SUPPLEMENTARY MATERIAL

Materials and Methods

Experimental challenge studies

For stable-state analyses of sputum hCAP18/LL-37 and other anti-microbial peptides of interest in COPD compared to healthy subjects, baseline (pre-infection) samples taken as part of two studies of experimental infection challenge in COPD(*36, 89*), were utilised. All subjects in these studies gave informed written consent and the protocols were approved by St Mary's NHS Trust Research Ethics Committee (study numbers 00/BA/459E and 07/H0712/138). In these studies, COPD subjects were all GOLD stage 0-II and the only permitted treatment was use of short-acting β_2 agonist therapy as required, thus minimising the confounding effect that therapies such as inhaled corticosteroids might have on anti-microbial peptides. From these studies, samples from a total of 37 subjects with COPD and 19 healthy non-smokers were available for analysis.

Bacteria

S. pneumoniae (serotype 2 [D39]) was cultured in Todd-Hewitt medium with 5% yeast at 37^0C and 5% CO_2. Growth in medium was assessed at an optical density (OD) of 600nM. Stocks were grown to midlog phase (OD at 600nM 0.5) and stored at -80^{o}C in 10% glycerol as 200uL single use aliquots. Bacterial CFU counts were calculated by serial dilution, plating, culture overnight on agar plates containing Columbia plus 5% defibrinated horse blood (Oxoid) and subsequent colony counting.

Bacterial infection and treatment of BEAS-2B cells or primary airway epithelial cells

BEAS-2B airway epithelial cells were cultured to 80% confluence in 12-well plates. Cells were treated with 1mL of 1nM or 10nM FP (Sigma-Aldrich) or vehicle dimethyl sulfoxide (DMSO) in 1mL medium for 1 hour. FP was then removed and replaced with 1mL of medium containing *S. pneumoniae* D39 (1 x 10^6 CFU/mL) and incubated for 24 hours at 37^0C. In separate experiments, cells were additionally treated with 100ng/mL of recombinant cathepsin D protein (*82*)). In other experiments, cells were stimulated with heat-killed *S.pneumoniae* D39 (incubation at 60^0C for one hour as described(*83*) with confirmation of sterility by subsequent

culture on blood agar plates) or agonists to TLR-2 (Pam3CSK; 1µg/mL(*84*)), TLR-9 (CpG ODN; 5µg/mL(*84*)) or NOD-2 (muramyldipeptide; 10 µg/mL(*85*))(all Invivogen).

Primary BECs: Primary BECs were obtained bronchoscopically from six patients with spirometrically confirmed COPD. All subjects gave written consent and the study was approved by Bromley Ethics Committee (REC reference 15/LO/1241). Primary cells were grown in LHC-9 medium (containing 10% sterile filtered fibroblast medium) in collagen coated T75 flasks. When cells were 80% confluent, they were seeded at 2.5 x 10^5 cells per well in 24 well plates. Cells were then treated with 500 µL of 10nM FP and infected with *S.pneumoniae* D39 (1 x 10^6 CFU/mL) as described above for BEAS-2B experiments.

RNA extraction, cDNA synthesis and quantitative PCR

Total RNA was extracted from cell lysates of airway epithelial cell cultures (RNAeasy kit, QIagen) and 2 µg was used for cDNA synthesis (Omniscript RT kit, Qiagen). Quantitative PCR to measure *CAMP* mRNA expression was carried out using the following primer and probe sequences: Forward primer: TCAC CAGA GGAT TGTG ACTT CAA. Reverse primer: TGAG GGTC ACTG TCCC CATA C; Probe: 5' -AAGG ACGG GCTG GTGA GCGG - 3' with normalization to 18S rRNA. Reactions were analyzed using ABI 7500 Fast Realtime PCR system (Applied Biosystems, Carlsbad, CA).

DNA extraction

Genomic DNA was extracted from human sputum and mouse lung tissue using the FastDNA Spin Kit for Soil (MP Biomedicals), according to manufacturer's instructions. Bead-beating was initially performed on sputum plugs or mouse lung tissue at 6800 rpm for two cycles of 30 seconds (Precellys, Bertin Technologies).

16S quantitative PCR

Total 16s bacterial loads were measured using quantitative PCR as previously described(*91*), performed in triplicate using the Viia7 Real Time PCR system (Life Technologies). In addition to template, each PCR reaction mix contained 0.2 µl of forward primer 520F (10µm; 5'-

AYTGGYDTAAAGNG -3'), 0.2 µl reverse primer 802R (10µm; 5'- TACNVGGGTATCTAATCC -3')(*91*), 5 µl SYBR Fast Universal master mix (Kapa Biosystems) and 3.6 µl H_2O. PCR cycling conditions were: Initial denaturation was performed at 90°C for 3 minutes, followed by 40 cycles of 20 seconds denaturation at 95°C, 30 seconds primer annealing at 50°C and a 30 second extension at 72°C. As default, melt curves were run at 15 minutes from 60 to 95°C. For creation of the standard curve, a 1:10 dilution series (10^4- 10^9 copies) of partially cloned 16S rRNA gene of *Vibrio natriegens* DSMZ 759 (Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany) was used.

16S rRNA gene sequencing and data processing

16S rRNA gene sequencing was performed dual barcoded fusion primers to target the V4 region of the 16S gene, as previously described(*91*). Samples were sequenced on the Illumina MiSeq platform (San Diego, USA) using the MiSeq V2 2 x 250bp cycle kit, according to manufacturer's instructions. Downstream processing was performed using QIIME (Quantitative Insights Into Microbial Ecology). Reverse primers and short reads, less than 150bp, were removed and reads were then joined with a minimum overlap of 200bp and less than 10% mismatch. Demultiplexing and quality filtering was carried out to ensure and average quality score of greater than 30 across 70% of the read. Any PhiX contamination was then then removed.

Open reference OTU picking was used to cluster sequences at 97% similarity. The most abundant sequence was chosen as a representative sequence for each OTU cluster. Sequences were aligned using PYNAST to implement the nearest alignment space termination (NAST) algorithm based on the Silva reference database (www.arb-silva.de) prior to chimera checking using Chimera slayer (http://microbiomeutil.sourceforge.net/). UCLUST was used to assign taxonomic identification using the Silva 115 NR database. Sequences were submitted to the European Nucleotide database, project number PRJEB28396. Statistical analyses were performed using R Studio (MA, USA) and GraphPad Prism 6.

***Streptococcus* quantitative PCR**

Quantitative PCR for *Streptococcus* was performed in triplicate using amplification primers for the component of the methionine aminopeptidase (*map*) gene that allows quantitation of members of the *Streptococcus* genus(*92*). The primers used were: *map*-up $^{5'}$ GCWGACTCWTGTTGGGCWTATGC $^{'3}$; *map*-down: $^{5'}$ TTARTAAGTTCYTTCTTCDCCTTG $^{'3.}$ For creation of a standard curve, a serial dilution of DNA isolated from *Streptococcus mitis* strain DSMZ-12643 was used (10^4 to 10^8 copies). PCR cycling conditions were: 90^{o}C for 3 minutes, followed by 40 cycles of 40^{o}C (20 seconds), 55^{o}C (30 seconds) and 72^{o}C (30 seconds).

Protein assays

A commercially available ELISA kit (HK321-01; Hycult Biotech, Netherlands) was used to measure LL-37/hCAP-18 concentrations in cell supernatants or sputum. This assay does not discriminate between LL-37 (the processed form of hCAP-18 protein) and full length, precleaved hCAP18 protein. The lower limit of detection for this assay is 0.14 ng/mL. Other cytokines, chemokine, AMP or surfactant protein concentrations in cell supernatants, mouse BAL or human sputum supernatants were assayed using commercially available ELISA kits, as previously described(*27, 87, 90*). MUC5AC protein in human sputum was measured after adhesion to a 96 well plate allowing samples to evaporate at 37^{0}C, using previously described in-house assays(*27, 87*). The detection antibody used was biotinylated anti-MUC5AC (400ng/mL; ThermoScientific). Standard curves for MUC5AC ELISAs were generated by serial 1:2 dilutions of supernatants taken from ionomycin stimulated H292 cells.

Western blotting was used to characterize hCAP18/LL-37 protein secreted in cell supernatants, using a protocol adapted from a previously published study(*33*). Samples were pooled and concentrated using a Concentrator Plus centrifuge (Eppendorf), resuspended in 60µL SDS-PAGE buffer, loaded onto 4-12% Bis-Tris SDS-PAGE gels. and transferred onto polyvinylidene difluoride (PVDF) membranes (Life Technologies). Positive controls of human COPD sputum supernatants analyzed in prior ELISA analyses, recombinant hCAP18 (Abcam) and LL-37 (Bio-techne) proteins were used. Membranes were blocked with 5% skimmed milk for 2 hours at room temperature. Membranes were then incubated with mouse monoclonal anti hCAP18/LL-37 antibody (1:200 diluted; clone 1.1.C12, Hycult Biotech) in 5% skimmed milk at 4^{0}C with shaking overnight followed by secondary antibody of goat anti-mouse-HRP

conjugated antibody (1:5000 diluted; Jackson Immunoresearch) in 5% skimmed milk for 1 hour at room temperature. Data were acquired using a Fusion FX7 image analyzer (Vilber Lourmat).

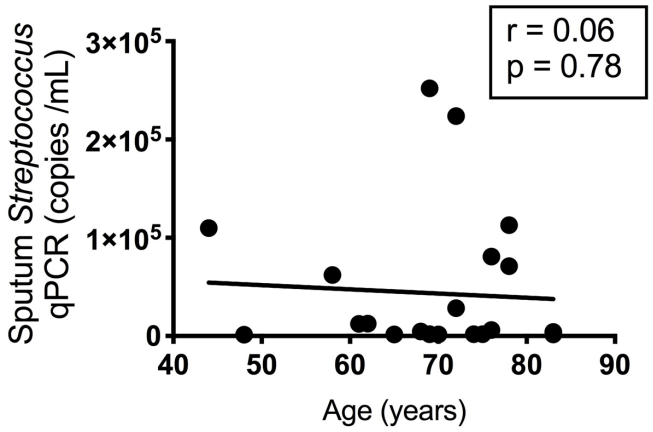

Figure S1: No correlation between Age and *Streptococcal* qPCR copies in subjects with COPD. Correlation analysis used was nonparametric (Spearman's correlation).

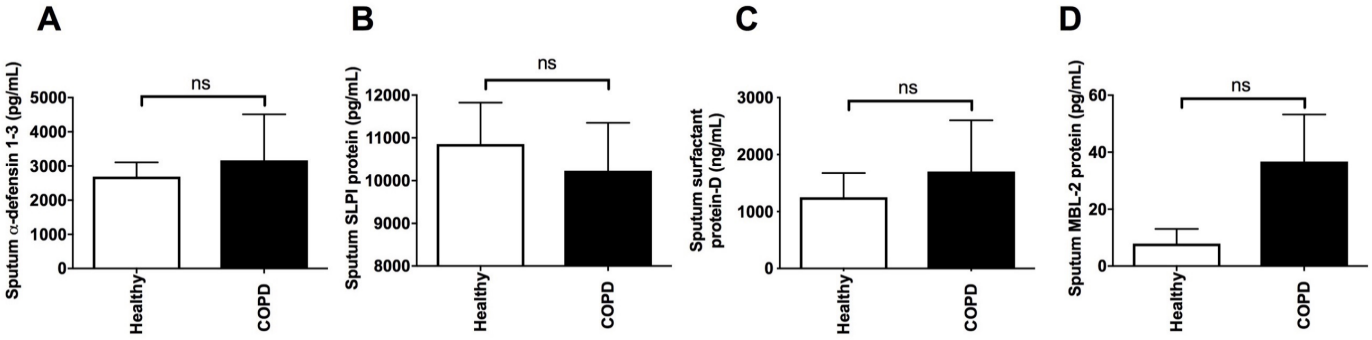

Figure S2: Anti-microbial peptide concentrations in subjects with COPD versus healthy controls. Stable state sputum concentrations of (A) alpha-defensin 1-3 (B) secretory leucocyte protease inhibitor (SLPI) (C) surfactant protein-D and (d) mannose-binding lectin (MBL)-2 were measured in 37 subjects with COPD (GOLD stage 0-2) and 19 healthy control subjects by ELISA. Data displayed as median (+/- IQR) and analysed by Mann Whitney U test. ns = non-significant.

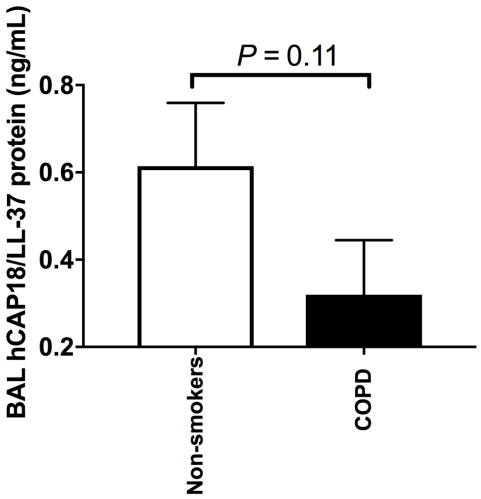

Figure S3: **Bronchoalveolar lavage concentrations of cathelicidin in COPD and healthy subjects.** Stable state bronchoalveolar lavage (BAL) hCAP18/LL-37 concentrations were measured in 15 subjects with COPD (GOLD stage 0-II) and 10 healthy control subjects by ELISA. Data are shown as median (IQR) and analysed by Mann Whitney *U* test.

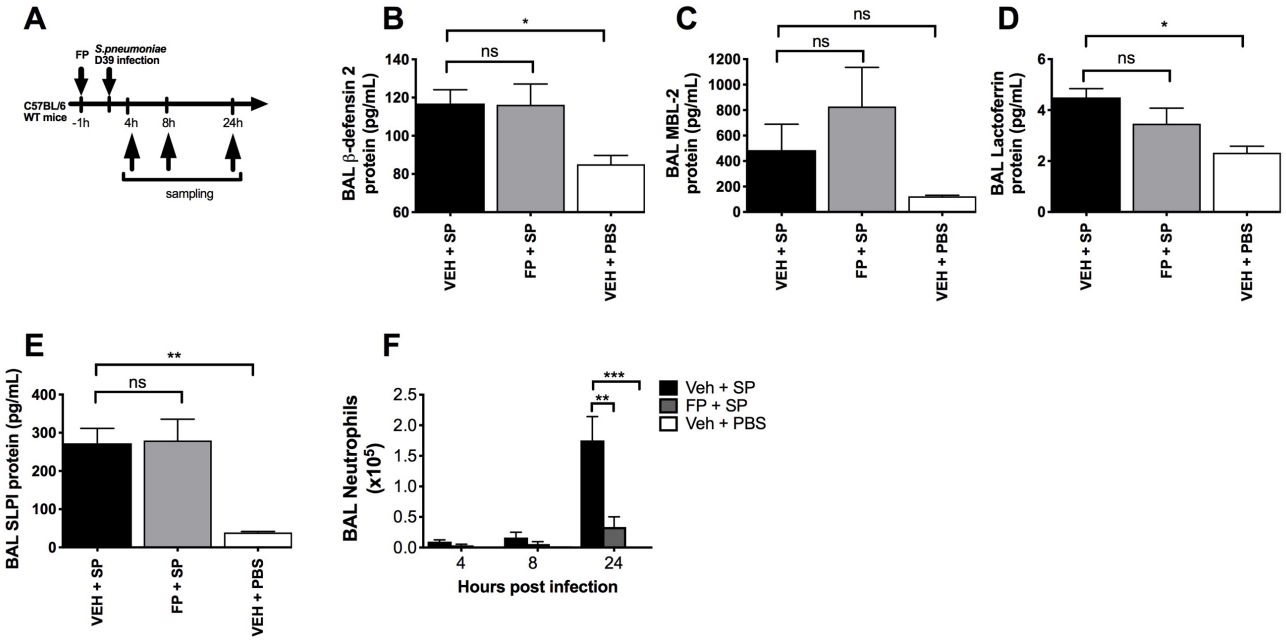

Figure S4: Effect of fluticasone propionate on anti-microbial peptides and neutrophilic inflammation in *S. pneumoniae* infected mice. (A) Experimental outline. C57BL/6 mice were treated intranasally with fluticasone propionate (20μg) or vehicle DMSO control and challenged intranasally with *S. pneumoniae* D39. BAL protein concentrations of (B) beta-defensin-2 (C) Mannose-binding lectin (MBL)-2 (D) Lactoferrin and (E) secretory leucocyte protease inhibitor (SLPI) were measured by ELISA at 8 hours post-infection.(F) Neutrophils numbers were enumerated in BAL by cytospin at the indicated timepoints. Data displayed as mean (+/- s.e.m) and analysed by one way ANOVA with Bonferroni post-test. n=5-6 mice per group representative of two independent experiments. ns = non-significant. *p<0.05 ***p<0.001.

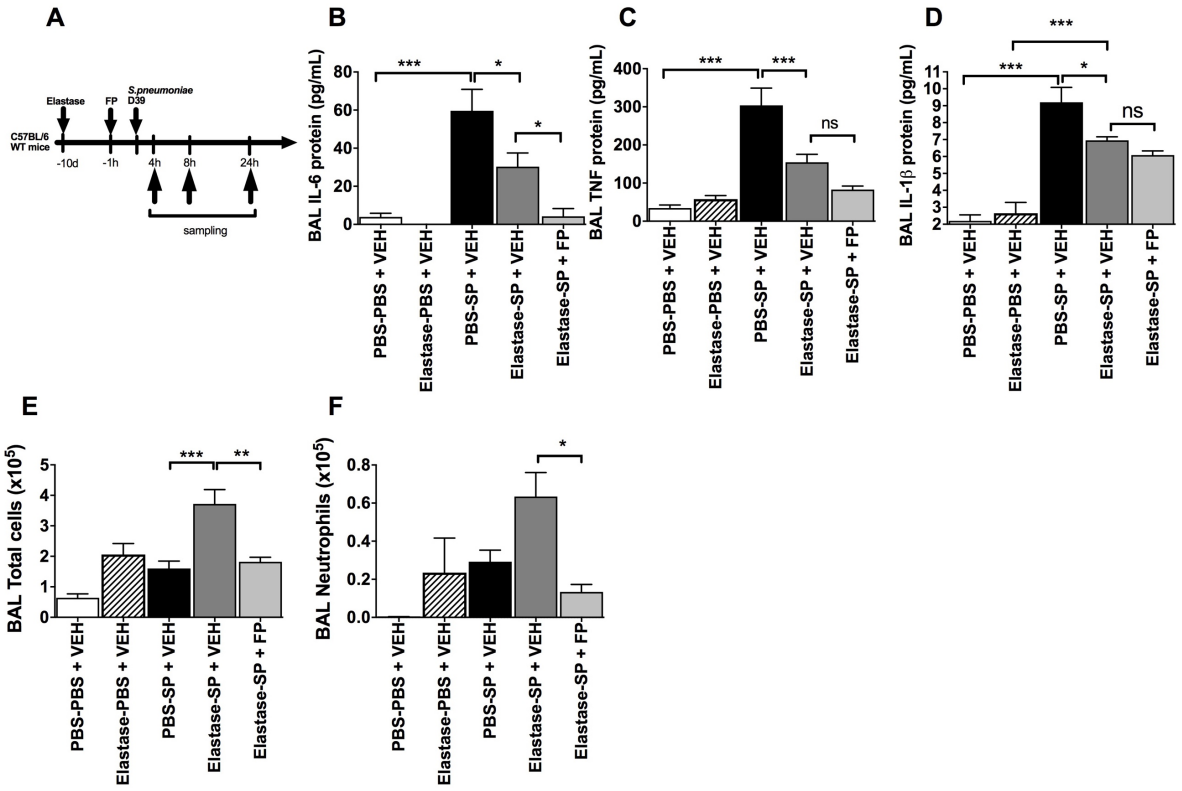

Figure S5: Effect of fluticasone propionate on pro-inflammatory responses in a mouse model of pneumococcal infection in COPD. (A) Experimental outline**.** C57BL/6 mice were treated intranasally with porcine pancreatic elastase or PBS control. Ten days later, mice were treated intranasally with fluticasone propionate (20μg) or vehicle DMSO and challenged with *S. pneumoniae* D39. (B) IL-6 (C) TNF and (D) IL-1β protein concentrations in BAL were.measured by ELISA at 8h post-infection. (E) Total cells and (F) Neutrophil numbers were enumerated by cytospin at 24h post-infection. Data shown as mean (+/- s.e.m) for n=5 mice per group, representative of at least 2 independent experiments. Data analysed by one way ANOVA with Bonferroni's post-test. n.s. non-significant *p<0.05 **p<0.01 ***p<0.001.

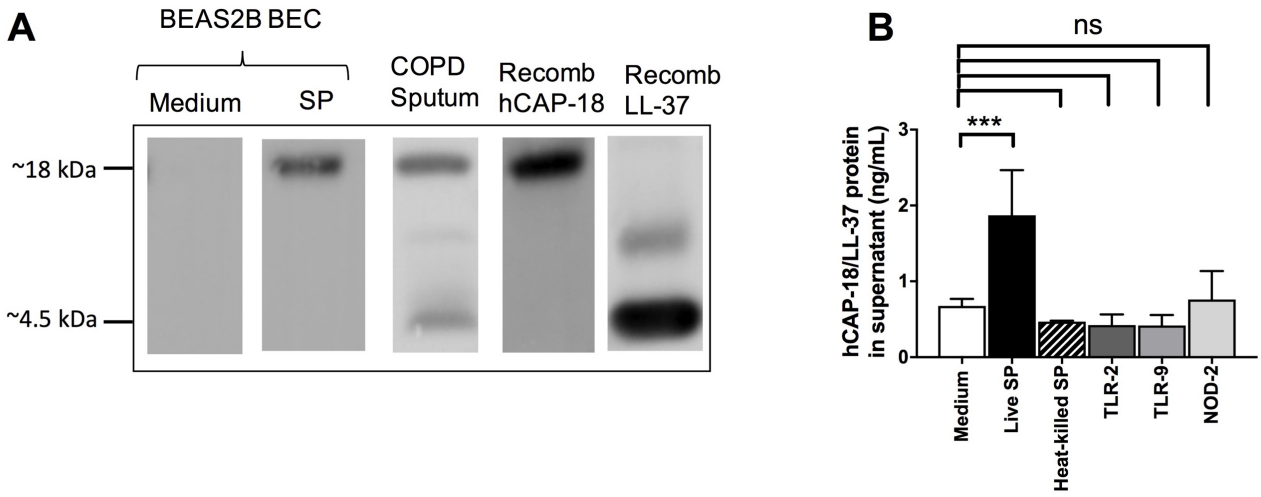

Fig S6: Western blot analysis to characterize secreted cathelicidin in airway epithelial cell cultures and evaluation of hCAP18/LL-37 induction following stimulation with live *S. pneumoniae* in comparison with heat-killed *S. pneumoniae* or agonists to TLR-2, -9 and NOD-2. (A) BEAS-2B cells were stimulated with *S. pneumoniae* D39 or medium control and hCA18/LL-37 release in cell supernatants was measured by Western blot analysis. Positive controls to show intact hCAP18 at 18kDa and/or LL-37 at 4.5kDa including human COPD sputum supernatants, recombinant hCAP18 protein and recombinant LL-37 protein are shown. Blot shown is representative of three independent experiments. (B) BEAS-2B cells were stimulated with live *S. pneumoniae* D39, heat-killed *S. pneumoniae* D39 or agonists to TLR-2 (Pam3CSK; 1µg/mL), TLR-9 (CpG ODN; 5µg/mL) or NOD-2 (muramyldipeptide; 10 µg/mL). hCAP18/LL-37 protein concentrations in cell supernatants were measured at 8 hours by ELISA. Data is shown as median (IQR) comprising three independent experiments and analysed by one-way ANOVA with Bonferroni post test. ***$p<0.001$; ns = non-significant.

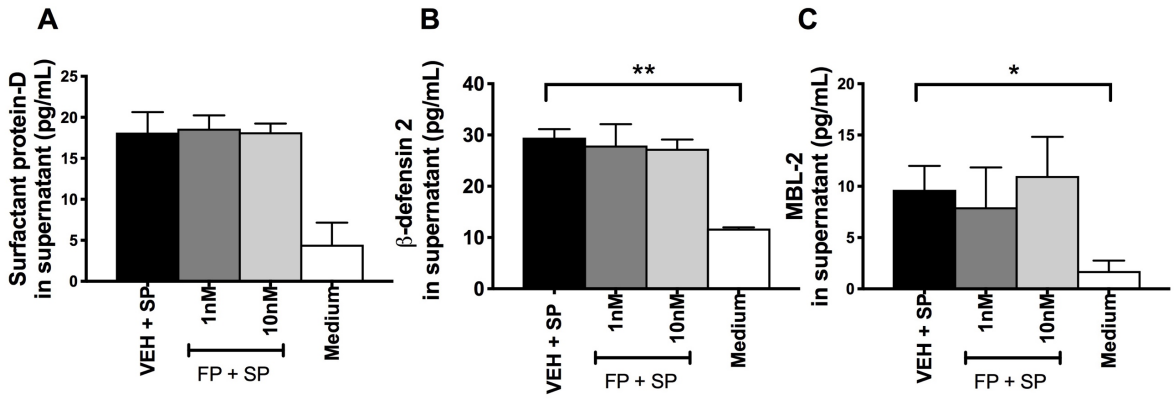

Figure S7: Effect of fluticasone propionate on induction of anti-microbial peptides by *Streptococcus pneumoniae* in airway epithelial cells. BEAS-2B cells were treated with 1 or 10nM fluticasone propionate and stimulated with *S. pneumoniae* D39. (A) surfactant protein-D (B) beta-defensin-2 and (C) MBL-2 concentrations in cell supernatants were measured by ELISA. Data displayed as median (+/- IQR) In (A-D) data analysed by Mann Whitney U test. Data comprises 4 independent experiments and analysed by one-way ANOVA with Bonferroni post-test. ns = non-significant. *p<0.05 **p<0.01.

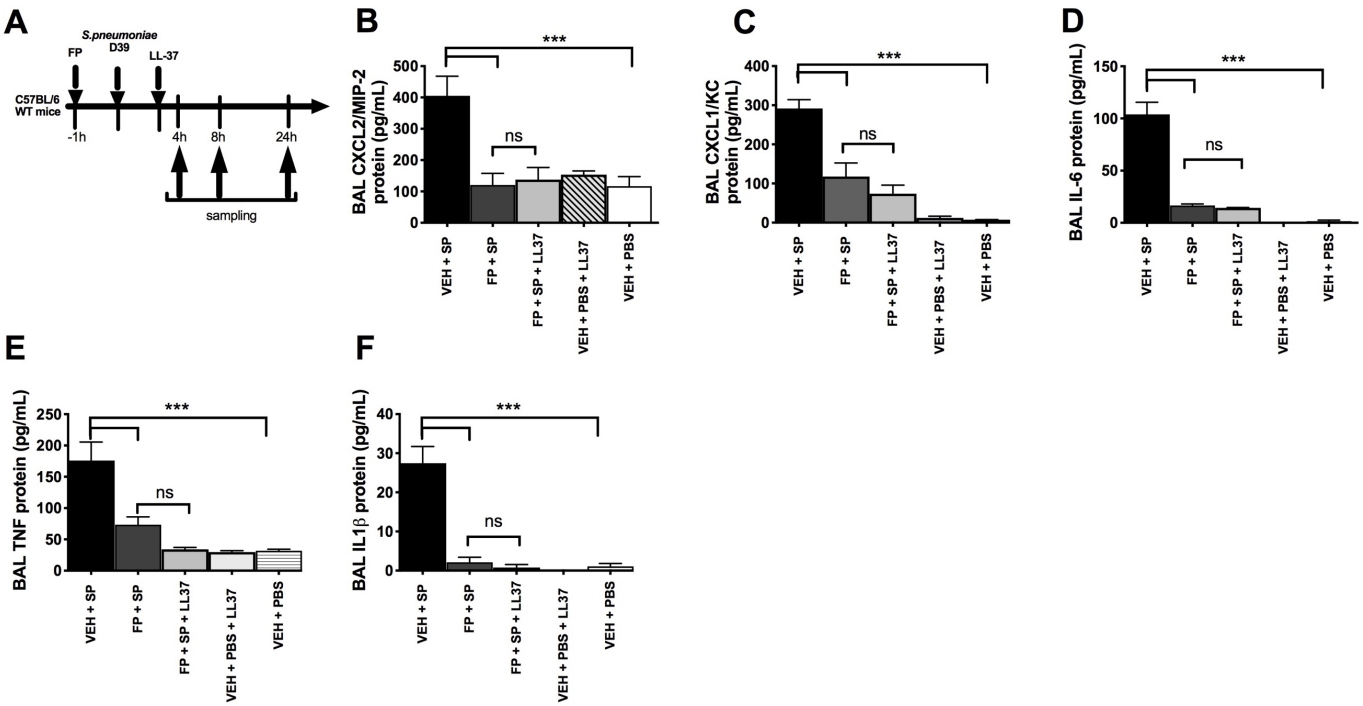

Figure S8: Exogenous cathelicidin does not restore pro-inflammatory responses suppressed by FP. (A) Experimental outline. C57BL/6 mice were treated intranasally with 20ug fluticasone propionate or vehicle control, challenged with *S. pneumoniae* D39 and additionally treated with 10 ug recombinant LL-37. (B) CXCL2/MIP-2. (C) CXCL1/KC, (D) IL-6 (E) TNF and (F) IL-1β were measured in bronchoalveolar lavage by ELISA. Data shown as mean (+/- s.e.m) for n=5 mice/group, representative of 2 independent experiments and analysed using one-way ANOVA with Bonferroni post-test. ns = non-significant. **p<0.01, ***p<0.001.

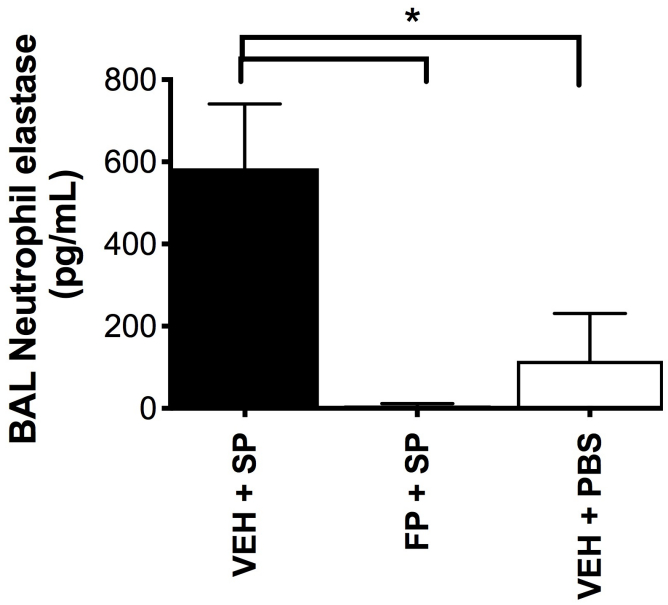

Figure S9: Fluticasone propionate suppresses neutrophil elastase induction by *S.pneumoniae* in mice.

C57BL/6 mice were treated intranasally with fluticasone propionate (20μg) or vehicle DMSO control and challenged intranasally with *S. pneumoniae* D39. Neutrophil elastase concentrations in bronchoalveolar lavage were measured at 24 hours post-infection by ELISA. Data shown as mean (+/- s.e.m) for n=5 mice/group, representative of 2 independent experiments and analysed using one-way ANOVA with Bonferroni post-test. ns = non-significant. *$p<0.05$.

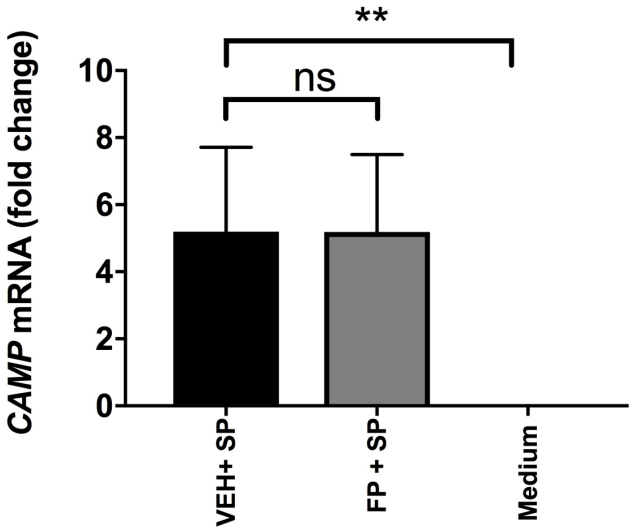

Figure S10: No effect of fluticasone propionate on *S.pneumoniae* induction of *CAMP* mRNA. BEAS-2B cells were treated with 10nM fluticasone propionate and stimulated with *S. pneumoniae* D39. Cell lysastes were collected at 8h post stimulation and relative *CAMP* mRNA was measured by quantitative PCR. Data is shown as median (IQR) comprising three independent experiments and analysed by one-way ANOVA with Bonferroni post-test. *p<0.05; ns = non-significant.

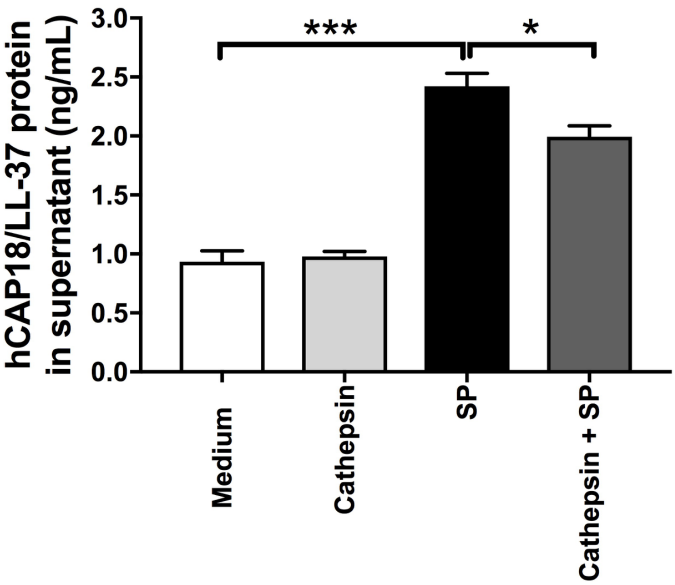

Figure S11: Exogenous Cathepsin D protein administration attenuates *S. pneumoniae* induction of cathelicidin in airway epithelial cells. BEAS-2B airway epithelial cells were treated with recombinant cathepsin-D protein (100ng) and stimulated with *S. pneumoniae* D39. hCAP18/LL-37 protein concentrations in cell supernatants were measured at 8 hours by ELISA. Data is shown as median (IQR) comprising four independent experiments and analysed by one-way ANOVA with Bonferroni post-test. *p<0.05 ***p<0.001; ns = non-significant

	COPD subjects (n = 37)	**Healthy subjects (n = 19)**	**p value**
Age	60 (53-67)	61 (53-64)	0.62
Male	24 (64.9%)	9 (47.4%)	0.26
FEV_1 L	2.07 (1.61 – 2.24)	2.68 (1.98 – 3.44)	<0.001
FEV_1 % predicted	66 (61.5 – 74.5)	105 (94-114)	<0.001
Short acting $beta_2$ agonist use	5 (13.5%)	0 (0%)	0.16

Table S1: Clinical characteristics of subjects included in stable state measurements of AMPs and cathepsin D in sputum (Figs 3a, 5b and S2). Data expressed as n(%) or median (IQR) and analysed by Fisher's exact test or Mann Whitney U test, Abbreviations: FEV1 = forced expiratory volume in 1 second.

	COPD subjects (n = 15)	**Healthy subjects (n = 10)**	**p value**
Age	61 (52-66)	61 (54-64)	0.95
Male	10 (66.6%)	5 (50%)	0.44
FEV_1 L	2.16 (1.74-2.39)	2.68 (2.56-3.39)	0.0043
FEV_1 % predicted	64 (60.5 -69.5)	101 (94-105)	<0.001
Short acting $beta_2$ agonist use	3 (20%)	0 (0%)	0.25

Table S2: Clinical characteristics of subjects included in stable state measurements of hCAP18/LL-37 in bronchoalveolar lavage (Fig S3). Data expressed as n(%) or median (IQR) and analysed by Fisher's exact test or Mann Whitney U test, Abbreviations: FEV1 = forced expiratory volume in 1 second.

	ICS users (n=11)	ICS non-users (n=16)	p value
Age (median (IQR))	72 (62-74)	67 (48-72)	0.20
Male	7 (63.6%)	10 (62.5%)	1.0
GOLD stage (median(IQR))	2 (2-2)	2(2-3)	0.36
Ischaemic heart disease	2 (18.2%)	1 (6.3%)	0.55
Diabetes mellitus	1 (9.1%)	1 (6.3%)	1.0
Cerebrovascular disease	2(18.2%)	0 (0%)	0.16
Prior annual exacerbation frequency (median (IQR))	3 (0-6)	1 (1-2)	0.45
Prior influenza vaccination	8 (72.7%)	11 (68.8%)	1.0
$Beta_2$ agonist inhaler use	10 (90.9%)	12 (75.0%)	0.62
Anti-muscarinic inhaler use	8 (72.7%)	11 (68.8%)	1.0
Prednisolone initiated at exacerbation	4 (36.4%)	1 (6.3%)	0.13
Antibiotics initiated at exacerbation	7 (63.6%)	7 (43.8%)	0.44

Table S3: Characteristics of patients with COPD exacerbations stratified according to current ICS use. Data expressed as n(%) or median (IQR) and analysed by Fisher's exact test or Mann Whitney U test. Abbreviations: GOLD: Global Initiative for chronic obstructive lung disease; ICS = inhaled corticosteroid

Patient	Age	Sex	Current smoker	Number of exacerbations in last year	Comorbidities	ICS use
1	72	Male	No	0	Hypertension, Diabetes	No
2	66	Male	No	0	Nil	Yes
3	65	Female	Yes	2	Hypertension	No
4	70	Female	No	2	Nil	No
5	72	Male	Yes	0	Hypertension Osteoporosis	Yes
6	70	Female	No	2	Nil	Yes

Table S4: Clinical characteristics of COPD patients included in primary airway epithelial cell experiments (figs 3h, 5f)