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Abstract

COPD patients have increased susceptibility to airway bacterial colonisation. *Haemophilus influenzae (H. influenzae), Moraxella catarrhalis (M. catarrhalis)* and *Streptococcus pneumoniae (S. pneumoniae)* are three of the most common respiratory bacterial species in COPD. *H. influenzae* colonisation, but not other bacteria, in COPD patients is associated with higher sputum neutrophil counts.

Alveolar macrophages are key in clearance of bacteria as well as releasing mediators to recruit and activate other immune cells in response to infection.

The aim was to characterise differences in COPD macrophage responses to *H. influenzae, M. catarrhalis* and *S. pneumoniae*, focusing on release of inflammatory and chemotactic mediators, and apoptosis regulation.

Lung macrophages and monocyte derived macrophages from COPD patients and control subjects were exposed to *H. influenzae, M. catarrhalis* or *S. pneumoniae*. Cytokine secretion (TNF-α, IL-6, CXCL8, CCL5 and IL-1β) were measured by ELISA and RT-qPCR, and apoptosis genes MCL-1, BCL-2, BAX and BAK1 by RT-qPCR. Apoptosis and ROS release were also measured.

Macrophages responded differentially to the bacterial species; with increased, prolonged production of the neutrophil chemoattractant CXCL8 in response to *H. influenzae* and *M. catarrhalis* but not *S. pneumoniae*. *S. pneumoniae* initiated macrophage apoptosis and ROS release, *H. influenzae* and *M. catarrhalis* did not, and increased anti-apoptosis gene (BCL-2 5.5 fold and MCL-1 2.4 fold respectively) expression.

Differential cytokine responses of macrophages to these bacterial species can explain neutrophilic airway inflammation associated with *H. influenzae,* but not *S. pneumoniae* in COPD. Furthermore, delayed macrophage apoptosis is a potential mechanism contributing to inability to clear *H. influenzae*.

Introduction

Persistent cigarette smoking causes neutrophilic airway inflammation, which is further increased in patients with chronic obstructive pulmonary disease (COPD) [1]. Neutrophilic airway inflammation causes tissue remodelling, mucus hyper-secretion and alveolar destruction [2]. Indeed, a recent study demonstrated an association between the magnitude of neutrophilic infiltration in the small airway walls and the degree of loss of alveolar attachments [3]. However, COPD is a heterogeneous disease, and the degree of neutrophilic inflammation varies considerably between individuals [2].

There is increased susceptibility to bacterial infection in COPD, with common pathogens being *Haemophilus influenzae (H. influenzae), Moraxella catarrhalis (M. catarrhalis)* and *Streptococcus pneumoniae (S. pneumoniae)* [4, 5]. Bacterial colonisation during the stable state in COPD patients is associated with higher neutrophil cell counts in sputum and broncho-alveolar lavage samples [6, 7]. Recent COPD cohort studies using quantitative polymerase chain reaction (qPCR) on sputum samples have shown an association between *H. influenzae* and sputum neutrophil counts, while this relationship with neutrophilia does not exist for other bacteria [8-11]. Similarly, 16S RNA-gene based microbiome analysis of sputum samples has demonstrated associations between neutrophil counts and both the Proteobacteria phylum and *Haemophilus* genus [12]. The clinical relevance of these findings was highlighted by a cohort study reporting a positive association between Proteobacteria abundance and mortality in COPD patients [13].

The underlying mechanisms responsible for the differential host (neutrophilic) immune response to *H. influenzae*, in contrast to other bacterial species, remains unclear. Alveolar macrophages may play a key role here, as these cells provide first-line host defence against

bacteria through phagocytosis and the release of mediators to recruit and activate other immune cells [14]. Alveolar macrophages exposed to bacterial ligands secrete chemokines, including CXCL8, that enable neutrophil chemotaxis [15]. Alveolar macrophages from COPD patients have reduced phagocytic ability [16, 17]; this has been demonstrated for both *H. influenzae* and *S. pneumoniae*. Studies in human and murine macrophages have shown that *S. pneumoniae* exposure causes downregulation of expression of the anti-apoptotic protein Mcl-1, which causes increased macrophage apoptosis and bacterial killing [18, 19]. We hypothesized that the observed associations between sputum neutrophilia and *H. influenzae* colonisation, but not other bacterial species, are due to differential alveolar macrophage responses to bacterial species, including induction of chemokines and regulation of macrophage apoptosis.

The aim of this study was to characterise COPD macrophage responses to *H. influenzae, M. catarrhalis* and *S. pneumoniae*, focusing on the release of chemotactic mediators and the expression of proteins involved in apoptosis regulation. The purpose of these investigations was to elucidate the mechanisms responsible for the observed association in COPD cohort studies between sputum neutrophil counts and *H. influenzae*, but not other bacterial species [8-11].

Methods

Subjects

Ten COPD patients and 10 healthy non-smoking (HNS) controls were recruited from the Medicines Evaluation Unit (Manchester University NHS Foundation Trust) and eight patients undergoing surgical resection for suspected lung cancer (Manchester University NHS Foundation Trust) were recruited (4 ex-smokers (ES) without airflow obstruction and 4

COPD patients) (Table 1). COPD patients had a smoking history of ≥10 pack years, and had evidence of airflow obstruction according to GOLD criteria [20]. All patients provided written informed consent using protocols approved by local Ethics Committees (10/H1016/25 and 20/NW/0302).

Cell culture

Experiments were initially performed using lung macrophages isolated from resected lung tissue as previously described [21] (see online supplement). However, due to the COVID-19 pandemic lung resection samples were unobtainable and subsequent experiments were carried out using monocyte derived macrophages (MDMs). MDMs were generated from PBMCs by culture with GM-CSF 10ng/ml for 7 days (full details online supplement). Macrophages were stimulated with unopsonised *H. influenzae* (National Collection of Type Cultures operated by Public Health England [NCTC] 12699), *M. catarrhalis* (NCTC 11020) or *S. pneumoniae* (NCTC 12977) at multiplicity of infection (MOI) stated in text or left unexposed for 4, 24 and 72 hours. See online supplement for bacterial preparation. Preliminary experiments showed 5:1 to be an optimal MOI for *H. influenzae, M. catarrhalis* and *S. pneumoniae* comparison. Also, no significant differences between MDM cytokine responses after exposure to opsonised or unopsonised bacteria were observed (Supplement Figure 2).

Supernatant cytokine levels [tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, C-X-C motif chemokine ligand (CXCL)-8 and chemokine (C-C motif) ligand 5 (CCL)-5] were measured by ELISA according to manufacturers' instruction (R & D system, UK). The lower limits of quantification were 15.6 pg·mL-1 for TNF-α, 9.4 pg·mL-1 for IL-6, 31.3 pg·mL-1 for CXCL8 and CCL5 and 3.9 pg·mL-1 for IL-1β. Supernatant levels of reactive oxygen species (ROS) were measured using Dihydrorhodamine 123 (D,123). Cells were harvested for mRNA extraction and concentrations measured (Supplement Figure 3). Samples were

normalised to generate equal concentrations of cDNA for qPCR analysis of TNF-α, IL-6, CXCL8, IL-1β, the anti-apoptotic genes: induced myeloid leukemia cell differentiation protein (MCL-1) and B-cell lymphoma 2 (BCL-2), pro-apoptotic genes: BCL2 associated x protein (BAX) and BCL2 homologous antagonist/killer (BAK1) and the endogenous control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (full methods see online supplement). The percentage of cells undergoing apoptosis was determined using *In Situ* Cell Death Detection Fluorescein kit (ROCHE, UK) (full methods see online supplement) with a minimum of 50 cells per condition counted across multiple fields of view to determine percentage positive staining.

Statistical analysis

Normality of data was assessed using Shapiro-Wilk normality test. For ELISA data two way ANOVA followed by Bonferroni's multiple comparison tests were used to compare between bacterial treatments and between subject groups. One way ANOVA followed by Tukey's test was also used to compare between bacterial treatments for apoptosis and ROS release data. Gene expression data was non-parametric and Kruskal-Wallis test with Dunn's post hoc analysis used to compare between bacterial treatments. $p < 0.05$ was considered statistically significant. All analyses were performed using GraphPad Prism version 5.02 (San Diego, USA).

<u>Results</u>

Bacterial stimulation of macrophage cytokine production

Protein secretion from COPD and Control MDMs and lung macrophages

MDMs from 10 HNS and 8 COPD patients and lung macrophages from 4 ES and 4 COPD patients were cultured with *H. influenzae, M. catarrhalis* or *S. pneumoniae* at increasing MOIs (0.005-5:1) for 24 hours (Supplementary Figure 3 and 4 respectively). Full MOI curve data showed that the highest levels of cytokine induction were reached at MOI 5:1. Figure 1 shows cytokine levels at MOI 5:1; *H. influenzae* and *M. catarrhalis* caused upregulation of TNF-α, IL-6 and CXCL8 secretion compared to control (cells not exposed to bacteria), while *S. pneumoniae* had no effect in either MDMs or lung macrophages. In both MDMs and lung macrophages, *M. catarrhalis* caused significant upregulation of all cytokines. *H. influenzae* caused significant upregulation of cytokine secretion in HNS MDMs and lung macrophages from both ES and COPD.

Comparisons between bacterial species showed significantly higher levels of TNF-α and IL-6 secretion for *H. influenzae* and *M. catarrhalis* and CXCL8 secretion for *M. catarrhalis* exposed cells compared to *S. pneumoniae*. TNF-α levels were significantly greater with *M. catarrhalis* exposure compared to *H. influenzae* in COPD MDMs, although this was not observed in lung macrophages.

Cytokine levels were similar in HNS compared to COPD patients from MDMs (n=10 and 8 respectively), and ES compared to COPD patients in lung macrophages (n=4 and 4 respectively). Subanalysis of the COPD group showed no differences in cytokine levels in patients treated with ICS compared to those without (Supplement Figure 5).

In a subset of COPD patients (n=7) CCL5 and IL-1β levels from MDMs were also measured after 24 hours exposure to all three bacteria at MOI 5:1. CCL5 and IL-1β levels were significantly increased after exposure to *H. influenzae* and *M. catarrhalis* but not *S. pneumoniae* (Supplement Figure 6).

Additional time-profile MDM experiments (n=5 COPD) showed that CXCL8 production was significantly greater after 72 hours versus 24 hours exposure to *H. influenzae* and *M. catarrhalis,* and CCL5 after exposure to *M. catarrhalis,* while TNF-α, IL-6 and IL-1β levels were similar at these timepoints (Supplement Figure 7).

The experiments already described used commercially available bacterial strains. We also cultured MDMs from 4 HNS and 4 COPD patients for 24 hours with *H. influenzae* (n=2 strains), *M. catarrhalis* (n=1 strain), and *S. pneumoniae* (n=1 strain) isolated from a single COPD patient (obtained over 2 clinic visits; Supplement Figure 8 and 9). The induction of TNF-α, IL-6 or CXCL8 were similar between the commercial strains and clinically isolated strains for each bacterial species.

Gene expression in MDMs from COPD patients

MDMs from COPD patients (n=5) were cultured with *H. influenzae*, *M. catarrhalis*, or *S. pneumoniae* at an MOI of 5:1 for 4, 24 and 72 hours (Figure 2). *H. influenzae* significantly upregulated expression of TNF-α, CXCL8 and IL-1β at all time-points, and IL-6 at 24 and 72 hours ($p < 0.05$ for all comparisons versus controls). Peak expression was reached at 24 hours for TNF-α and IL-6, and at 72 hours for CXCL8 and IL-1β. The effects of *M. catarrhalis* were numerically lower in comparison to *H. influenzae*, with significant upregulation of gene expression observed at fewer time-points. *S. pneumoniae* had no significant effect on expression of any cytokine.

Apoptosis in MDMs from COPD patients

MDMs from COPD patients (n=6) were cultured with *H. influenzae, M. catarrhalis* or *S. pneumoniae* at an MOI of 5:1 for 24 hours (Figure 3). *H. influenzae* and *M. catarrhalis* caused 7% of cells to undergo apoptosis, with positive staining for TUNEL. Culture with *S. pneumoniae* significantly increased apoptosis compared to control (p=0.017) with 98% of cells staining positive.

MDMs from COPD patients (n=5) were cultured with *H. influenzae*, *M. catarrhalis*, or *S. pneumoniae* at an MOI of 5:1 for 4, 24 and 72 hours (Figure 4). The anti-apoptosis genes MCL-1 and BCL-2 were significantly increased at 24 hours by *M. catarrhalis* 2.4 fold (p=0.04) and *H. influenzae* 5.5 fold (p<0.001) respectively. *H. influenzae*, and *M. catarrhalis* also down regulated pro-apoptosis gene BAX expression at 4 hours (p<0.01 both comparisons) but had no significant effect on BAK1 expression. *S. pneumoniae* significantly down regulated MCL-1 and BAK1 expression after 4 (p=0.04 and 0.036 respectively) and 72 hours (p<0.01 both comparisons) and BAX after 72 hours (p<0.01), with no effect on BCL-2 expression.

Reactive oxygen species (ROS) release in MDMs from COPD patients

MDMs from COPD patients (n=7) were cultured with *H. influenzae*, *M. catarrhalis*, or *S. pneumoniae* at an MOI of 5:1 for 24 hours and supernatant levels of ROS were determined (Figure 5). *H. influenzae* or *M. catarrhalis* caused no increase in ROS release. *S. pneumoniae* significantly increased ROS release from MDMs (p=0.0067).

Discussion

We demonstrate that human lung macrophages and MDMs show differential responses to the respiratory pathogens *H. influenzae, M. catarrhalis* and *S. pneumoniae. H. influenzae* and *M. catarrhalis* induce larger inflammatory cytokine responses compared to *S. pneumoniae*. Importantly *S. pneumoniae* exposure resulted in reduced expression of the anti-apoptotic marker MCL-1, associated with high levels of apoptosis (measured by TUNEL staining) and ROS release. In contrast, *H. influenzae* and *M. catarrhalis* exposure caused gene expression changes that favoured delayed apoptosis.

These differential cytokine responses, particularly the neutrophil chemoattractant CXCL8, provide a mechanistic explanation for the clinical observations that *H. influenzae* is associated with increased neutrophil numbers in COPD patients. In contrast, *S. pneumoniae* caused greater macrophage apoptosis which is likely to be the cause of lower chemokine secretion. The effects of *H. influenzae* and *M. catarrhalis* with regard to delayed apoptosis provides a mechanism by which these bacteria may undergo intracellular survival within the macrophage [22] by evading the microbial killing mechanisms associated with cellular apoptosis [18], and thereby persist as colonising bacteria in the airways.

The time profile of gene and protein expression showed that CXCL8 and IL-1β had a much later peak (at least 72 hours) compared to TNF-α and IL-6 which peaked much earlier. We have previously reported similar patterns of prolonged secretion of CXCL8, in contrast to other inflammatory mediators, from alveolar macrophages [23-25]. CXCL8 plays a major role as a neutrophil chemoattractant in neutrophilic lung inflammation in COPD [15, 26], and the results here demonstrate a mechanism by which *H. influenzae* and *M. catarrhalis*, but not

S. pneumoniae, can cause excessive neutrophil recruitment into the lungs via macrophage activation.

COPD cohort studies have demonstrated a stronger association for neutrophilic inflammation with *H. influenzae* compared to *M. catarrhalis* during the stable state [4, 9, 27]. *M. catarrhalis* has been linked to increased interferon signalling while *H. influenzae* presence is associated with neutrophilia [4] and increased levels of TNF-α, CXCL8, IL-1β and MPO in sputum [9, 27]. However, we did not observe clear differences between *H. influenzae* and *M. catarrhalis* for TNF-α, IL-6, CXCL8 and IL-1β protein secretion, although there was evidence of greater TNF-α, CXCL8 and IL-1β gene expression for *H. influenzae.* These cytokine protein results do not provide an explanation for the greater neutrophilic inflammation profile observed in COPD cohort studies with *H. influenzae* compared to *M. catarrhalis*, when sampling was performed in the stable state. A possible explanation is that *M. catarrhalis* is present in less COPD patients in the stable state, but is a cause of exacerbations when it may cause excessive inflammation [4]. *M. catarrhalis* has shorter periods of colonisation [28], while some *H. influenzae* strains give rise to long-term persistence [29], and the potential to upregulate further the neutrophilic inflammation that exists in COPD.

Although levels of TNF-α, IL-6 and CXCL8 are increased in the airways of COPD patients [30, 31], we observed no differences between COPD and control macrophages. This is consistent with previously published data utilising lung macrophages from COPD patients with no differences in base line or LPS stimulated levels of TNF-α, IL-6 or CXCL8 [21] and *H. influenzae* antigen stimulated TNF-α and CXCL8 in MDMs [32]. However, the absolute numbers of alveolar macrophages are increased in the airways of COPD patients compared to

controls [33] which ex-vivo cell cultures do not take into account. Therefore the increased macrophage numbers may account for the overall raised cytokine levels in the lungs of COPD patients. Comparisons between COPD and control macrophages were limited to cytokine protein levels. It would also be of interest to assess potential differences between groups in cytokine gene expression, apoptosis or ROS release.

We observed similar effects with *H. influenzae* and *M. catarrhalis* on apoptosis and anti-apoptosis gene expression profiles, suggesting that this is not the mechanistic reason for differences between these bacterial species in their ability persist in the airways by evading the host immune response. Other potential explanations are that *H. influenzae* reduces expression of the bacterial recognition markers CD36, CD206 and CD163 in alveolar macrophages [23], which may allow evasion of host defence. Additionally, *H. influenzae* strains identified as persistent colonisers in COPD show phase variation in simple sequence repeats in genes encoding virulence functions, including adhesins (HMW1A/HMW2A), modifications of lipooligosaccharide, and iron uptake (haemoglobin-haptoglobing binding protein) [34]

A previous study investigated alveolar macrophage responses to respiratory bacteria [35]; Although no direct comparisons or statistical analysis were performed, visualisation of the figures suggests lower levels of both CXCL8 and TNF-α were induced in response to *S. pneumoniae* compared to *H. influenzae* or *M. catarrhalis* [35]. Here, we confirm statistical significance for these differences in COPD macrophages.

The lack of stimulatory effect of *S. pneumoniae* on cytokine release could potentially be due to macrophage death and a reduction in cell numbers. Indeed, we observed a reduction in the quantity of mRNA collected from *S. pneumoniae* exposed cells at 24 and 72 hours suggesting

fewer cells. However, the mRNA collected was normalised to generate equal concentrations of cDNA for the PCR assays and the expression data presented is relative to housekeeping genes which would also be reduced. Therefore while it is likely that there were less cells present at the later time points for *S. pneumoniae* exposure, those which were present were expressing lower levels of cytokines. Also the mRNA concentrations at 4 hours were similar between the control and all experiments with bacteria, highlighting that the differences seen for cytokine mRNA expression at 4 hours is not due to a reduced number of cells.

The contributions of different TLR stimulations varies between different bacterial wall components and bacterial species [35]. *S. pneumoniae* is a Gram-positive bacteria with a different wall structure to the Gram-negative *H. influenzae* and *M. catarrhalis.* TLR2 has been shown to play a major part in Gram-positive bacterial recognition [36] while TLR2 and TLR4 mediated cytokine responses are diminished in COPD alveolar macrophages leading to impaired *H.influenzae* clearance [35]. It is likely that differential TLR signalling plays a role in the mechanisms involved in our findings and it would be valuable to investigate specific TLR signalling mechanisms involved in the differential bacterial responses we observed.

It is interesting that *Streptococcus* is one of the most abundant genera in healthy individuals [37], while *S. pneumoniae* levels measured by PCR in the sputum of healthy controls is higher compared to both *H. influenzae* and *M. catarrhalis* [11]. It is possible that the lower induction of pro-inflammatory mediators allows the presence of some *S. pneumoniae* strains to be tolerated at relatively high levels in the airways.

S. pneumoniae induces apoptosis in alveolar macrophages [38, 39], which is required for effective bacterial clearance [18]. Mcl-1 is a key anti-apoptotic protein involved in the switch

from cell viability to apoptosis in alveolar macrophages [18]. Mcl-1 forms heterodimers with pro-apoptotic Bcl-2 family members such as Bax and Bak to inhibit mitochondrial membrane permeabilisation [40]. The decrease of Mcl-1 expression in macrophages after 14-16 hours exposure to *S. pneumoniae* causes apoptosis and therefore killing and clearance [18]. Over expression of MCL-1 in a murine model decreases the caspase-dependent mitochondrial ROS production associated with this late-phase intracellular killing of *S. pneumoniae*, and thereby increases neutrophil recruitment [41]. While we observed a decrease in the pro-apoptotic gene BAK-1 upon *S. pneumoniae* exposure, it is possible this is not a true reflection of apoptotic state and the ratio of BAX/Bcl-2 protein may have been a better measurement. However consistent with these previous reports, we showed a decrease in the expression of MCL-1, and importantly an increase in macrophage apoptosis and ROS production following exposure to *S. pneumoniae*.

COPD macrophages are defective in apoptosis associated killing and clearance of *S. pneumoniae* infection regulated by Mcl-1 [19]. MCL-1 expression in alveolar macrophages from COPD patients is higher both at baseline and following exposure to *S. pneumoniae* leading to decreased apoptosis compared to controls. Higher levels of Mcl-1 in COPD also reduced mROS production after bacterial challenge [19]. We did not study regulation of Mcl-1 in COPD versus controls, as this has been well documented as above. However, we demonstrate differential regulation of apoptosis and anti-apoptosis genes by *S. pneumoniae*, in comparison to *H. influenzae* and *M. catarrhalis*, that can contribute to different outcomes with regard to the host immune response.

A limitation of this analysis is that due to the COVID-19 pandemic the availability of surgical samples and as such alveolar macrophages was limited leading to the use of MDMs for many of the experiments. While MDMs are not truly identical to alveolar macrophages, MDMs from COPD patients show dysfunctional behaviour compared to controls [42, 43] and therefore represent a relevant model. Also, although we showed differential bacterial effects on the release of ROS by the macrophages, the assay utilised here was not specific and it would be of interest to further investigate the effects on caspase-1 induced mitochondrial ROS which is key in mechanism of bacterial clearance and dysfunctional in COPD macrophages [19, 43].

We show differential macrophage cytokine secretion in response to the common bacterial species found in COPD which can explain the excessive neutrophilic airway inflammation observed with *H. influenzae,* but not *S. pneumoniae*. Furthermore, the regulation of macrophage apoptosis provides a potentially important mechanism contributing to persistent *H. influenzae* colonisation in COPD.

Data Availability Statement

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Author Contributions

SL, JB, RG, DP and ADB performed experimentation. RS was the lead surgeon for the study. SL, AB and DS designed the study. All authors were involved in analysis and interpretation of the data, and preparation of the manuscript with major contributions from SL and DS All authors read and approved the final manuscript.

Tables

	Monocyte derived macrophages (MDMs)			Lung Macrophages		
	HNS	COPD	p value	Ex-Smoker	COPD	p value
n	10	10	-	4	4	-
Age (Years)	32 [23-56]	67 [53-73]	<0.0001	74 [71-83]	67 [59-78]	0.49
Gender: M/F	6/4	5/5	-	0/4	2/2	-
FEV$_1$ (L)	3.4 (0.6)	1.5 (0.8)	<0.0001	2.3 (1.1)	1.7 (0.4)	0.30
FEV1 % predicted	99 (11)	53 (21)	<0.0001	82 (20)	77 (10)	0.60
FVC (L)	4.5 (1.1)	3.3 (1.1)	0.03	3.3 (1.5)	2.6 (0.6)	0.4
FEV1/FVC Ratio (%)	76 (7)	37 (16)	<0.0001	77 (6)	65 (2)	0.1
Current smokers (%)	N/A	20	-	0	50	-
Pack years	N/A	39 (11)	-	12 (14)	50 (26)	0.2
ICS usage (%)	N/A	60	-	N/A	25	-

Table 1 –Demographics for COPD subjects and Ex-Smoking and Healthy Non-Smoking controls: FEV$_1$ forced expiratory volume in 1 second; FVC forced vital capacity; ICS inhaled corticosteroids. Data presented as median [range] or mean (SD) as appropriate.

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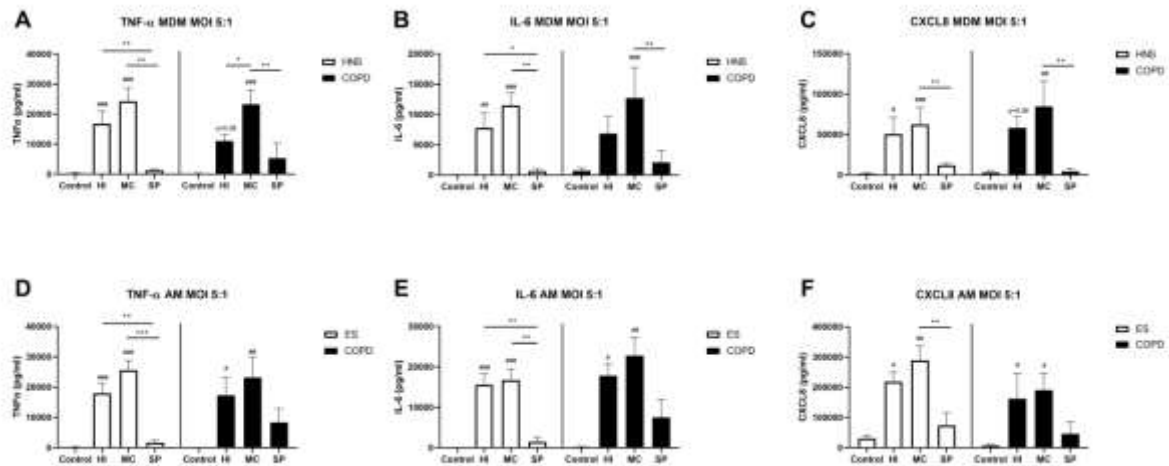
Figure 1. Effects of bacterial exposure on macrophage cytokine production

Monocyte derived macrophages (MDMs) from 10 healthy non-smokers (HNS) and 8 COPD patients (A-C) or lung macrophages from 4 ex-smokers (ES) and 4 COPD patients (D-F) were exposed to *Haemophilus influenzae* (HI), *Moraxella catarrhalis* (MC) or *Streptococcus pneumoniae* (SP) at a multiplicity of infection (MOI) of 5:1 or left unexposed (Control) for 24 hours. Supernatant levels of TNF-α (A&D), IL-6 (B&E) and CXCL8 (C&F) were measured by ELISA. Data presented as mean + SEM. Levels were compared between conditions using two way ANOVA followed by a Bonferroni's multiple multiple comparisons test.

#,##,### = significantly above unexposed control (p<0.05, 0.01, 0.001 respectively)

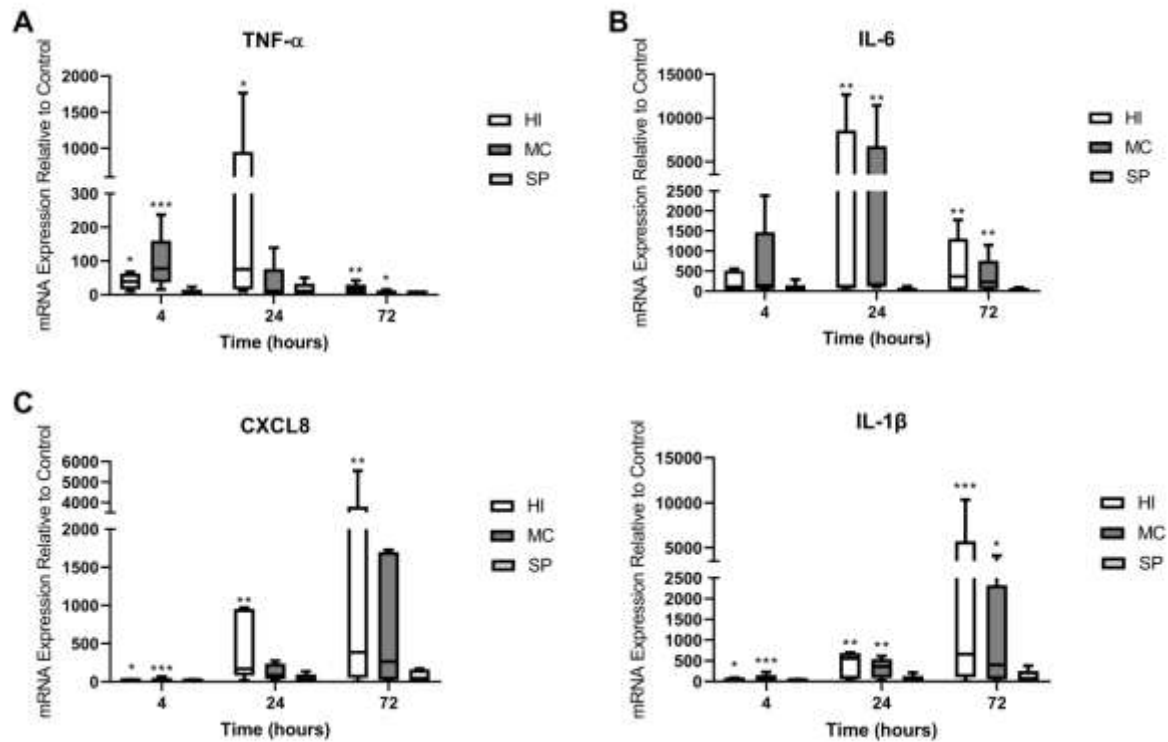*,**,*** = significant difference between bacterial species (p<0.05, 0.01, 0.001 respectively)

Figure 2 Effects of bacterial exposure on monocyte derived macrophage cytokine gene expression

Monocyte derived macrophages (MDMs) from 5 COPD patients were exposed to *Haemophilus influenzae* (HI), *Moraxella catarrhalis* (MC) or *Streptococcus pneumoniae* (SP) at a multiplicity of infection (MOI) of 5:1 or left unexposed (Control) for 4, 24 or 72 hours. mRNA expression levels of TNF-α (A), IL-6 (B), CXCL8 (C) and IL-1β (D) were measured by RT-qPCR. Data presented as median ± range. RT-qPCR data expression relative to endogenous control ($2^{-\Delta\Delta C_t}$).

*,**,*** = significantly above unexposed control (p<0.05, 0.01, 0.001 respectively)
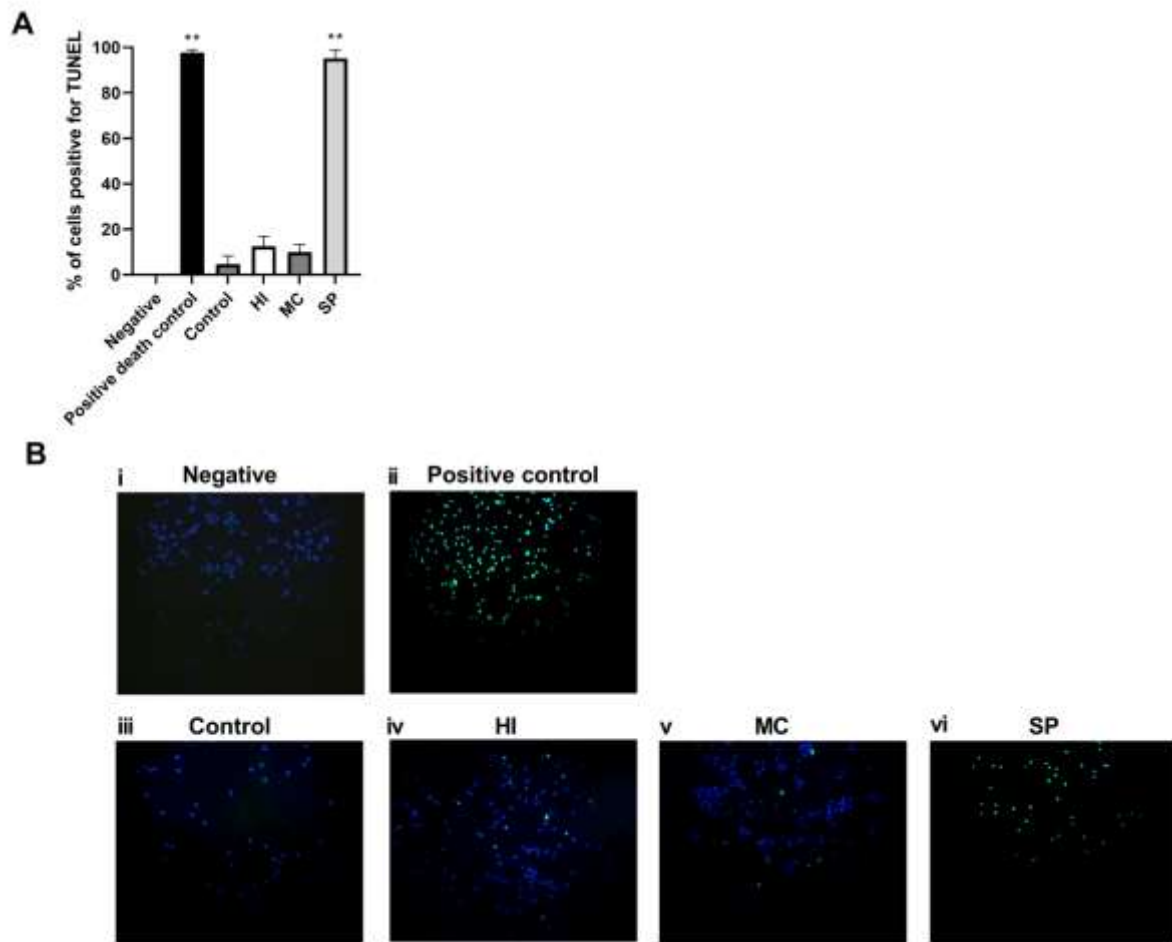
Figure 3 Effects of bacterial exposure on monocyte derived macrophage apoptosis

Monocyte derived macrophages (MDMs) from 6 COPD patients were exposed to *Haemophilus influenzae* (HI), *Moraxella catarrhalis* (MC) or *Streptococcus pneumoniae* (SP) at a multiplicity of infection (MOI) of 5:1, Triton-X 0.1% (Positive death control) or left unexposed (Control) for 24 hours. Cells were stained for nuclear stain DAPI and the apoptosis marker TUNEL or left unstained (Negative). The percentage of positive stained cells was determined with a minimum of 50 cells per condition counted across multiple fields

of view. Data represent mean + SEM percentage cells positive for TUNEL (A) and representative overlay images of TUNEL (green) and DAPI (blue) shown (B).

*,**,*** = significantly above unexposed control (p<0.05, 0.01, 0.001 respectively)
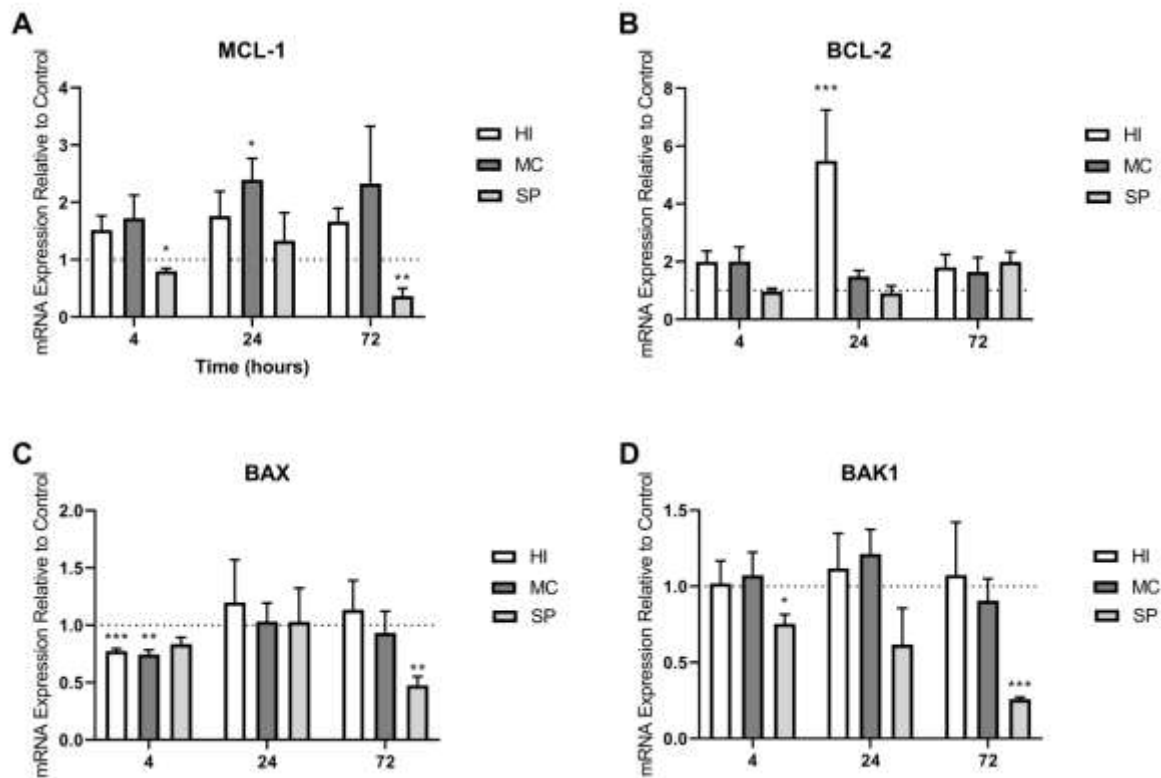
Figure 4 Effects of bacterial exposure on monocyte derived macrophage apoptosis gene expression

Monocyte derived macrophages (MDMs) from 5 COPD patients were exposed to *Haemophilus influenzae* (HI), *Moraxella catarrhalis* (MC) or *Streptococcus pneumoniae* (SP) at a multiplicity of infection (MOI) of 5:1 or left unexposed (Control) for 4, 24 or 72 hours. mRNA expression levels of anti-apoptosis genes MCL-1 (A) and BCL-2 (B) and pro-apoptosis genes BAX (C) and BAK1 (D) were measured by RT-qPCR. Data presented as mean + SEM. RT-qPCR data expression relative to endogenous control ($2^{-\Delta\Delta C_t}$).

*,**,*** = significantly different compared to unexposed control (p<0.05, 0.01, 0.001 respectively)
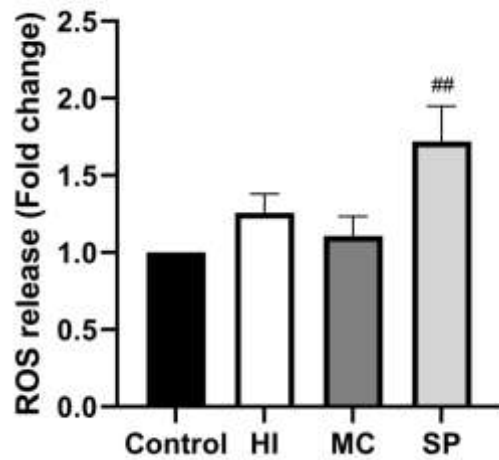
Figure 5 Effects of bacterial exposure on monocyte derived macrophage ROS release

Monocyte derived macrophages (MDMs) from 7 COPD patients were exposed to *Haemophilus influenzae* (HI), *Moraxella catarrhalis* (MC) or *Streptococcus pneumoniae* (SP) at a multiplicity of infection (MOI) of 5:1 or left unexposed (Control) for 24 hours. Supernatant levels of ROS were measured. Data presented as mean + SEM fold change of absorbance compared to unexposed control.

= significantly above unexposed control ($p < 0.01$)

Differential responses of COPD macrophages to respiratory bacterial pathogens

Simon Lea[1], Augusta Beech[1,2], James Baker[1], Rosemary Gaskell[1], Dharmendra Pindolia[1], Aisha Baba Dikwa[1], Rajesh Shah[3], Dave Singh[1,2]

Isolation of Lung macrophages

Lung macrophages (LM) were isolated from resected lung tissue, airways were flushed with sterile normal saline, and the washout fluid was layered on Ficoll gradient (GE Healthcare Life Sciences, UK). LM viability was confirmed by Trypan blue exclusion, cells then re-suspended at a concentration of $1x10^6$ cell/ ml in RPMI 1640 medium (Sigma Aldrich, UK) supplemented with 10% foetal bovine serum (Invitrogen, Paisley, UK), 1% penicillin/streptomycin (Sigma Chemical, Poole, Dorset, UK), and 1% L-glutamine (Invitrogen). Cells were cultured on appropriate culture plates and incubated at 37°C and 5% CO_2 for a minimum of 18 hours, non-adherent cells were washed next day with supplemented RPMI 1640 medium (without antibiotic) before infection. Cells were then infected with *Haemophilus influenzae (H. influenzae), Moraxella catarrhalis (M. catarrhalis)* or *Streptococcus pneumoniae (S. pneumoniae)* and left for 24 hours before supernatants were collected.

Monocyte-derived macrophage generation

Monocyte derived macrophages (MDMs) were generated from peripheral blood mononuclear cell (PBMCs). Heparinized blood was layered onto Ficoll Gradient (GE Healthcare Sciences, UK) and centrifuged in order to induce phase separation and the PBMCS were collected and washed. Viability was confirmed by trypan blue exclusion and cells were then re-suspended at a concentration of $1x10^6$ cell/ ml in RPMI 1640 medium (Sigma Aldrich, Poole, UK) supplemented with 10% foetal bovine serum (Invitrogen, Paisley, UK), 1% penicillin/streptomycin (Sigma Aldrich, Poole, UK), and 1% L-glutamine (Invitrogen, Paisley, UK).

Cells were seeded at a concentration of $100x10^4$ per well in a 96-well plate (Greiner Bio-One, Gloucester, UK). To stimulate macrophage differentiation, Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF (Peprotech, London UK) was added at a concentration of

10ng/ml. A breathe-easy membrane (Sigma Aldrich, Poole, UK) was added to seal the plate and cells were incubated at 37℃, 5% CO_2 for at least 7 days.

24 hours prior to bacterial infection, cells were washed with supplemented RPMI 1640 medium (without antibiotic).

Cells were then infected with *H. influenzae, M. catarrhalis* or *S. pneumoniae* and left at 37℃, 5% CO_2 for 24 hours before supernatants were collected.

Bacterial culture

Bacteria was cultured in supplemented brain heart infusion broth (Sigma-Aldrich, Poole, UK). 500ml of broth was made with distilled water and supplemented with 10μg/ml each of Hemin and β-Nicotinamide dinucleotide (both Sigma-Aldrich, Poole, UK). The broth was aliquotted into 50ml tubes and stored at $4^{o}C$.

To generate bacterial stocks, a frozen bead of *Heamophilus influenza (NCTC 12699), Morrexella catharils* (NCTC 11020) or *Streptococcus pneumonaie* (NCTC 12977) was transferred to 10ml of supplemented brain heart infusion broth (Sigma-Aldrich, Poole, UK) and left overnight at 37°C, 5% CO_2.

Bacterial suspension was set to OD_{600} of 1.2±0.02 by plate reader (PoLAR Star Omega, BMG LABTECH). Bacterial viability and count were confirmed each time by plate counting. Bacterial suspension with 1.2 OD (~$1x10^9$cfu/ml) was diluted to give the range of multiplicity of infection (MOI) 0.005:1-50:1(bacteria:macrophage).

Bacterial quantification: Miles Misra assessment

Quantification of viable bacteria was determined by Miles-Misra protocol. Eight 10-fold serial dilutions were prepared from a 1ml aliquot of fresh PBS-suspended bacterial stock. 20μl of bacterial stock and 180μl of PBS was used to create the following dilution series: $1x10^1$, $1x10^2$, $1x10^3$, $1x10^4$, $1x 10^5$, $1x10^6$, $1x10^7$, $1x10^8$. Chocolate agar plates were split into 8 equal quadrants and 2 drops of each serial dilution were plated on a quadrant. Drops were left to dry then plates inverted and incubated overnight at 37°C, 5% CO_2.

Developed colonies were counted in the quadrant with the highest number of colonies where individual colonies were discernible. The following calculation was performed: number of colonies/number of drops x dilution factor x 100 to give the number of colony forming units per millilitre (CFU/ml). Using the CFU assessment, volume required for a multiplicity of infection (MOI) of 5 was calculated.

Multiplicity of infection (MOI) calculation

In order to keep the bacterial load consistent in experiments, MOI was used. This is the ratio of bacteria per cell (macrophages, neutrophils or total cell counts depending on experiment type). MOI of 5 was used in all experiments.

From the Miles Misra assessment, MOI was determined by calculating CFU/ml and working out how much bacteria were required. Cell numbers were $1x10^4$ so 100,000/ CFU/ml x1000 was used to determine how much bacterial stock to add in microlitres. A Miles Misra of the bacterial MOI was also taken at each experiment to check bacterial numbers.

In-situ cell death assay

Sputum cell viability was measured by *In Situ* Cell Death Detection Fluorescein kit (ROCHE, UK). Cells were re-suspended in RMPI antibiotic-free media (Sigma-Aldrich, Poole, UK) at $1x10^6$ per ml and plated in chamber slides ($0.1x10^6$ cells per well) and either exposed to *H. influenzae, M. catarrhalis* or *S. pneumoniae* 5:1 MOI or Triton-X 0.1% as a positive death control or left unexposed (negative control and stained unexposed control) for 24 hours at 37°C, 5% CO_2. Cells were the fixed with 4% paraformaldehyde in PBS (Sigma Aldrich) for 1 hour. Slides were rinsed with PBS and permeablised with freshly prepared permeablisation buffer (0.01%Triton X in 0.1% sodium citrate) for 2 minutes on ice. 50 µl of label solution was added to unexposed cells as the negative control; and the TUNEL mix was freshly prepared according to manufacturer's instruction and added to the pre-treated cells. Slides were protected from light and incubated for 1 hour at 37^0 C. Slides were mounted in mounting media containing the nuclear stain DAPI and immunofluorescence was detected by fluorescence microscopy. The percentage of TUNEL positive cells was calculated.

RT-PCR

50 ng/µl of RNA in 20µl reaction mix, was used for cDNA synthesis by TaqMan reverse transcription-PCR (RT-PCR) using the Verso^{TM} 2-Step QRT-PCR kit (Thermo Scientific, Surry, UK). cDNA (50 ng) was used in 25 µl reaction mix containing primer probes for gene expression TNF-α, IL-6, CXCL8, IL-1β, the anti-apoptotic genes: induced myeloid leukemia cell differentiation protein (MCL-1) and B-cell lymphoma 2 (BCL-2), pro-apoptotic genes: BCL2 associated x protein (BAX) and BCL2 homologous antagonist/killer (BAK1) and the endogenous control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (catalogue no.: 4352934E) (Applied Biosystems). Thermal cycling was carried out on a Stratagene MX3005P (Agilent Technologies, West Lothian, UK). Relative expression levels were determined using the ΔCt method for sputum expression normalizing to GAPDH endogenous control or ΔΔCt method for MDM expression normalizing to GAPDH endogenous control and to unstimulated levels.

Cytokine assays

Supernatants were analysed by ELISA, performed according to manufacturer's instructions (R and D systems, Abingdon, UK), to quantify TNFα, IL-6, CXCL8, IL-1β and CCL5 levels. The lower limits of quantification were 15.6 pg·mL-1 for TNFα, 9.4 pg·mL-1 for IL-6, 31.3 pg·mL-1 for both CXCL8 and CCL5 and 3.9 pg·mL-1 for IL-1β.

Supplement Table 1

	Monocyte derived macrophages (MDMs)			Lung Macrophages		
	HNS	**COPD**	**p value**	**Ex-Smoker**	**COPD**	**p value**
n	10	8	-	4	4	-
Age (Years)	32 [23-56]	68 [63-73]	<0.0001	74 [71-83]	67 [59-78]	0.49
Gender: M/F	4/6	4/4	-	0/4	2/2	-
FEV_1 (L)	3.4 (0.6)	1.4 (0.9)	<0.0001	2.3 (1.1)	1.7 (0.4)	0.30
FEV1 % predicted	99 (11)	52 (24)	<0.0001	82 (20)	77 (10)	0.60
FVC (L)	4.5 (1.1)	3.4 (1.3)	0.03	3.3 (1.5)	2.6 (0.6)	0.4
FEV1/FVC Ratio (%)	76 (7)	40 (11)	<0.0001	77 (6)	65 (2)	0.1
Current smokers (%)	N/A	25	-	0	50	-
Pack years	N/A	39 (11)	-	12 (14)	50 (26)	0.2
ICS usage (%)	N/A	50	-	N/A	25	-

Supplement Table 1 –Demographics for COPD subjects and Ex-Smoking and Healthy Non-Smoking controls in cytokine protein experiments: FEV_1 forced expiratory volume in 1 second; FVC forced vital capacity; ICS inhaled corticosteroids**.** Data presented as median [range] or mean (SD) as appropriate.

Supplement Table 2

	Monocyte derived macrophages (MDMs)	
	COPD No ICS	**COPD ICS**
n	4	4
Age (Years)	68 [63-73]	70 [64-73]
Gender: M/F	3/1	1/3
FEV$_1$ (L)	1.6 (0.7)	0.9 (0.4)
FEV1 % predicted	54 (15)	52 (24)
FVC (L)	3.9 (1.3)	3.4 (1.3)
FEV1/FVC Ratio (%)	40 (7)	35 (10)
Current smokers (%)	25	25
Pack years	35 (16)	40 (5)
ICS usage (%)	0	100

Supplement Table 2 –Demographics for COPD subjects on inhaled corticosteroid treatment or not in cytokine protein experiments: FEV$_1$ forced expiratory volume in 1 second; FVC forced vital capacity; ICS inhaled corticosteroids. Data presented as median [range] or mean (SD) as appropriate.

Supplement Table 3

	Monocyte derived macrophages (MDMs)
	COPD
n	5
Age (Years)	66 [53-73]
Gender: M/F	2/3
FEV$_1$ (L)	1.7 (0.8)
FEV1 % predicted	63 (19)
FVC (L)	3.4 (1.1)
FEV1/FVC Ratio (%)	39 (23)
Current smokers (%)	0
Pack years	43 (8)
ICS usage (%)	80

Supplement Table 3 –Demographics for COPD subjects in qPCR and 24 hour v 72 hour cytokine experiments: FEV$_1$ forced expiratory volume in 1 second; FVC forced vital capacity; ICS inhaled corticosteroids**.** Data presented as median [range] or mean (SD) as appropriate.

Supplement Table 4

	Monocyte derived macrophages (MDMs)
	COPD
n	7
Age (Years)	66 [30-73]
Gender: M/F	2/5
FEV$_1$ (L)	1.7 (0.9)
FEV1 % predicted	64 (22)
FVC (L)	3.2 (0.9)
FEV1/FVC Ratio (%)	45 (26)
Current smokers (%)	0
Pack years	34 (18)
ICS usage (%)	57

Supplement Table 4 –Demographics for COPD subjects in ROS, CCL5 and IL-1β protein experiments: FEV$_1$ forced expiratory volume in 1 second; FVC forced vital capacity; ICS inhaled corticosteroids**.** Data presented as median [range] or mean (SD) as appropriate.
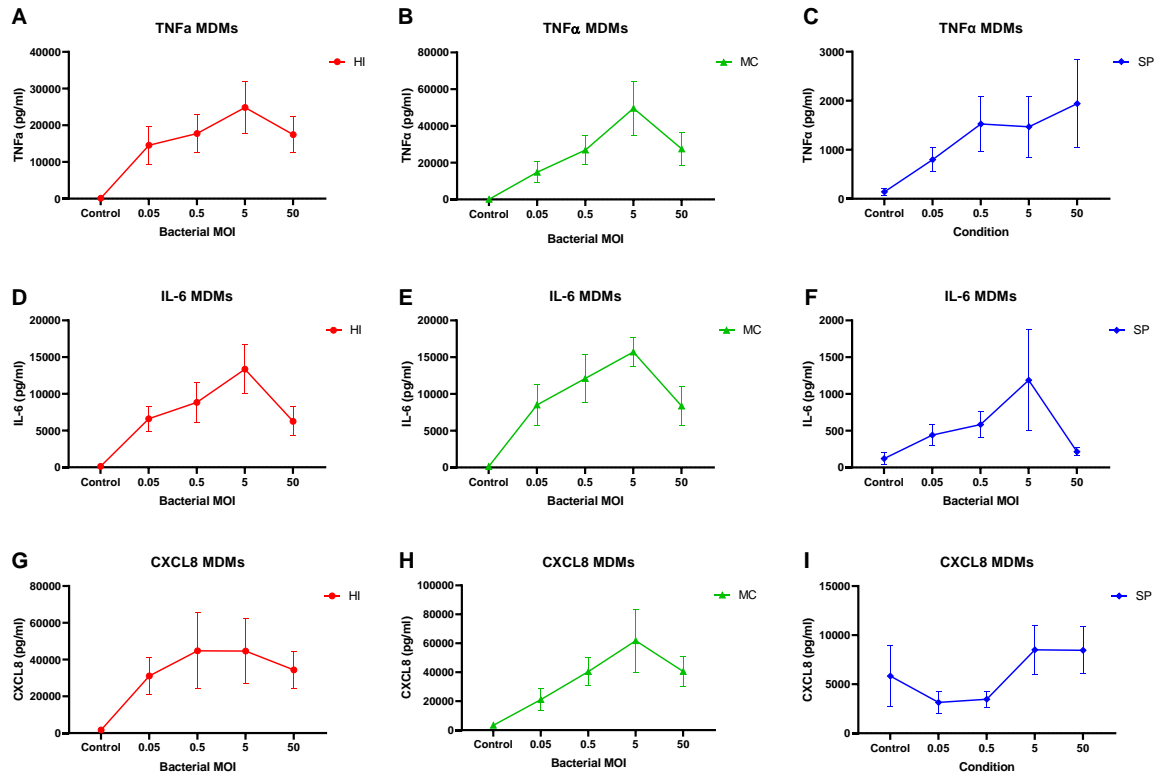
Supplement Figure 1. Multiplicity of infection cytokine dose response curves

Monocyte derived macrophages (MDMs) from 6 subjects were exposed to *Haemophilus influenzae* (HI), *Moraxella catarrhalis* (MC) or *Streptococcus pneumoniae* (SP) at a multiplicity of infection (MOI) of 50:1-0.05:1 or left unexposed (Control) for 24 hours. Supernatant levels of TNF-α (A, D & G), IL-6 (B, E & H) and CXCL8 (C, F & I) were measured by ELISA. Data presented as mean + SEM.
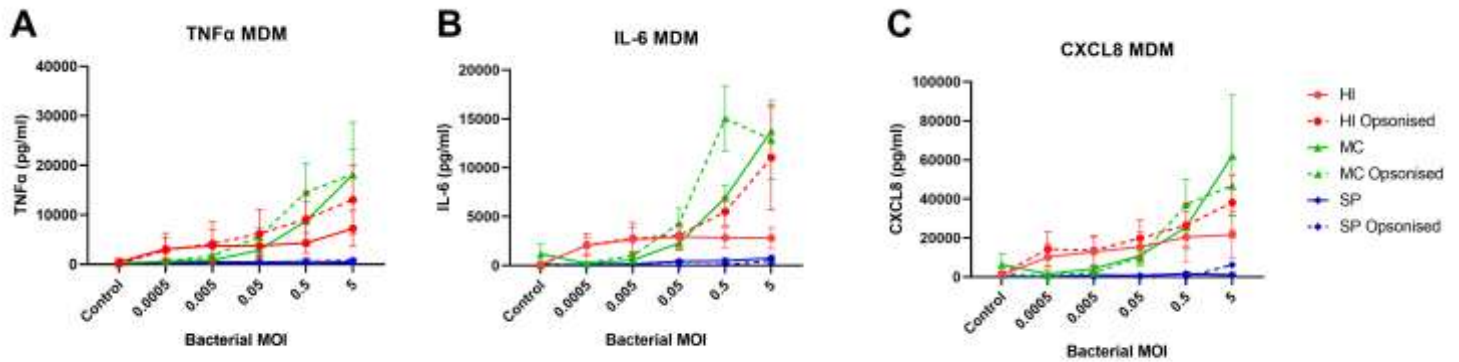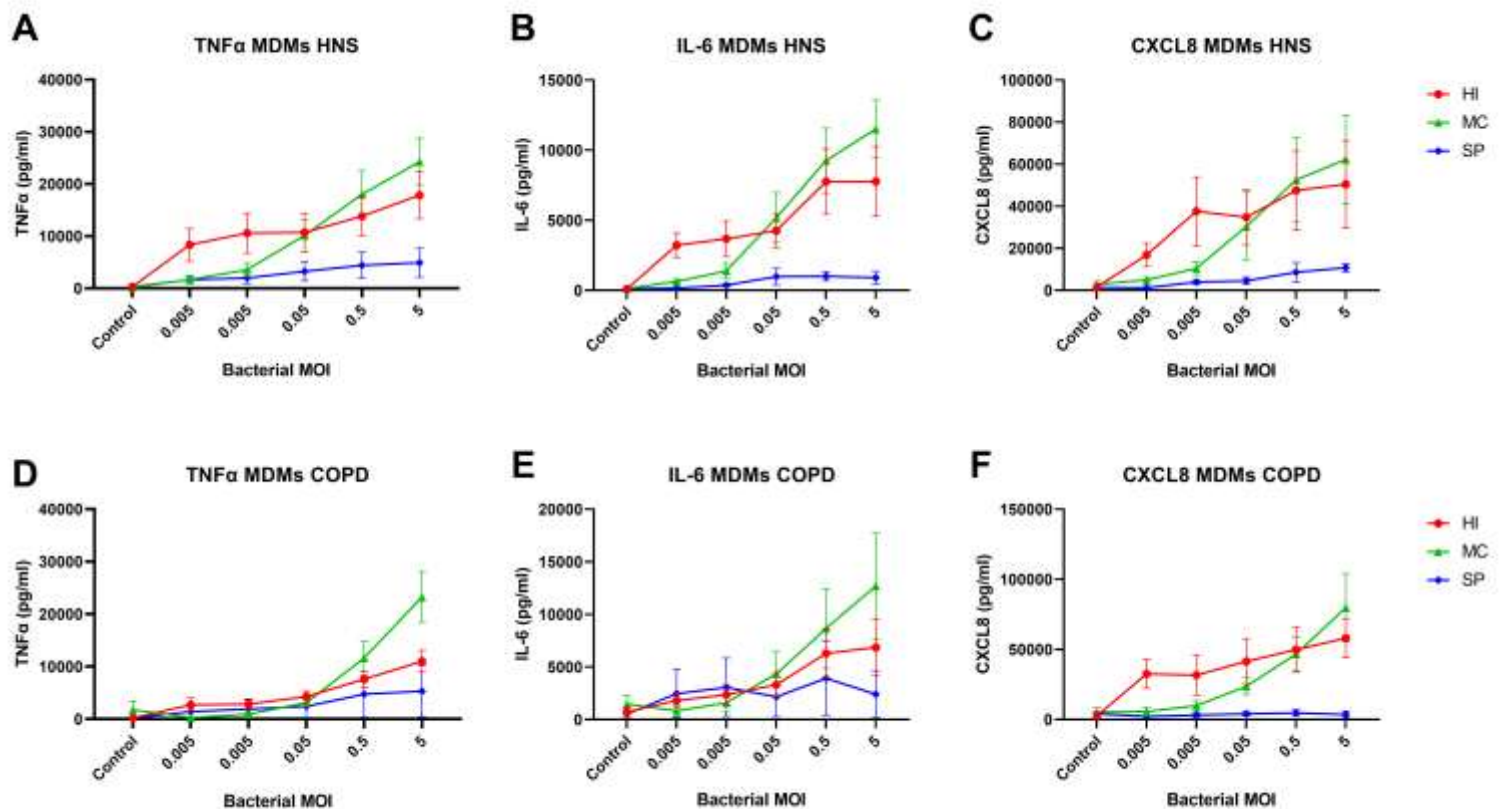
Supplement Figure 2. Effects of opsonised bacterial exposure on macrophage cytokine production

Monocyte derived macrophages (MDMs) from 6 subjects were exposed to opsonised or non-opsonised *Haemophilus influenzae* (HI), *Moraxella catarrhalis* (MC) or *Streptococcus pneumoniae* (SP) at a multiplicity of infection (MOI) of 5:1-0.0005:1 or left unexposed (Control) for 24 hours. Supernatant levels of TNF-α (A), IL-6 (B) and CXCL8 (C) were measured by ELISA. Data presented as mean + SEM.
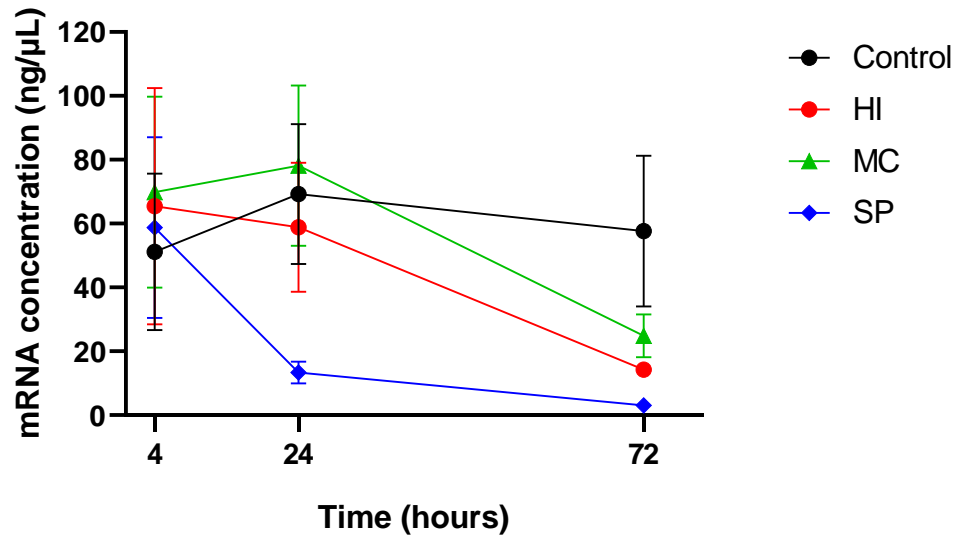
Supplement Figure 3 mRNA concentrations from bacteria exposed monocyte derived macrophage cultures.

Monocyte derived macrophages (MDMs) from 5 COPD patients were exposed to *Haemophilus influenzae* (HI), *Moraxella catarrhalis* (MC) or *Streptococcus pneumoniae* (SP) at a multiplicity of infection (MOI) of 5:1 or left unexposed (Control) for 4, 24 or 72 hours. Cell were lysed, mRNA isolated and concentrations analysed. Data presented as mean ± SEM.
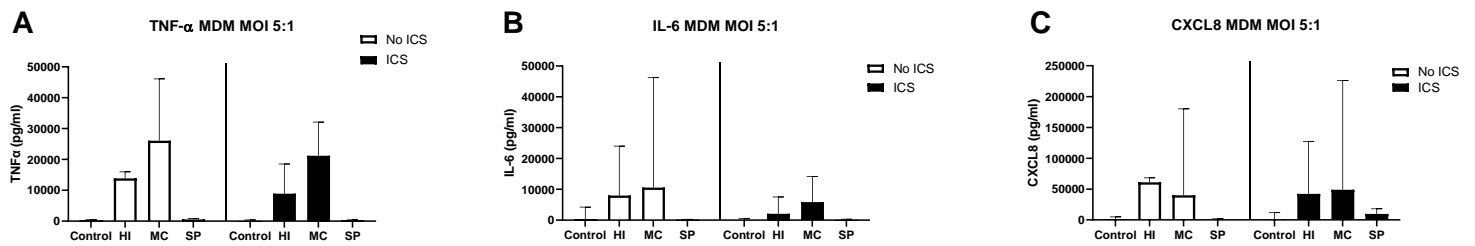
Supplement Figure 4. Effects of bacterial exposure on monocyte derived macrophage cytokine production

Monocyte derived macrophages (MDMs) from 10 healthy non-smokers (HNS) (A-C) and 8 COPD patients (D-F) were exposed to *Haemophilus influenzae* (HI), *Moraxella catarrhalis* (MC) or *Streptococcus pneumoniae* (SP) at a multiplicity of infection (MOI) of 5:1-0.005:1 or left unexposed (Control) for 24 hours. Supernatant levels of TNF-α (A&D), IL-6 (B&E) and CXCL8 (C&F) were measured by ELISA. Data presented as mean + SEM.
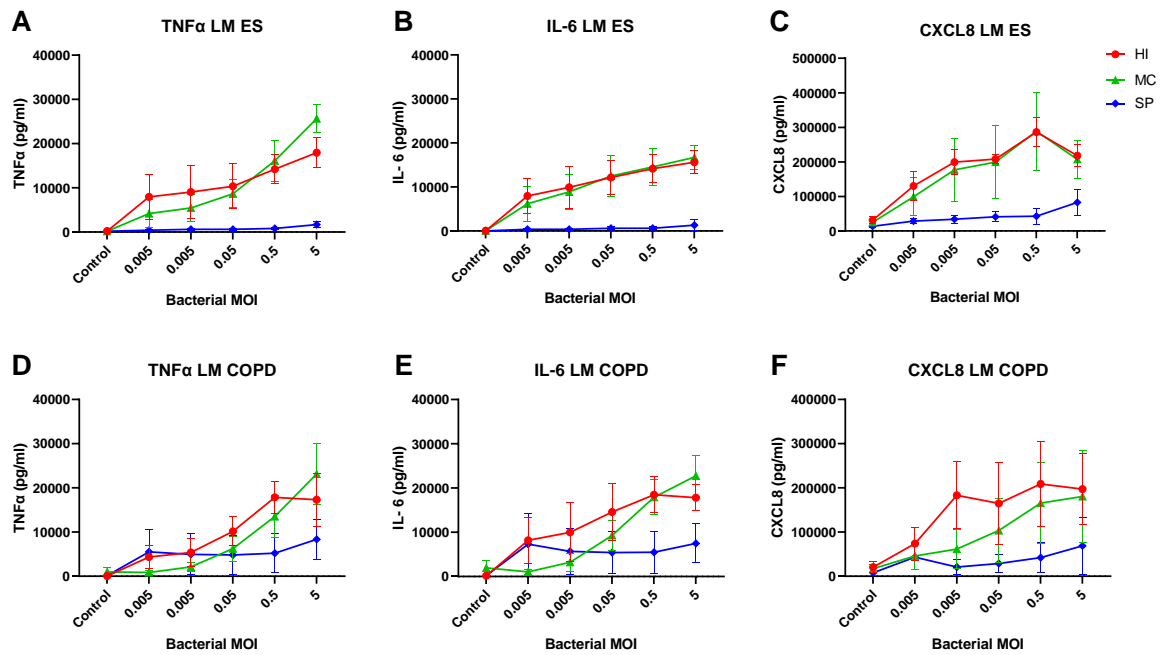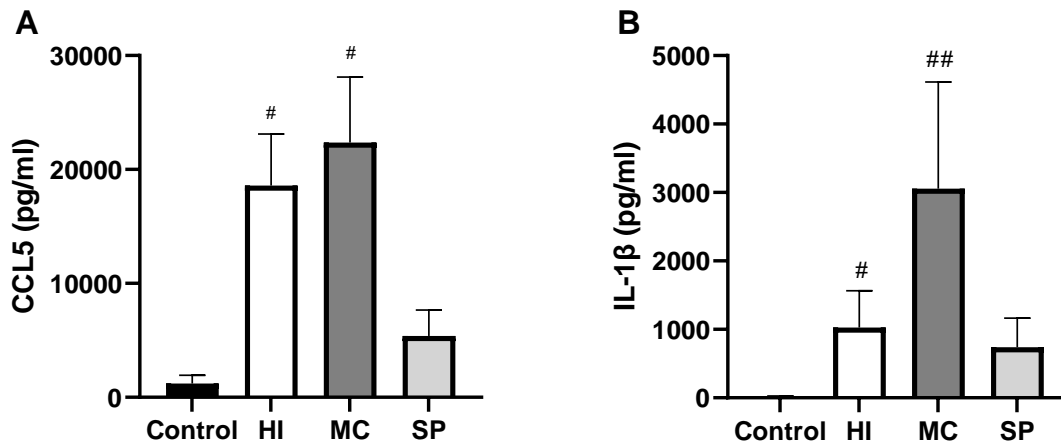
Supplement Figure 5. Effects of bacterial exposure on lung macrophage cytokine production

Lung macrophages from 4 ex-smokers (ES) (A-C) and 4 COPD patients (D-F) were exposed to *Haemophilus influenzae* (HI), *Moraxella catarrhalis* (MC) or *Streptococcus pneumoniae* (SP) at a multiplicity of infection (MOI) of 5:1-0.005:1 or left unexposed (Control) for 24 hours. Supernatant levels of TNF-α (A&D), IL-6 (B&E) and CXCL8 (C&F) were measured by ELISA. Data presented as mean + SEM.

Supplement Figure 6. Effects of bacterial exposure on cytokine production in monocyte derived macrophages from COPD patients with or without inhaled corticosteroid treatment
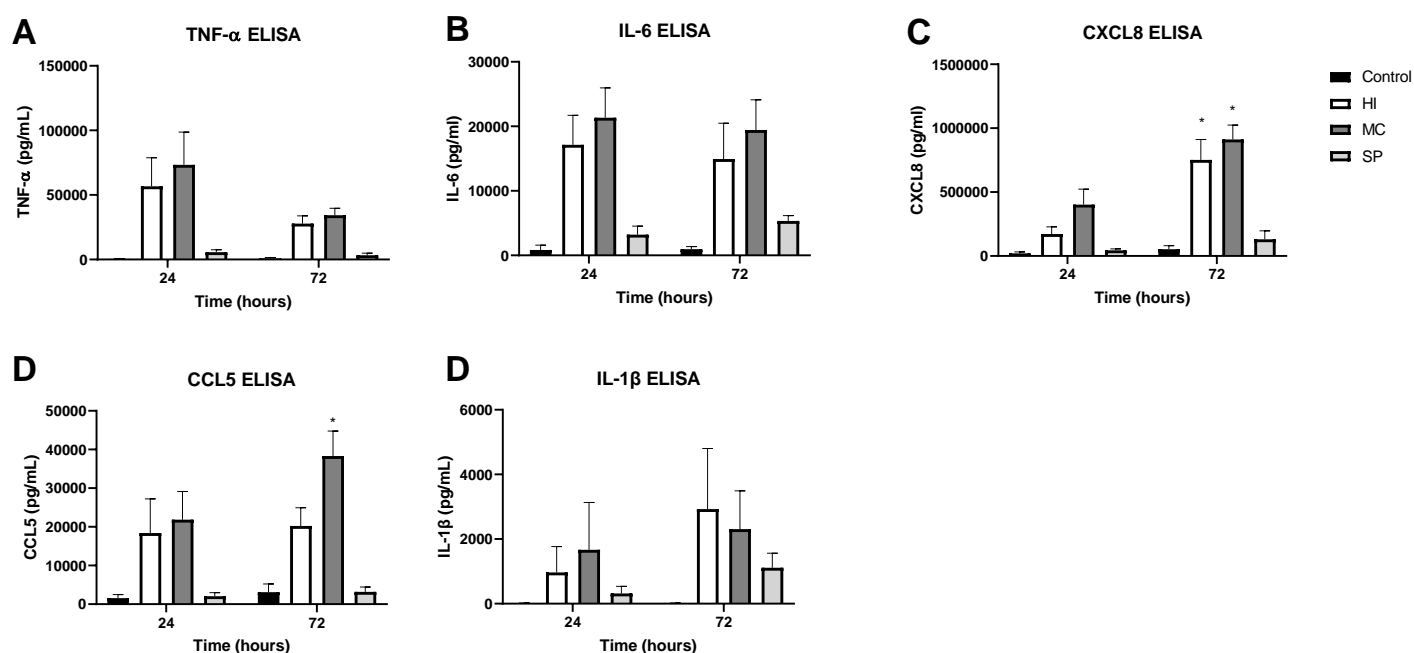
Monocyte derived macrophages (MDMs) from 4 COPD patients without inhaled corticosteroid (ICS) treatment and 4 COPD patients with ICS treatment (A-C) exposed to *Haemophilus influenzae* (HI), *Moraxella catarrhalis* (MC) or *Streptococcus pneumoniae* (SP) at a multiplicity of infection (MOI) of 5:1 or left unexposed (Control) for 24 hours. Supernatant levels of TNF-α (A), IL-6 (B) and CXCL8 (C) were measured by ELISA. Data presented as mean + SEM. Levels were compared between patient groups using two way ANOVA followed by a Bonferroni's multiple multiple comparisons test.

Supplement Figure 7 Effects of bacterial exposure on monocyte derived macrophage CCL5 and IL-1β production

Monocyte derived macrophages (MDMs) from 7 COPD patients were exposed to *Haemophilus influenzae* (HI), *Moraxella catarrhalis* (MC) or *Streptococcus pneumoniae* (SP) at a multiplicity of infection (MOI) of 5:1 or left unexposed (Control) for 24 hours. Supernatant levels of CCL5 (A) and IL-1β (B) were measured by ELISA. Data presented as mean + SEM. Levels were compared between conditions using ANOVA and Tukey's multiple comparisons test.
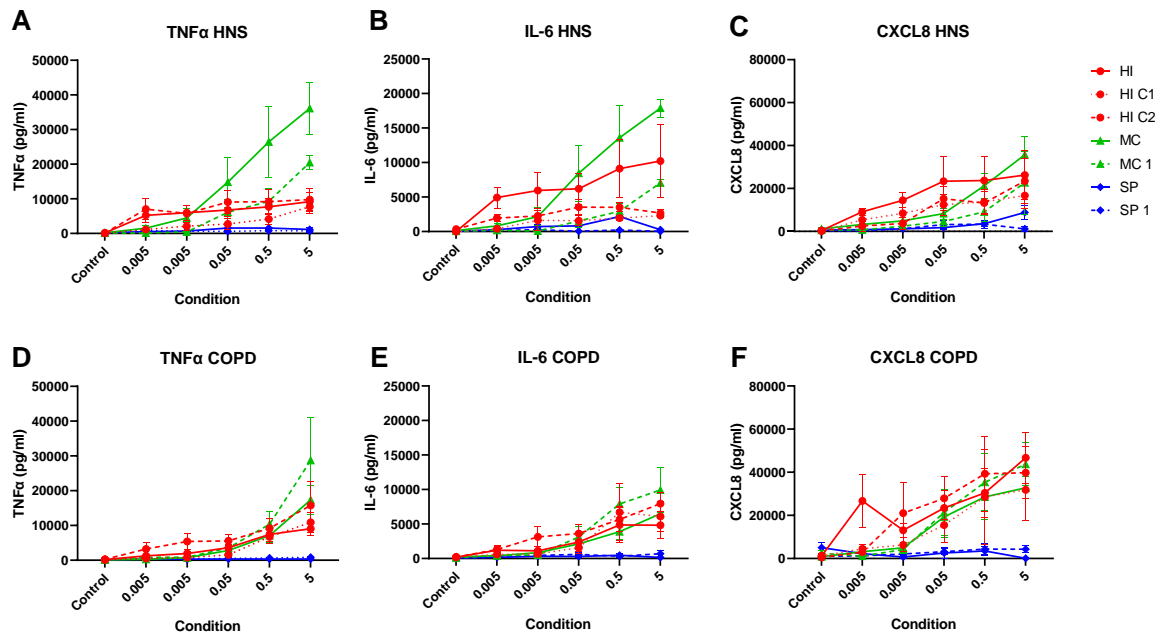
= significantly above unexposed control (p<0.05).

Supplement Figure 8 Effects of bacterial exposure on monocyte derived macrophage cytokine production

Monocyte derived macrophages (MDMs) from 5 COPD patients were exposed to *Haemophilus influenzae* (HI), *Moraxella catarrhalis* (MC) or *Streptococcus pneumoniae* (SP) at a multiplicity of infection (MOI) of 5:1 or left unexposed (Control) for 24 or 72 hours. Supernatant levels of TNF-α (A), IL-6 (B), CXCL8 (C), CCL5 (D) and IL-1β were measured by ELISA. Data presented as mean + SEM. Levels were compared between time points for each condition using two way ANOVA followed by a Bonferroni's multiple multiple comparisons test.

* = significantly above 24 hours (p<0.05)

Supplement Figure 9. Effects of clinically isolated bacteria on monocyte derived macrophage cytokine production

Monocyte derived macrophages (MDMs) from 4 healthy non-smokers (HNS) (A-C) and 4 COPD patients (D-F) were exposed to reference strains and clinical isolates of *Haemophilus influenzae* (HI), *Moraxella catarrhalis* (MC) or *Streptococcus pneumoniae* (SP) at a multiplicity of infection (MOI) of 5:1-0.005:1 or left unexposed (Control) for 24 hours. Supernatant levels of TNF-α (A&D), IL-6 (B&E) and CXCL8 (C&F) were measured by ELISA. Data presented as mean + SEM.