response.

We initially studied expression of AMPs in baseline sputum samples from a cohort of patients with mild to moderate severity COPD (GOLD stage 0-2)(clinical details shown in table s1). We found that the human hCAP18/LL-37 protein was reduced in COPD patients (n=37) versus healthy non-smokers (n=19) ($P<0.05$; Fig. 3A) but no differences were observed in concentrations of other AMPs/surfactant proteins including α-defensin1-3, secretory leucocyte protease inhibitor (SLPI), surfactant protein-D or mannose-binding lectin 2 (fig. S2a-d). We additionally observed no differences in bronchoalveolar lavage(BAL) hCAP18/LL-37 protein concentrations in a sub-group of COPD patients (n=15) versus healthy non-smokers (n=10)($P=0.11$; fig S3)(Clinical details shown in Table S2). Consistent with reduced airway cathelicidin in human COPD, we observed that experimental induction of COPD-like disease using elastase administration in mice also led to reduced baseline concentrations of the ortholog cathelicidin-related anti-microbial peptide (CRAMP) compared to PBS-treated control mice ($P<0.01$; Fig. 3B).

To determine whether cathelicidin expression is related to bacterial control in COPD airways, we examined relationships between sputum hCAP18/LL-37 concentrations and presence of *Streptococcus* assessed by qPCR in stable-state samples from the COPD subjects used to evaluate the microbiome (n=23, clinical characteristics are shown in Table 1). There was a significant negative correlation between sputum hCAP18/LL37 and *Streptococcus* qPCR copies ($P<0.05$; Fig. 3C).

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Having observed that stable-state airway cathelicidin concentrations are reduced in COPD, we next determined whether ICS suppress this AMP. FP administration suppressed early CRAMP induction by *S. pneumoniae* in mice at 8 hours post-infection ($P<0.05$; Fig. 3D) but had no effect on *S. pneumoniae* induction of other AMPs β-defensin 2, mannose-binding lectin 2, lactoferrin or SLPI (fig. S4a-e). FP also suppressed neutrophils at 24 hours post-infection ($P<0.01$; fig. S4f). In the elastase COPD mouse model, induction of CRAMP by *S. pneumoniae* was deficient in elastase- versus PBS-treated mice and further impaired by FP administration ($P<0.01$ and $P<0.05$ respectively; Fig. 3E). Elastase-treated mice infected with *S. pneumoniae* (fig. S5A) also had deficient induction of pro-inflammatory cytokines IL-6 ($P<0.05$; fig S5B), TNF (P<0.001; fig S5c) and IL-1β in BAL ($P<0.05$; fig. S5D) compared to PBS-treated mice. Conversely, elastase-treated mice had increased cellular airway inflammation ($P<0.001$; BAL total cells, fig. S5E) in response to *S. pneumoniae* infection. FP administration suppressed IL-6 concentrations ($P<0.05$; fig S5B) and BAL total ($P<0.001$; fig S5E) and neutrophil cell counts ($P<0.05$; fig S5F) but had no effect on TNF or IL-1β concentrations (fig. S5C and D).

To confirm the clinical relevance of our findings, we measured sputum cathelicidin concentrations in a cohort of patients reporting COPD exacerbations (30)(with pathogen detection at exacerbation as follows: virus alone n=14, bacteria alone n=4; virus/bacteria co-infection n=4; no pathogen identified n=5). Patients were stratified according to current use (n=11) or non-use (n=16) of ICS with samples assessed during exacerbation (at onset and 2 weeks) and at resolution (6 weeks). Clinical characteristics of exacerbating patients are shown in Table S3. In keeping with findings in animal models, ICS users had suppressed sputum supernatant concentrations of hCAP18/LL-37 versus ICS non-users at exacerbation onset ($P<0.01$; Fig. 3F) but not at 2 and 6 weeks post-exacerbation. To further investigate the clinical importance of cathelicidin during exacerbation, we examined relationships between sputum hCAP18/LL-37 and markers of exacerbation severity. Sputum hCAP18/LL-37 correlated negatively with peak acute FEV_1 decline from baseline and additionally with sputum concentrations of the mucin glycoprotein MUC5AC ($P<0.05$ respectively; Fig. 3G). There was no correlation between sputum hCAP18/LL-37 and 16S qPCR bacterial loads during exacerbation ($P= 0.073$; Fig. 3G).

Combined, these data confirm that ICS use impairs cathelicidin production in vivo and additionally suggest that cathelicidin might be an important determinant of bacterial clearance and clinical severity during exacerbations.

ICS exert inhibitory effects on cathelicidin responses at the bronchial epithelium

We next sought to understand how ICS use dampens lung Streptococcal defences. Cathelicidin can be produced by the bronchial epithelium(31) and also by airway inflammatory cells, particularly neutrophils(32). Given our observations that FP-mediated impairment in bacterial clearance occurred at 8 hours ($P<0.01$; Fig. 2D), an earlier timepoint than airway neutrophil recruitment occurred in *S. pneumoniae*-infected mice (24 hours, fig. S4F) and since FP could induce microbiota disruption in unchallenged mice, where neutrophil recruitment does not occur, we reasoned that FP was not acting through effects on neutrophil-produced cathelicidin in our models. We hypothesised that ICS mediates its

inhibitory effects on early cathelicidin production by the pulmonary epithelium. BEAS2B bronchial epithelial cells treated with FP prior to *S. pneumoniae* infection had reduced hCAP18/LL-37 production compared to non-FP treated controls infected with *S. pneumoniae* (P<0.05; Fig. 3H). Western blot analysis confirmed that cathelicidin is secreted in the uncleaved form (18kDa) in airway epithelial cell culture supernatants (Fig S6A), as previously reported(33). We observed no induction of hCAP18/LL-37 by heat-killed *S. pneumoniae* or individual agonists for the major pneumococcal pattern recognition receptors(PRR) TLR-2, TLR-9 or NOD-2 in epithelial cell cultures, indicating that viable pneumococci are required to stimulate cathelicidin production in this experimental system (Fig. S6B). FP had no effect on the induction of surfactant protein-D, β defensin-2 or mannose-binding lectin-2 (Fig S7A to C). Other AMPs evaluated including α-defensin1-3, SLPI and elafin were not detectable in cell supernatants.

In keeping with the observed effect in an airway epithelial cell line, FP also impaired ex vivo production of hCAP18/LL-37 by primary bronchial epithelial cells from patients with COPD (*P*<0.05; Fig. 3H)(clinical characteristics are shown table S4). This confirms that ICS can impair cathelicidin responses in cells taken directly from COPD patients and indicates that these effects are likely to be important clinically.

Impairment of pulmonary immunity by FP is dependent on suppression of cathelicidin

To confirm the functional importance of FP-mediated suppression of cathelicidin, we examined whether FP had effects on lung bacterial control in mice with gene-targeted deletion of cathelicidin (*Camp*-/-). In contrast to wild-type mice, FP administration had no effect on bacterial loads in *Camp*-/- mice, thereby confirming that FP suppression of cathelicidin plays a major role in effects on bacterial control (Fig. 4A). We additionally found that exogenous replacement of cathelicidin using recombinant protein administration (Fig. 4B) restored the disrupted lung microbiota associated with FP administration by reversing FP-mediated increases in *Streptococcus* bacterial load in mice (*P*<0.01; Fig. 4C). Exogenous cathelicidin administration in FP-treated *S. pneumoniae*-infected mice (Fig. 4D) also significantly reduced bacterial loads (*P*<0.05; Fig. 4E) without affecting suppression of BAL neutrophil chemokines CXCL2/MIP-2 or CXCL1/KC (Fig. S8A to C) or pro-inflammatory cytokines IL-6, TNF or IL1β, (Fig. S8D to F). These experiments confirmed that cathelicidin is both necessary and sufficient for ICS to mediate effects on lung bacterial control in vivo.

Airway concentrations of the protease Cathepsin D are enhanced in COPD and further augmented by ICS during bacterial infection

Next, we sought to understand how FP reduces airway cathelicidin. Previous studies have suggested that proteolytic cleavage by enzymes such as neutrophil elastase and cathepsin D may contribute to LL-37 degradation in the airway(34, 35). Given that airway concentrations of neutrophil elastase and cathepsin D are known to be augmented in COPD(36, 37), we hypothesized that elevations in these enzymes may drive the reduced cathelicidin observed in our models. We observed suppression rather than enhancement of neutrophil elastase by FP in the mouse *S. pneumoniae* infection model (*P*<0.05; Fig. S9), suggesting that

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neutrophil elastase-mediated cleavage of cathelicidin is unlikely to account for the reduced concentrations observed with ICS administration in COPD.

Cathepsin D concentrations were significantly increased in sputum from COPD subjects versus healthy subjects at clinical stability ($P<0.05$; Fig. 5A). Characteristics of patients included in this analysis are shown in Table S1. Mice treated with elastase to induce COPD-like disease also had increased BAL concentrations of cathepsin D ($P<0.01$; Fig. 5B). FP administration augmented BAL cathepsin D concentrations in *S. pneumoniae*-infected mice ($P<0.05$; Fig. 5C) and also increased BAL cathepsin D concentrations in elastase-treated *S. pneumoniae*-infected mice ($P<0.05$; Fig. 5D). To further investigate relationships between ICS treatment and cathepsin D during COPD exacerbations, we measured sputum concentrations from the COPD exacerbation cohort (see Table S3). ICS users had increased sputum supernatant cathepsin D concentrations versus ICS non-users at exacerbation onset ($P<0.05$; Fig. 5E).

Given our prior observations that FP exerts inhibitory effects on cathelicidin production by the bronchial epithelium, we evaluated FP effects on cathepsin D production by epithelial cell cultures following *S. pneumoniae* infection. FP administration augmented cathepsin D protein induction by *S. pneumoniae* in BEAS2B airway epithelial cells ($P<0.05$; Fig. 5F). Similar effects occurred in COPD cells with augmentation of ex vivo *S. pneumoniae* induction of cathepsin D by FP ($P<0.05$; Fig .5F). Further evidence that FP exerts inhibitory effects on cathelicidin through increased cathepsin-D mediated degradation rather than impairment of gene transcription was demonstrated by lack of an effect of FP administration on *S. pneumoniae* upregulation of *CAMP* mRNA in airway epithelial cells (fig S10). Combined, these observations indicate that cathepsin D, a known negative regulator of cathelicidin, is enhanced in COPD and augmented by ICS during bacterial lung infection.

Exogenous Cathepsin D protein administration attenuates *S. pneumoniae* induction of cathelicidin in airway epithelial cells

To further confirm that cathepsin D can degrade cathelicidin released by the airway epithelium in response to bacterial infection, we administered recombinant cathepsin D protein in *S. pneumoniae* treated airway epithelial cells. Cathepsin D administration had no effects in the absence of infection but significantly attenuated hCAP18/LL-37 induction in response to *S. pneumoniae* infection in BEAS2B airway epithelial cell cultures ($P<0.05$; fig S11).

Inhibition of cathepsin D reverses suppressed cathelicidin and restores impaired bacterial control associated with FP administration

To confirm that cathepsin D contributes to impaired cathelicidin responses associated with ICS administration in COPD, we assessed the effect of inhibiting its action during *S. pneumoniae* infection using the lysosomal protease inhibitor pepstatin-A. Pepstatin-A administration prior to *S. pneumoniae* infection in mice (Fig. 6A) reversed FP-suppression of BAL CRAMP protein at 8 hours post-infection ($P<0.001$; Fig. 6B) with a concomitant reduction in FP-mediated increases in bacterial loads ($P<0.05$;Fig. 6C). These observations indicate that FP regulates cathelicidin through effects on cathepsin D and suggest that

enhanced cathepsin D plays a mechanistic role in the suppression of pulmonary immunity associated with ICS use in COPD.

Discussion

The underlying mechanisms involved in susceptibility to bacterial infection in COPD and how the disease, treatment and microbiota interact to promote exacerbations and pneumonia represents a crucial research question in the field. Our study fits with an emerging conceptual framework whereby ICS use contributes to impaired anti-bacterial host-defence in COPD through deleterious effects on innate immunity. Here, we provide mechanistic insight into how patients with COPD treated with ICS are at increased risk of bacterial infection. Using a combination of mouse and human COPD models, we identify that ICS-mediated suppression of cathelicidin drives expansion of Streptococci within the microbiota and that cathelicidin is necessary and sufficient for ICS impairment of bacterial control. Our studies indicate a mechanism for impairment of cathelicidins by ICS through augmentation of the protease cathepsin D. Using human studies, we additionally demonstrate that lower cathelicidin concentrations are associated with increased clinical severity during COPD exacerbations.

Molecular culture-independent techniques have revealed the existence of a lower respiratory tract microbiome consisting of complex bacterial communities(13) which are altered in chronic respiratory diseases such as COPD(14, 15). The COPD microbiota varies according to the population studied but is broadly characterised by an outgrowth of Proteobacteria phylum and an increase in Streptococci and Staphylococci within Firmicutes phylum(13–15). Microbiota shifts including increases in *Streptococcus* occur during COPD exacerbations (38). Our data indicate that ICS may further accentuate expansion of a bacterial genus that is already increased within the COPD microbiota. A previous clinical trial similarly reported increased Firmicutes following 12 months ICS treatment in COPD(39) and another study showed that systemic corticosteroid administration in mice can perturb the intestinal microbiota with expansions in disease-relevant genera(40).

Within the *Streptococcus* genus, *S. pneumoniae* is commonly implicated in colonization, exacerbations and pneumonia in COPD(1, 2, 28). COPD is, additionally, a risk factor for severe pneumonia and invasive pneumococcal disease(41, 42). Using mouse models of Streptococcal expansion with *S. pneumoniae*, we demonstrate that ICS further impairs pulmonary bacterial clearance in vivo. Our findings confirm those of previous studies showing impaired clearance of *Klebsiella pneumoniae*(43) and *Pseudomonas aeruginosa*(25) with ICS administration in mice and advance our understanding by indicating that ICS-mediated effects on bacterial control also, importantly, occur with pneumococcal infection in COPD and are thus likely to be of relevance to the reported clinical pneumonia signal.

Mechanisms of susceptibility to bacterial infection in COPD are poorly understood and a number of contributory abnormalities in pulmonary defences have been postulated including altered PRRs expression(44–46), immune cell dysfunction(47, 48) and mucociliary impairment(49, 50). However, many of these defects are unaffected or corrected, rather than worsened, by corticosteroids(43, 47, 51–53) and therefore do not provide adequate

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explanation for the increased pneumonia risk shown in clinical studies for a range of ICS agents(8, 9, 54–56). We focused on AMPs and surfactant proteins, soluble molecules present in the airway that form an important first-line of defence against pathogens. These mediators have been shown to be reduced in airway samples from COPD patients or smokers(16–20) and are also susceptible to corticosteroid suppressive effects(24–27). We found that concentrations of the AMP cathelicidin were reduced in COPD airways, that FP administration impairs cathelicidin induction by *S. pneumoniae* in experimental models and that ICS users have reduced cathelicidin at time of COPD exacerbation. Therefore, in contrast to a range of other AMPs/surfactant proteins shown here to be unaffected by FP, cathelicidin is suppressed by ICS use in COPD. Studies have similarly reported corticosteroid impairment of cathelicidin induction by vitamin D3 in vitro(24) and to *Pseudomonas aeruginosa* infection in mice(25) and we again extend these prior observations to confirm that similar effects specifically occur in the context of *S. pneumoniae* infection in COPD.

Using loss and gain of function studies in mice, we show that impairment of pulmonary immunity by FP is dependent on cathelicidin suppression, thereby demonstrating a major mechanistic role in ICS-related pneumonia in COPD. There is interest in the potential to therapeutically manipulate the microbiome and influence lung immunity. We show here that an AMP applied exogenously to boost anti-bacterial immunity can beneficially modulate the lung microbiota and, theoretically, reduce risk of a therapy-associated infective complication. Recombinant LL-37 administration has been shown to alter gut microbiota in mice(21) and systemic cathelicidin concentrations correlate with microbiota composition in infants hospitalised with bronchiolitis(57). Combined with our findings, this highlights an emerging role for cathelicidins in microbiota regulation. Cathelicidins have wide ranging anti-bacterial effector functions including bacterial killing, modulation of PRR-mediated responses(58) and inflammatory cell chemotaxis(59). These functions could all theoretically affect microbiota composition. Cathelicidins also have host-defence roles against other pathogens including *Klebsiella*(60), *Pseudomonas*(61), respiratory syncytial virus(62) and influenza(63), suggesting that the importance of this peptide may extend beyond its role in *S. pneumoniae* infection, to be involved in immunity to numerous other COPD-relevant infections.

Airway regulation of cathelicidins in COPD is likely to be complex; our findings are consistent with studies showing reduced cathelicidin concentrations in sputum from patients with severe COPD (GOLD stage III-IV) versus healthy individuals(16) and reduced serum concentrations in frequent exacerbators versus healthy individuals(64) but contradictory studies report increased airway hCAP18/LL-37 in COPD(65, 66). Reasons for discrepancies between existing studies is likely to be multifactorial with a number of confounders potentially affecting airway cathelicidin including ICS, inflammatory profile, microbiota composition and vitamin D concentrations. Our finding that sputum hCAP18/LL-37 negatively correlates with severity measures provides additional evidence that cathelicidin responses may be important during COPD exacerbations. Combined with the observation that exogenous LL-37 improves bacterial control, this implicates a central role for cathelicidin during COPD bacterial infections and raises speculation that therapies aimed at boosting cathelicidin responses might provide an effective strategy. Administration of

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bioengineered cathelicidin-secreting probiotic bacteria has been shown to protect against intestinal bacterial colonization in animal studies(67) and it is plausible that similar approaches could be effective in treating infections associated with chronic lung diseases. In the current study, we also observed that, in addition to cathelicidin, FP administration impaired other anti-bacterial responses to *S. pneumoniae* in the mouse model of elastase-induced COPD including BAL IL-6 concentrations and neutrophils, effects which may also contribute to increased infection susceptibility. Our finding that exogenous cathelicidin administration restores bacterial clearance without affecting FP-suppression of these other responses gives support to the relative importance of cathelicidin. In contrast to the suppressive effects of ICS on epithelial cathelicidin production reported here, other studies have also shown that corticosteroids can increase some host-defence mediators including lactotransferrin(68), CCL20(69) and surfactant protein-A(70). However, our finding that no effect of FP on bacterial loads was observed in *Camp*-deficient mice and that exogenous cathelicidin blocked effects of FP on bacterial loads suggests that cathelicidin suppression is more important than effects on other mediators.

We identified a mechanism for impairment of cathelicidin responses by FP in COPD through augmentation of cathepsin D, a lysosomal protease shown to be increased in COPD(37) and can inactivate AMPs such as cathelicidin through proteolytic cleavage(34, 35). Cathepsin D is known to be a steroid-inducible gene and glucocorticoids can increase expression and activity of this protease in a range of tissues.(71–73) Administration of pepstatin-A, an inhibitor of cathepsin D activity, reconstituted suppressed cathelicidin responses and reversed impaired bacterial control associated with FP administration in mice. Pepstatin-A has also previously been shown to reduce dissemination and mortality in a *Candida albicans* infection mouse model,(74) and this compound is also under investigation as a therapy for other diseases such as breast cancer(75). In contrast to our findings in COPD, cathepsin D expression has been shown to be unchanged in asthma(76). This may explain why the risk of pneumonia associated with ICS use in clinical studies has been shown to occur exclusively in COPD but not asthma(77, 78), where cathepsin D concentrations may be lower and thus have lesser inhibitory effects on cathelicidin and subsequent anti-bacterial immunity. Our data supports further investigation into development of cathepsin D inhibitors as potential therapeutic agents in COPD.

Calverley *et al* reported that ICS-related pneumonia episodes may occur either following a protracted symptomatic exacerbation or ‘de novo’ (44.8% and 55.2% of pneumonic episodes respectively)(79). We have recently reported that ICS can suppress innate anti-viral immune responses to rhinovirus infection leading subsequently to impaired production of the AMP SLPI and increased bacterial loads(27). These effects provide a mechanism to explain secondary pneumonias that follow an initial virus-induced exacerbation but since >50% of ICS-related pneumonic episodes may occur de novo, distinct mechanisms other than effects on virus-induced secondary bacterial infection may be involved. In the current study, we utilised direct experimental models of bacterial infection and, in contrast to effects seen in virus infection, we found no suppressive effect of FP on SLPI in these models but instead found that cathelicidin was the major AMP involved. We speculate that two distinct mechanisms of ICS-induced pneumonia may occur depending on whether or not a preceding virus infection is involved. Our data adds to the increasingly recognised importance of anti-

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microbial peptides in COPD anti-bacterial host defence and further suggest that ICS impairment of these peptides could be important clinically.

This study has some limitations. The effects of ICS on the resident lung microbiota were evaluated in a cross-sectional human study combined with analyses following direct administration of fluticasone in mice. Definitive evidence that ICS therapy can alter the lower respiratory tract microbiota in man would require sequential analyses before and after initiation of fluticasone in patients. Additionally, the in vivo functional effects of ICS on cathelicidin and pneumococcal clearance in COPD were demonstrated in our study using a mouse model of elastase-induced disease. We have previously reported that this model recapitulates features of human COPD exacerbation when combined with rhinovirus infection (29). However, neither elastase models nor other models such as smoke exposure in mice can completely recapitulate the complexities and heterogeneity of human disease. Given that cigarette smoking has previously been shown to affect the airway microbiota (80, 81), animal studies using smoke exposure models are required to confirm our findings.

In conclusion, our study identifies a central role for cathelicidin in COPD anti-bacterial host-defence and demonstrates that suppression by ICS mediates microbiota dysregulation and impaired pulmonary immunity. Therapeutic restoration of cathelicidin responses to enhance antibacterial host-defence, either using recombinant protein administration or through inhibition of negative regulator cathepsin D, might beneficially modulate the lung microbiota and improve bacterial control, providing an effective strategy for treating COPD.

Materials And Methods

Study design

The primary objective of this study was to define the effects of inhaled corticosteroids on the lung microbiota and pulmonary immune responses to bacterial infection in COPD. All animal experiments were performed under the authority of the UK Home Office outlined in the Animals (Scientific Procedures) Act 1986 after ethical review by Imperial College London Animal Welfare and Ethical Review Body (project licence PPL 70/7234). The number of animals in each treatment group was determined by power calculations based on extensive previous experience with the model systems and is shown in the respective figure legends. For analyses from human subjects, sample sizes were opportunistic and carried out using historical samples from previously conducted studies. Authors were blinded for analyses of hCAP-18/LL-37 and other immune mediators. All studies were ethically approved and are detailed in the relevant section within the methods. No samples or animals were excluded from any analyses.

Streptococcus pneumoniae infection and treatment of mice

Female C57BL/6 mice of 6-8 weeks age, purchased from Charles River Laboratories, were used for animal studies. *Camp*-/- mice of 6-8 weeks age on a C57BL/6 background were purchased from Jackson Laboratories. Mice were housed in individually ventilated cages under specific pathogen-free conditions.

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FP powder (Sigma-Aldrich) was resuspended in DMSO at a concentration of 357µg/mL, followed by dilution 1:1000 in PBS. Mice were lightly anaesthetized with isofluorane then treated intranasally with 50 µl FP solution (equating to 20µg dose) or vehicle DMSO diluted 1:1000 in PBS as control. One hour following FP administration, mice were intranasally infected with 50 µl containing 5 x 105 CFU *S. pneumoniae* D39 or PBS control. In experiments to evaluate the effect of ICS administration on the lung microbiota in mice, FP was administered intranasally, as detailed above, without *S. pneumoniae* infection. In separate experiments, one hour following *S. pneumoniae* infection, mice were additionally treated intranasally with 50µl PBS containing 10µg recombinant human LL-37(Bio-Techne), a similar dose to that used previously in animal infection studies(61, 62). In additional experiments, mice were treated intraperitoneally with 60mg/kg pepstatin-A, a dose previously shown to inhibit cathepsin D activity in mouse lung(86), 24 hours prior to *S. pneumoniae* infection.

For evaluation of the effect of FP in a mouse model of COPD bacterial infection, mice were treated intranasally with 1.2 units of porcine pancreatic elastase (Merck) to induce emphysematous lung changes(29) and additionally treated intranasally with 20 µg FP, one hour prior to infection with 5 x 10^5 CFU *S. pneumoniae* D39 or PBS control.

Mice were culled at 4, 8 or 24h post administration of fluticasone and/or *S. pneumoniae* infection. BAL fluid, whole lung and blood were taken and prepared for analyses, as previously described(87).

The St. Mary's Hospital naturally-occurring human COPD exacerbation cohort

A cohort of 40 subjects was recruited to a prospective study investigating naturally-occurring exacerbations in patients with COPD between June 2011 and December 2013, as previously reported(30). All subjects were confirmed to have COPD by spirometry and all treatments were permitted. A full medication history was taken at recruitment and subjects were classified as current ICS users or non-users. Subjects gave informed written consent and the study was approved by the East London Research Ethics Committee (Protocol number 11/LO/0229). All subjects had an initial baseline visit during clinical stability for medical assessment, peak expiratory flow rate measurement, spirometry (forced expiratory volume in 1 second (FEV 1), forced vital capacity (FVC) and clinical sample collection, including spontaneous or induced sputum, taken as previously described(36, 88).

Subjects had repeat visits at three monthly intervals when clinically stable and were followed up for a minimum of 6 months. Subjects reported to the study team when they developed symptoms of an acute exacerbation defined using the East London cohort criteria(5). Subjects were reviewed by the study team within 48 hours of symptom onset. Sputum samples were collected at the onset of exacerbation, at 2 weeks during and 6 weeks after exacerbation. Viruses were detected in sputum using polymerase chain reaction as described previously (36).

Statistical analysis

Experiments in mouse models involved 5-10 animals per treatment condition and data is presented as mean+/-SEM, representative of at least two independent experiments. In vitro

experiments in BEAS-2B cells were performed 3-5 times and data were analyzed using one-way ANOVA with significant differences between groups assessed by Bonferroni's multiple comparison test. In vitro primary airway epithelial cell experiments were performed on cells from n=6 patients and data were analyzed using one one-way ANOVA with significant differences between groups assessed by Bonferroni's multiple comparison test. Data from the human samples is shown as median +/- IQR and analyzed using the Mann-Whitney U test. Correlations between datasets were examined using Spearman's rank correlation coefficient. All statistics were performed using GraphPad Prism 6 software. Differences were considered significant when $p<0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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One Sentence Summary

Inhaled corticosteroids promote bacterial infection in chronic obstructive pulmonary disease by suppressing the anti-microbial peptide cathelicidin.

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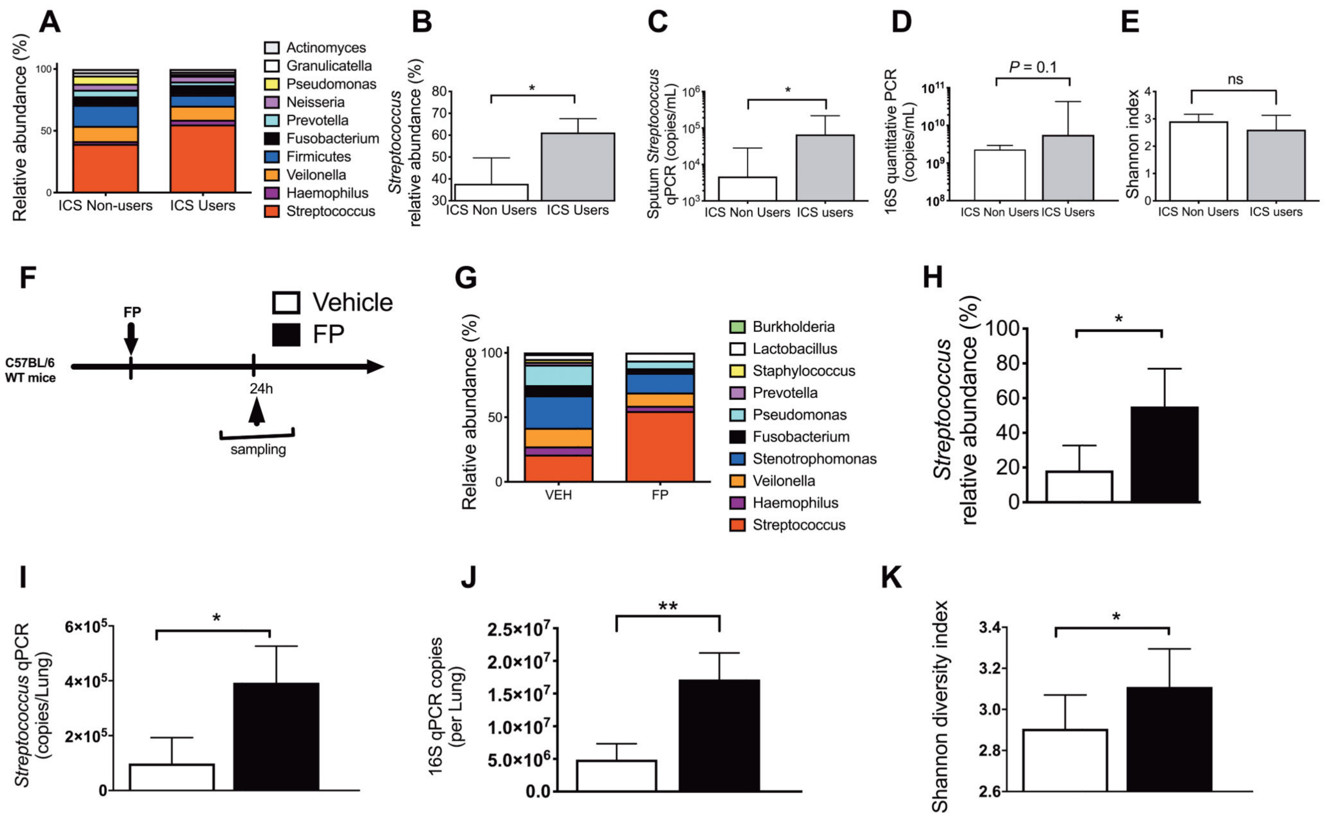

Figure 1. Inhaled corticosteroids alter the lower respiratory tract microbiota inducing proliferation of *Streptococcus* genera.

(A-E) Evaluation of the lung microbiota in sputum samples from patients with COPD (n=10 ICS users and n=13 ICS non-users). (a) Relative abundance of the top ten operational taxonomic units (OTUs) in ICS users and non-users. (B) Relative abundance of *Streptococcus* (C) *Streptococcus* qPCR copies (D) total bacterial loads assessed by 16S qPCR and (E) Shannon diversity index in sputum samples from ICS users versus non-users. (F) Experimental outline. C57BL/6 mice were treated intranasally with 20ug fluticasone propionate or vehicle control. Lung tissue was harvested at 24 hours post-administration and lung microbiota was evaluated by 16S rRNA sequencing. (G) Relative abundance of the top ten operational taxonomic units (OTUs) in FP and vehicle treated mice. (H) Relative abundance of *Streptococcus* (I) *Streptococcus* qPCR copies (J) total bacterial loads assessed by 16S qPCR and (K) Shannon diversity index in FP and vehicle control treated mice. In (f)-(k) experiments comprise n=6-8 mice/group. Data shown as median (+/- IQR) and analyzed using Mann Whitney *U* test. n.s. non-significant *p<0.05 **p<0.01 ***p<0.001.

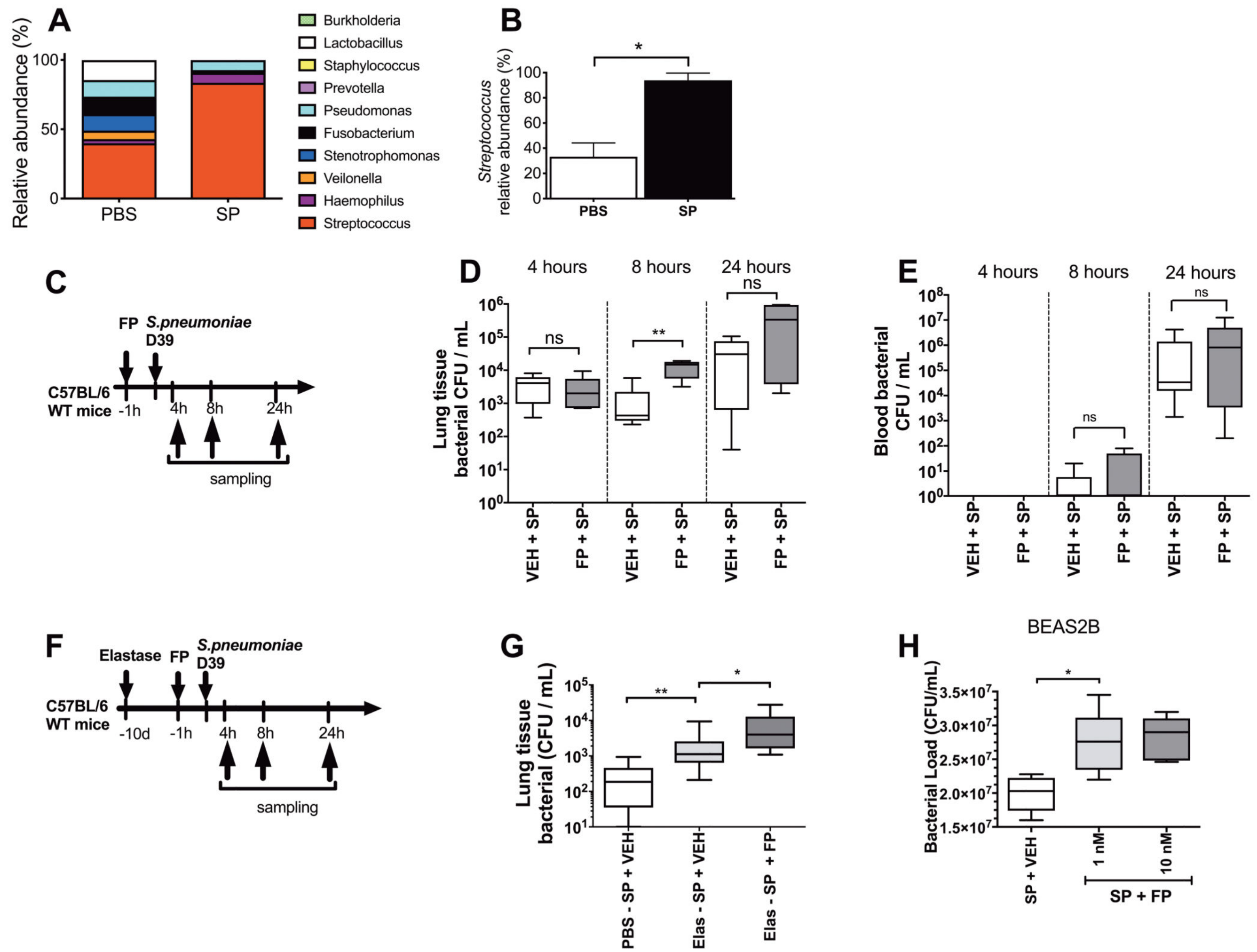

Figure 2. Fluticasone propionate impairs pulmonary clearance of *Streptococcus pneumoniae*.

(A-B) Lung microbiota was evaluated by 16S rRNA sequencing in lung tissue from mice challenged with *S. pneumoniae* (SP) and PBS treated controls at 8 hours post-challenge. (A) Relative abundance of the top ten operational taxonomic units (OTUs) (B) Relative abundance of *Streptococcus*. (C) Experimental outline. C57BL/6 mice were treated with 20ug FP or vehicle DMSO control and additionally infected with *S. pneumoniae* D39. Bacterial loads in (D) lung tissue and (E) blood were measured at the indicated timepoints post-infection by quantitative culture. (F) Experimental outline. C57BL/6 mice were treated intranasally with a single dose of elastase or PBS as control. Ten days later, mice were treated intranasally with fluticasone propionate (20μg) or vehicle DMSO control and challenged with *S. pneumoniae* D39. (G) Bacterial loads were measured at 8 hours post-infection. (H) BEAS-2B cells were treated with 1 or 10nM fluticasone propionate, stimulated with *S. pneumoniae* D39 and bacterial loads were measured in cell supernatants by quantitative culture at 24 hours post-infection. Bacterial load data are displayed as box and whisker plots showing median (line within box), IQR (box) and minimum to maximum (whiskers). Experiments comprise n=6-8 mice/group, representative of at least two

independent experiments. Data analyzed using Mann Whitney *U* test or one-way ANOVA with Bonferroni post-test. n.s. non-significant *$p<0.05$ **$p<0.01$ ***$p<0.001$.

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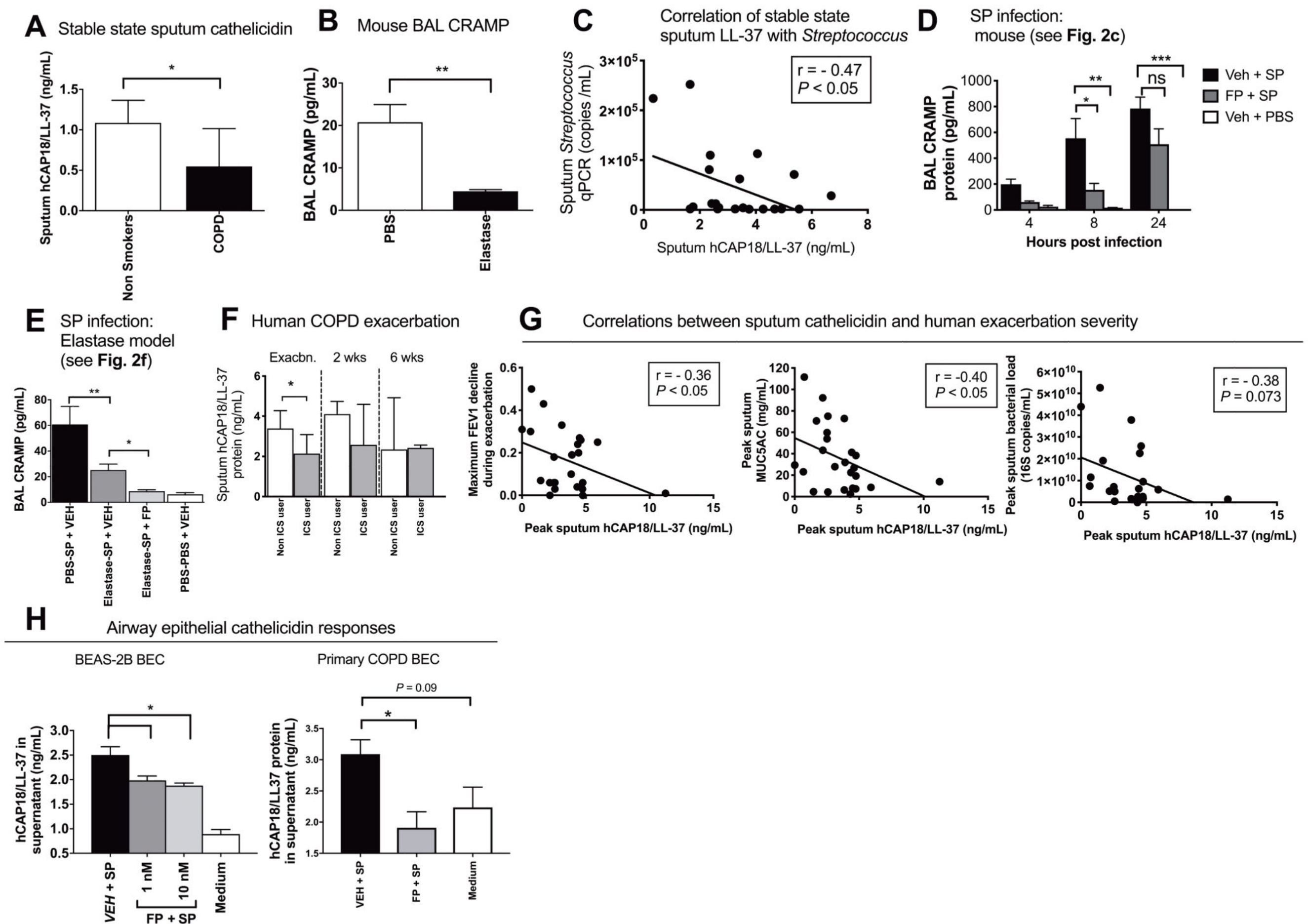

Figure 3. Cathelicidin responses to bacterial infection are impaired by inhaled corticosteroid and negatively correlate with COPD exacerbation severity.

(A) Stable state sputum hCAP18/LL-37 concentrations were measured in 37 subjects with COPD (GOLD stage 0-II) and 19 healthy control subjects by ELISA. (B) Cathelicidin-related anti-microbial peptide (CRAMP) concentrations were measured in mice at 10 days following intranasal treatment with 1.2 units of porcine pancreatic elastase or PBS control. (C) Correlation between sputum hCAP18/LL-37 and *Streptococcal* qPCR copies in 23 subjects with COPD. (D) C57BL/6 mice were treated intranasally with fluticasone propionate (20μg) or vehicle DMSO control and challenged intranasally with *S. pneumoniae* D39. CRAMP concentrations in BAL were measured by ELISA at the indicated timepoints. (E) C57BL/6 mice were treated intranasally with porcine pancreatic elastase or PBS control. Ten days later, mice were treated intranasally with fluticasone propionate, challenged with *S.pneumoniae* D39 and CRAMP concentrations in BAL measured at 8 hours post-infection. (F) Subjects with COPD (n=27) were monitored prospectively and sputum samples taken during exacerbation. Sputum hCAP18/LL-37 concentrations were measured by ELISA at the indicated timepoints. (G) Correlation between sputum hCAP18/LL-37 and FEV_1 decline, sputum MUC5AC concentrations and sputum bacterial loads. (H, left) BEAS-2B cells were treated with 1 or 10nM fluticasone propionate, stimulated with *S. pneumoniae* D39 and hCAP18/LL-37 concentrations in cell supernatants were measured at 8 hours by

ELISA. (h, right) Primary bronchial epithelial cells from 6 subjects with COPD were cultured, treated with 10nM FP, stimulated ex vivo with *S. pneumoniae* and hCAP18/LL-37 concentrations in cell supernatants were measured at 8 hours. In panels (B), (D), (E) and (H) data shown as mean (+/- sem) and analyzed by one-way ANOVA with Bonferroni's post-test. For human sputum analyses in (A) and (F) data are shown as median (IQR) and analyzed by Mann Whitney *U* test. In (C) and (G), correlation analysis used was nonparametric (Spearman's correlation). Animal experiments comprise n=5-8 mice/group, representative of at least two independent experiments. BEAS2B experiments comprise n=4 independent experiments. n.s. non-significant *p<0.05 **p<0.01 ***p<0.001

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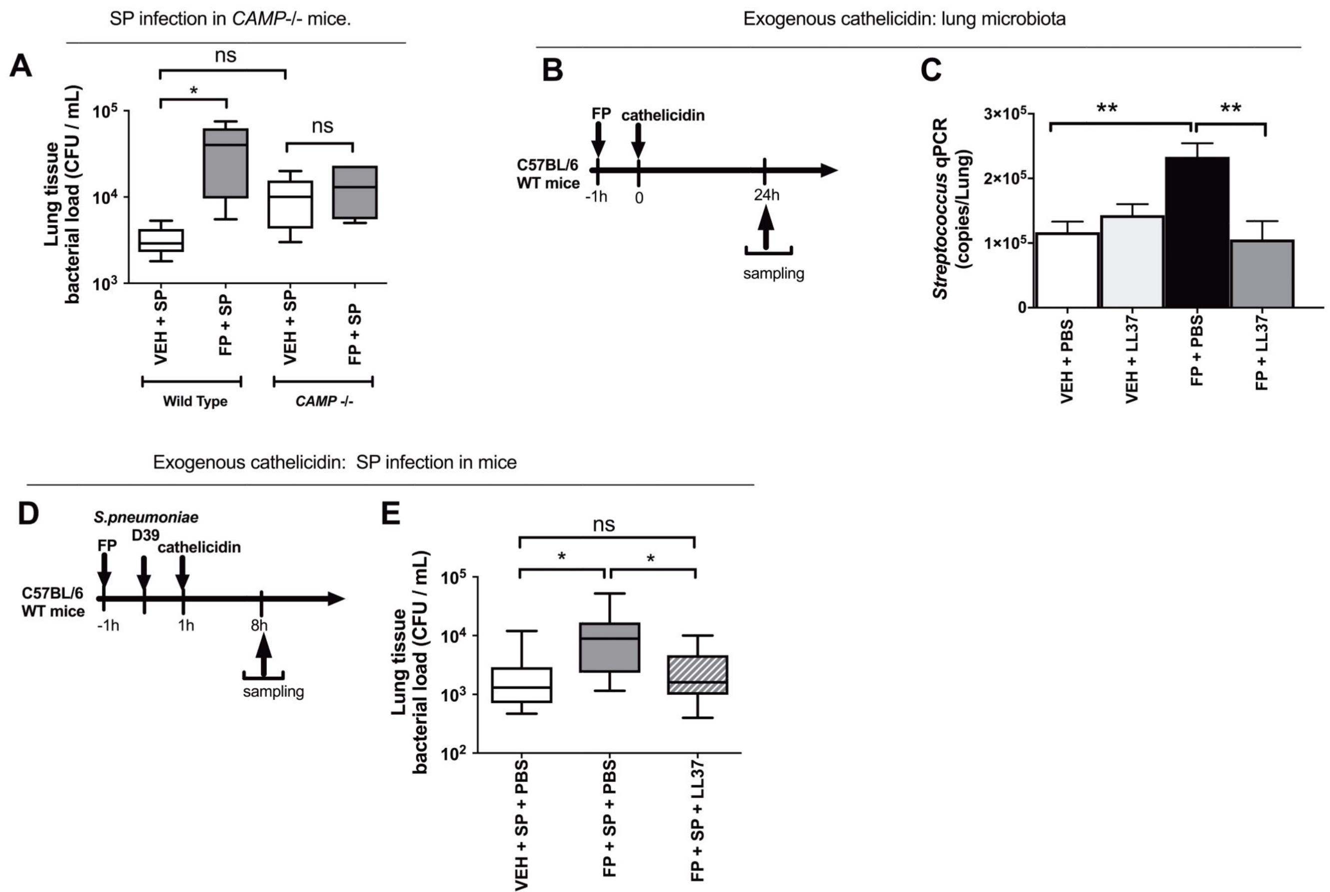

Figure 4. Impairment of pulmonary immunity by fluticasone propionate is dependent on cathelicidin.

(A) Wild type or *CAMP* -/- C57BL/6 mice were treated with 20ug fluticasone propionate or vehicle DMSO control and challenged intranasally with *S. pneumoniae* D39. Lung bacterial loads were measured by quantitative culture at 8h post-infection (B) Experimental outline. C57BL/6 mice were treated intranasally with 20ug fluticasone propionate or vehicle control and additionally with 10 ug recombinant LL-37. Lung tissue was harvested at 24h post-administration. (C) Lung *Streptococcus* was measured by qPCR (D) Experimental outline. C57BL/6 mice were treated intranasally with 20ug fluticasone propionate or vehicle DMSO control, challenged with *S. pneumoniae* D39 and additionally treated with 10 ug recombinant LL-37. Lung tissue was harvested at 8h post-administration. (E) Lung bacterial loads measured by quantitative culture. Data in (C) shown as mean (+/- S.E.M). Bacterial load data displayed as box and whisker plots showing median (line within box), IQR (box) and minimum to maximum (whiskers). Animal experiments comprise n=5-10 mice/group, representative of at least two independent experiments. Data analyzed by one-way ANOVA with Bonferroni post-test. n.s. non-significant *p<0.05 **p<0.01.

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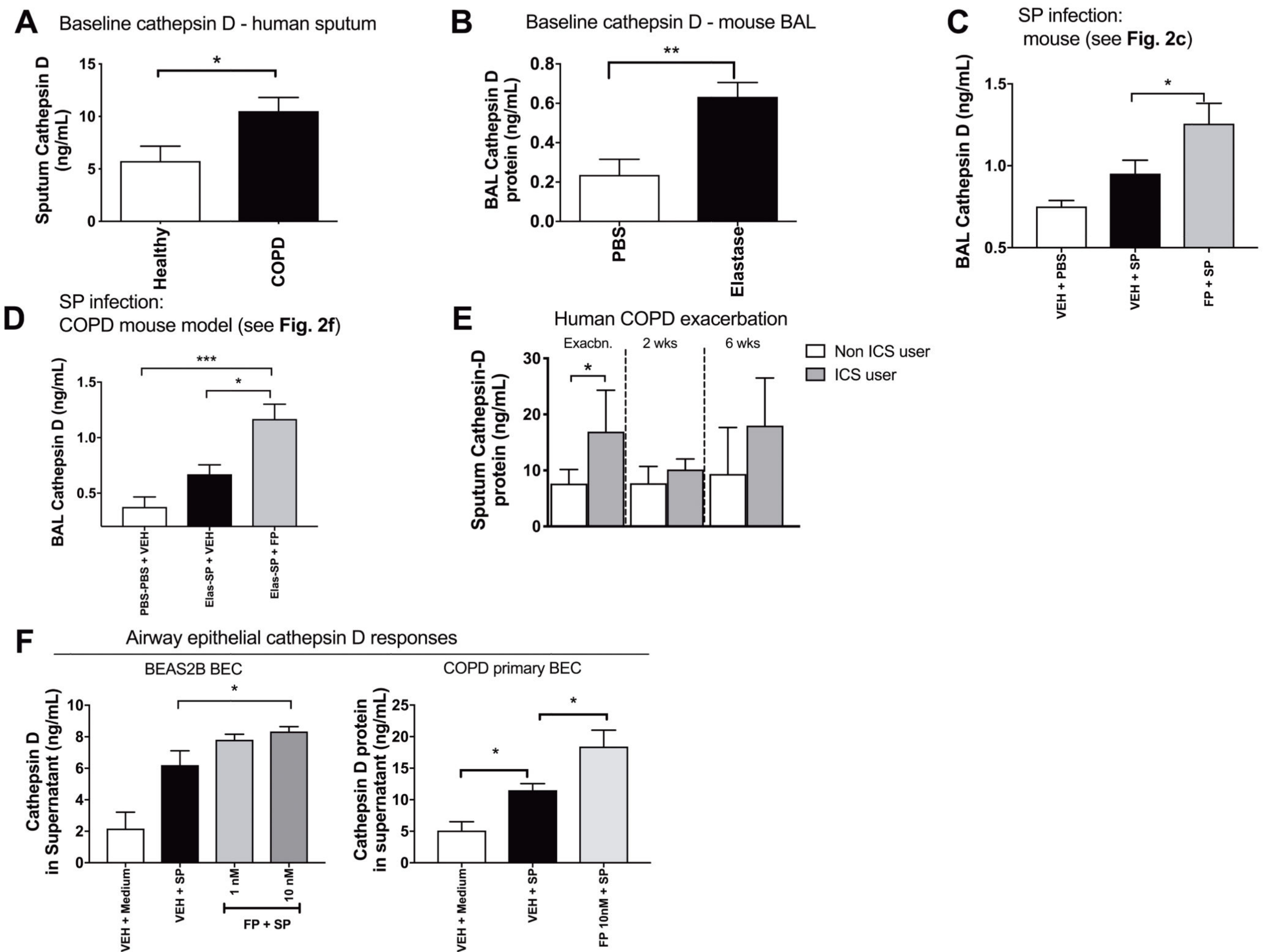

Figure 5. Airway cathepsin D is increased in COPD and further enhanced by inhaled corticosteroid administration during bacterial infection.

(A) Stable state sputum cathepsin D concentrations were measured in 37 subjects with COPD (GOLD stage 0-II) and 19 healthy control subjects by ELISA. (B) Cathepsin D concentrations were measured in mice at 10 days following intranasal treatment with 1.2 units of porcine pancreatic elastase or PBS control. (C) C57BL/6 mice were treated intranasally with fluticasone propionate (20μg) or vehicle DMSO control and challenged with *S. pneumoniae* D39. Cathepsin D concentrations in BAL were measured by ELISA at 8 hours post-infection. (D) C57BL/6 mice were treated intranasally with porcine pancreatic elastase or PBS control. Ten days later, mice were treated intranasally with fluticasone propionate, challenged with *S.pneumoniae* D39 and cathepsin D concentrations in BAL measured at 8 hours post-infection. (E) Subjects with COPD (n=27) were monitored prospectively and sputum samples taken during exacerbation. Sputum cathepsin-D concentrations were measured by ELISA at the indicated timepoints. (F, left) BEAS-2B cells were treated with 1 or 10nM fluticasone propionate, stimulated with *S. pneumoniae* D39 and cathepsin D concentrations in cell supernatants were measured at 8 hours. (F, right) Primary airway epithelial cells from 6 subjects with COPD were cultured, treated with 10nM FP,

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stimulated ex vivo with *S. pneumoniae* and cathepsin D concentrations in cell supernatants were measured at 8 hours. In panels (B)-(D) and (F) data shown as mean (+/- sem) and analyzed by one-way ANOVA with Bonferroni's post-test. For human sputum analyses in (A) and (E), data are shown as median (IQR) and analyzed by Mann Whitney *U* test. Animal experiments comprise n=6-8 mice/group, representative of at least two independent experiments. n.s. non-significant *p<0.05 **p<0.01 ***p<0.001.

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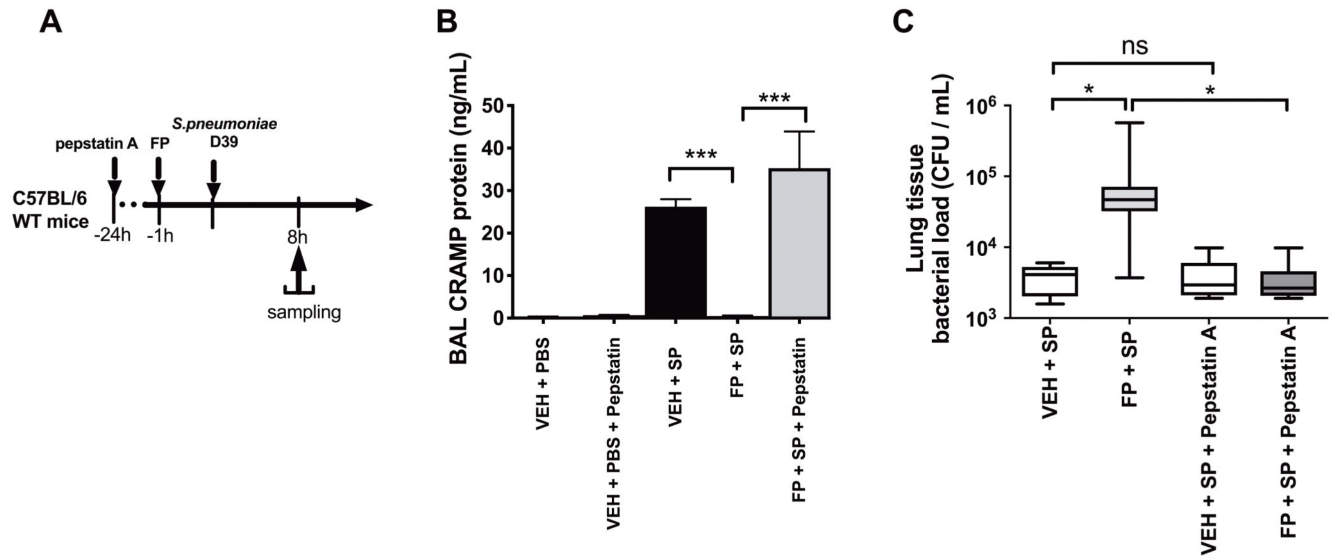

Figure 6. Inhibition of cathepsin D reverses FP-mediated suppression of cathelicidin and restores lung bacterial control.

(A) Experimental outline. C57BL/6 mice were treated with 60mg/kg intraperitoneal pepstatin-A, 24 hours prior to intranasal treatment with 20μg fluticasone propionate or vehicle control and challenge with *S. pneumoniae* D39. (B) CRAMP concentrations in BAL were measured by ELISA at 8h post-infection. (C) Lung bacterial loads were measured by quantitative culture at 8 hours post-infection. Data in (B) shown as mean (+/- S.E.M). Bacterial load data in (C) displayed as box and whisker plots showing median (line within box), IQR (box) and minimum to maximum (whiskers). Animal experiments comprise n=8-10 mice/group, representative of at least two independent experiments. Data analyzed by one-way ANOVA with Bonferroni post-test. n.s. non-significant *p<0.05 **p<0.01 ***p<0.001.

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Table 1
Clinical characteristics of COPD patients included in analyses in figure 1 and 3c.

Data expressed as n(%) or median (IQR) and compared by Fisher's exact test or Mann Whitney U test. Abbreviations: BODE =score comprising parameters of body Mass index, airflow obstruction, dyspnea and exercise; FEV1 = forced expiratory volume in 1 second; GOLD = Global Initiative for chronic obstructive lung disease; ICS = inhaled corticosteroid

	ICS Users (n=10)	ICS Non Users (n=13)	p value
Age	74 (69-78)	68 (61-69)	0.013
Male sex	7 (70%)	9 (69.2%)	1.0
GOLD stage I/II/III/IV	2 (1.8-2)	2 (1.5-2.5)	0.91
FEV_1 % predicted	65 (53.0-80.3)	67 (55.5-78.5)	0.77
BODE index	4 (1.0-5.3)	1 (0.5-3.0)	0.11
Long term oxygen therapy	1 (10.0%)	0 (0%)	0.43
Body Mass Index	26.5 (22.9-28.7)	23.8 (21.9-26.9)	0.50
Current Smoking	1 (10%)	3 (23.1%)	0.60
Comorbidities			
Diabetes Mellitus	0 (0%)	1 (7.7%)	1.0
Hypertension	1 (10.0%)	0 (0%)	0.43
Ischemic Heart Disease	2 (20.0%)	0 (0%)	0.18
Osteoporosis	2 (20.0%)	3 (23.1%)	1.0
Number of exacerbations in preceding year	1 (0-3)	1 (0-1)	0.53
Treatments			
Fluticasone propionate	5 (50%)	n/a	-
Budesonide	4 (40%)	n/a	-
Beclomethasone dipropionate	1 (10%)	n/a	-
Muscarinic antagonist	6 (60.0%)	3 (23.1%)	0.10
Short or Long-acting $Beta_2$ agonist	9 (90.0%)	9 (69.2%)	0.33