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°C, 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°C, 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°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

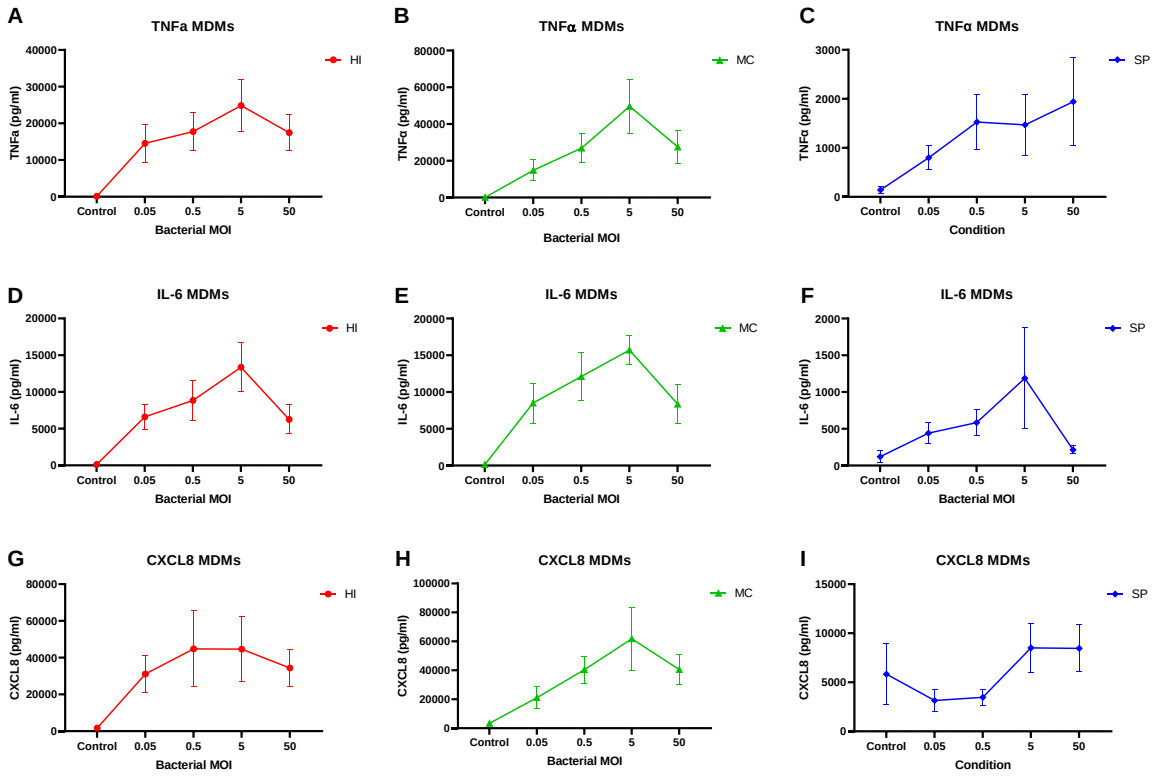
	Monocyte derived macrophages (MDMs)
	COPD
n	5
Age (Years)	66 [53-73]
Gender: M/F	2/3
FEV_1 (L)	1.7 (0.8)
FEV1 % predicted	63 (19)
FVC (L)	3.4 (1.1)
FEV1/FVC Ratio (%)	39 (23)
Current smokers (%)	0
Pack years	43 (8)
ICS usage (%)	80

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

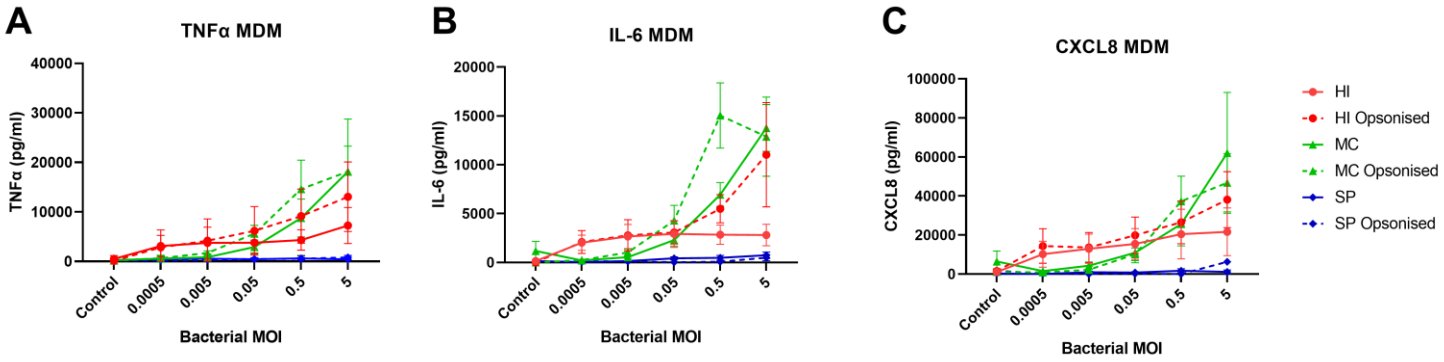
	Monocyte derived macrophages (MDMs)
	COPD
n	7
Age (Years)	66 [30-73]
Gender: M/F	2/5
FEV_1 (L)	1.7 (0.9)
FEV1 % predicted	64 (22)
FVC (L)	3.2 (0.9)
FEV1/FVC Ratio (%)	45 (26)
Current smokers (%)	0
Pack years	34 (18)
ICS usage (%)	57

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.



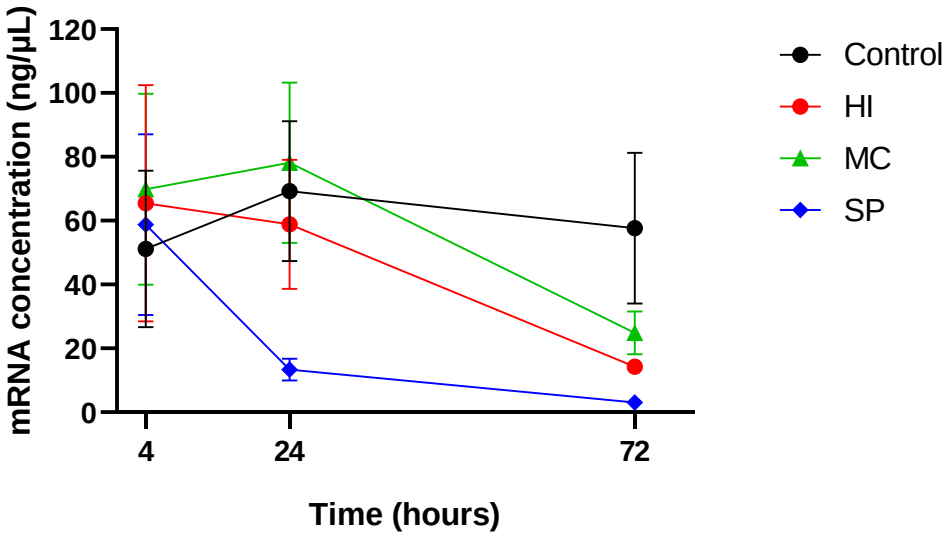
Supplement Figure 1. Multiplicity of infection cytokine dose response curves

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.



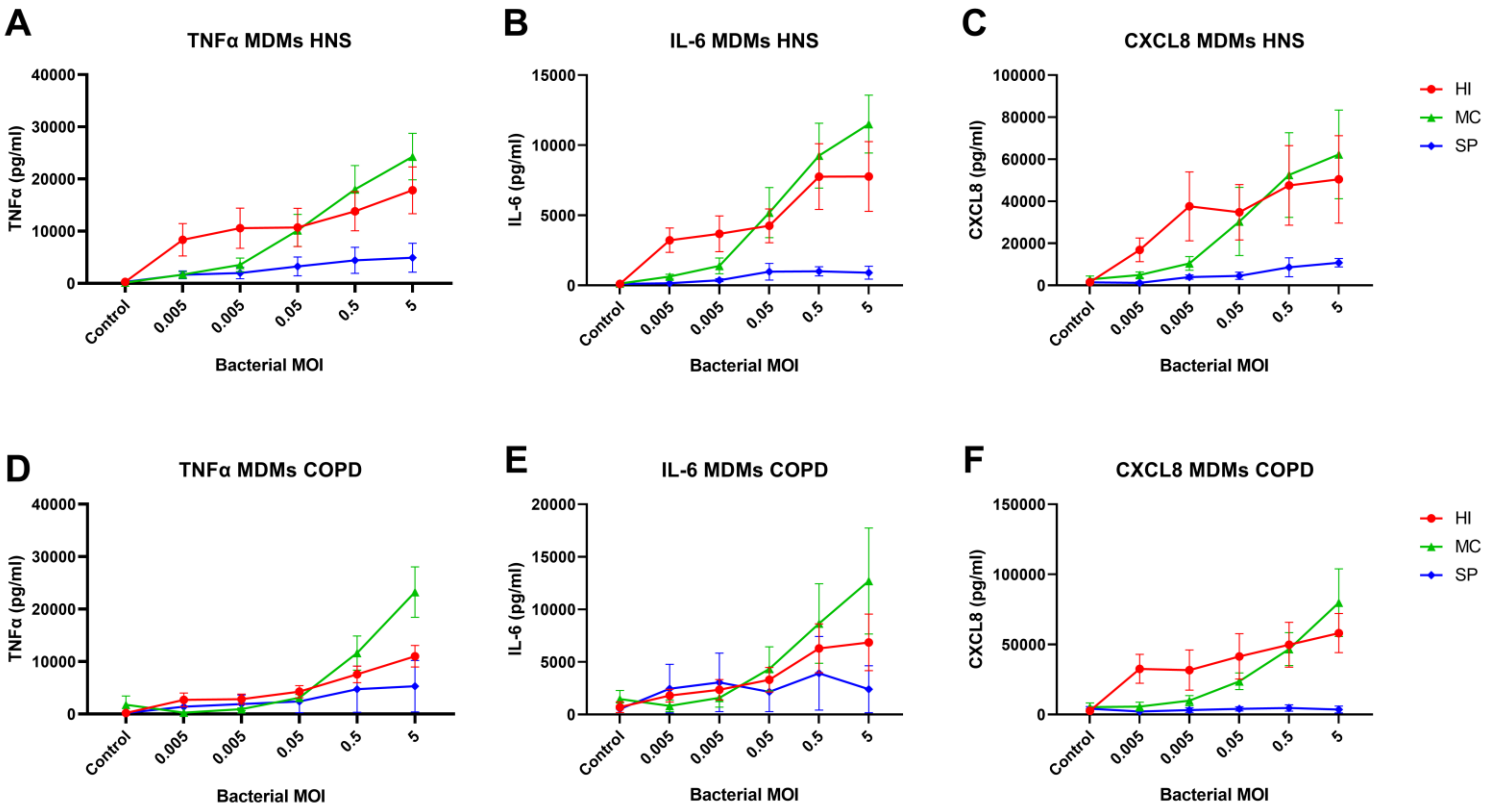
Supplement Figure 2. Effects of opsonised bacterial exposure on macrophage cytokine production

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.



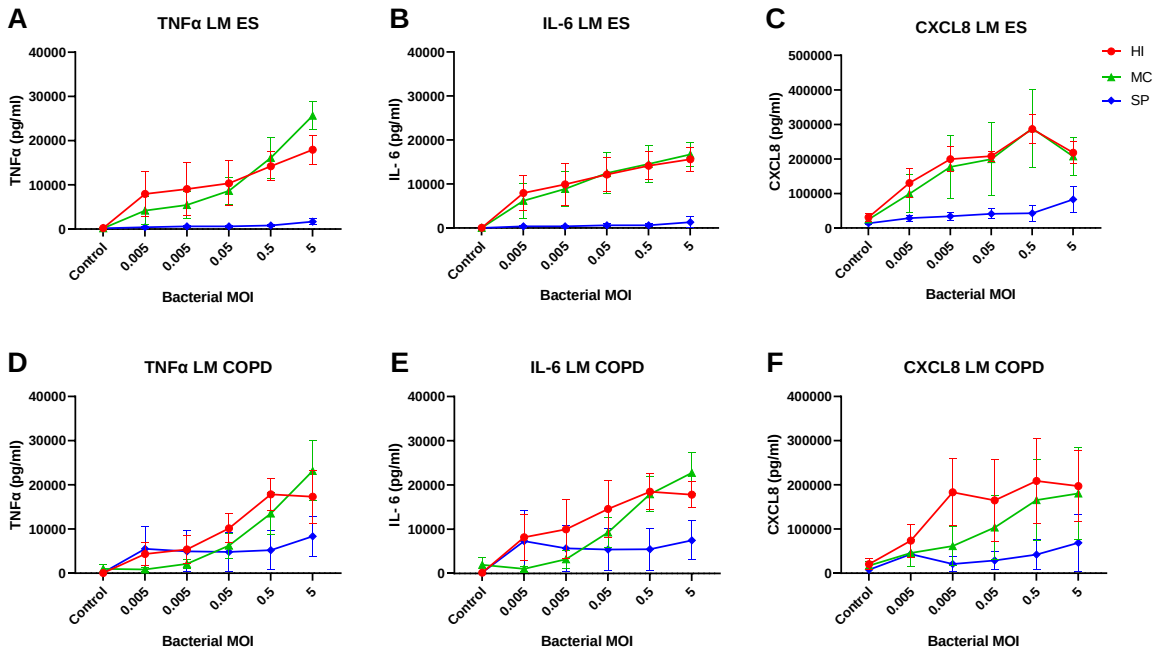
Supplement Figure 3 mRNA concentrations from bacteria exposed monocyte derived macrophage cultures.

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.



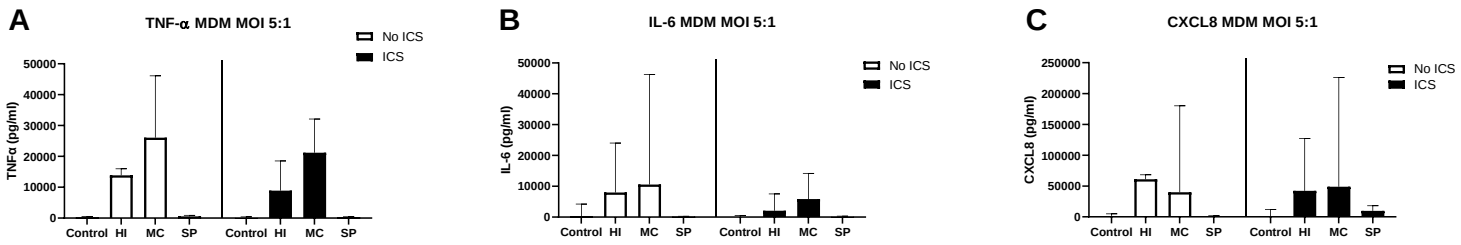
Supplement Figure 4. Effects of bacterial exposure on monocyte derived macrophage cytokine production

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.



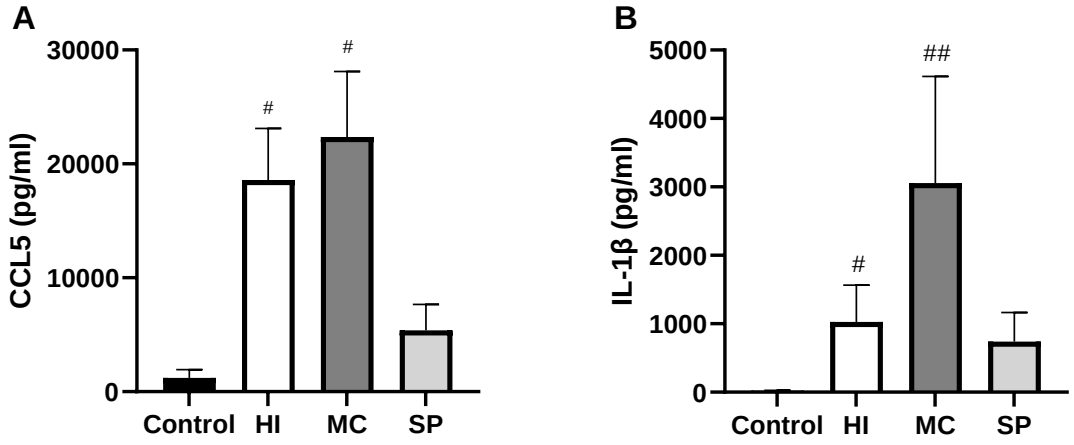
Supplement Figure 5. Effects of bacterial exposure on lung macrophage cytokine production

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

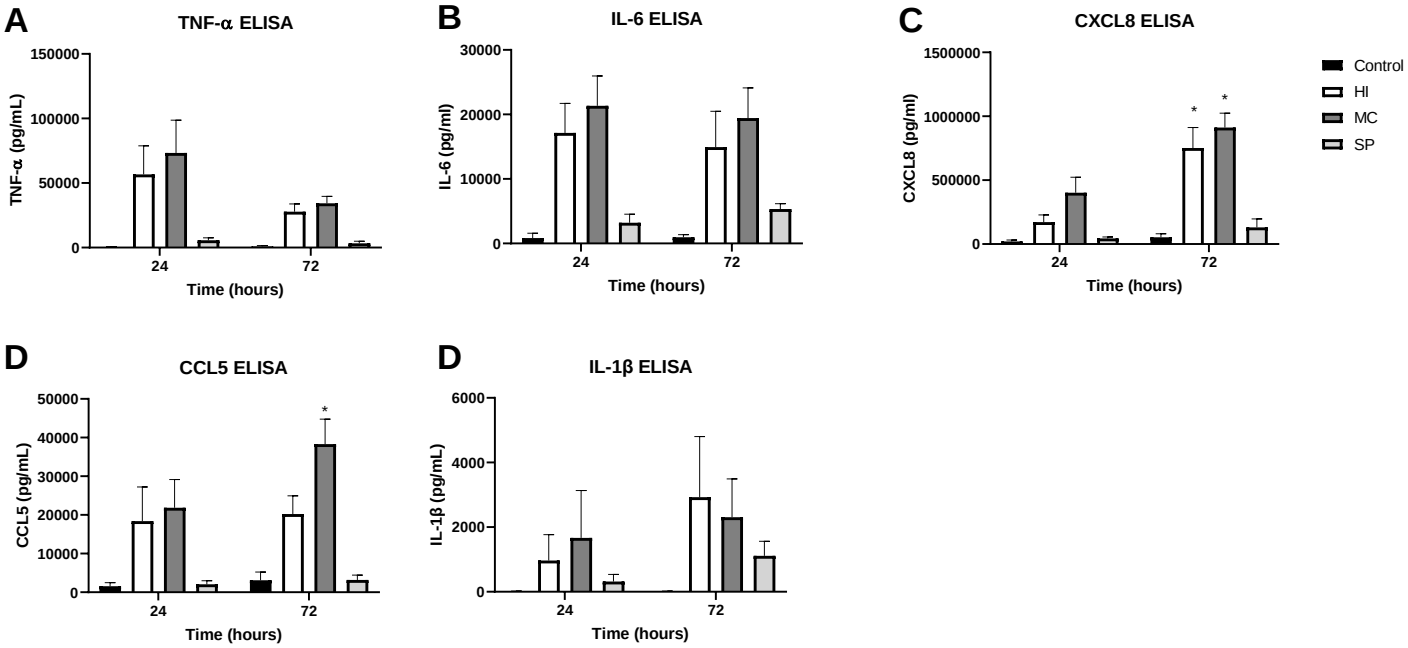
Monocyte derived macrophages (MDMs) from 4 COPD patients without inhaled corticosteroid (ICS) treatment and 4 COPD patients with ICS treatment (A-C) exposed to *Haemophilus influenzae* (HI), *Moraxella catarrhalis* (MC) or *Streptococcus pneumoniae* (SP) at a multiplicity of infection (MOI) of 5:1 or 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.

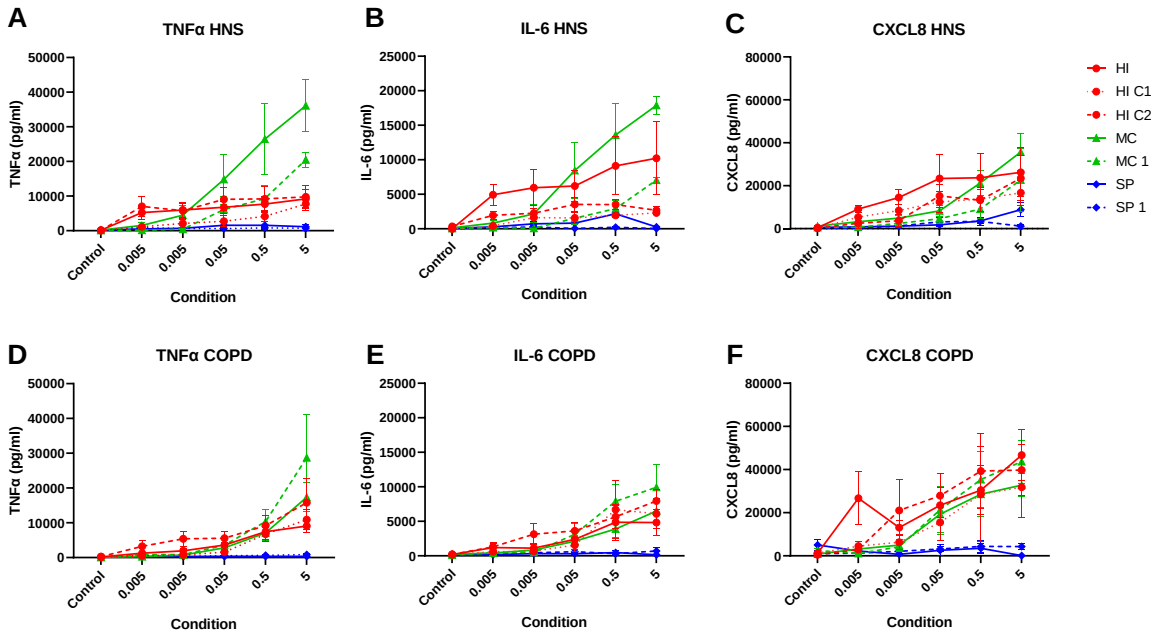
= significantly above unexposed control ($p<0.05$).



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.