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Take home Message

Defective phagocytosis in COPD macrophages is worsened by oxidative stress and is linked to altered mitcohondiral function.

Abstract

Background: Increased reactive oxygen species (ROS) have been implicated in the pathophysiology of chronic obstructive pulmonary disease (COPD).

Objective: This study examined the effect of exogenous and endogenous oxidative stress on macrophage phagocytosis in patients with COPD.

Methods: Monocyte-derived macrophages (MDM) were generated from non-smoker, smoker and COPD subjects, differentiated in either GM-CSF (G-Mφ) or M-CSF (M-Mφ). Alveolar macrophages were isolated from lung tissue or bronchoalveolar lavage. Macrophages were incubated +/- 200μM H_2O_2 for 24 hours, then exposed to fluorescently-labelled *H. influenzae* or *S. pneumoniae* for 4 hours, after which phagocytosis, mitochondrial ROS (mROS), and mitochondrial membrane potential ($\Delta\Psi$m) were measured.

Results: Phagocytosis of bacteria was significantly decreased in both G-Mφ and M-Mφ from COPD patients, compared to non-smoker controls. In non-smokers and smokers, bacterial phagocytosis did not alter mROS or $\Delta\Psi$m, however in COPD, phagocytosis increased early mROS and decreased $\Delta\Psi$m in both G-Mφ and M-Mφ. Exogenous oxidative stress reduced phagocytosis in non-smoker and COPD alveolar macrophages, and non-smoker MDM, associated with reduced mROS production.

Conclusion: COPD macrophages show defective phagocytosis, which is associated with altered mitochondrial function and an inability to regulate mROS production. Targeting mitochondrial dysfunction may restore the phagocytic defect in COPD.

Keywords

Chronic obstructive pulmonary disease, macrophage, oxidative stress, mitochondria

Abbreviations

COPD – Chronic obstructive pulmonary disease

GM-CSF – Granulocyte macrophage colony stimulating factor

M-CSF – Macrophage colony stimulating factor

G-Mφ- GM-CSF differentiated macrophage

M-Mφ- M-CSF differentiated macrophage

mROS – Mitochondrial reactive oxygen species

$\Delta\Psi m$ – Mitochondrial membrane potential

Introduction

Chronic obstructive pulmonary disease (COPD) comprises three pathological conditions, namely emphysema, chronic bronchitis, and small airways disease [1]. COPD is characterized by a progressive decline in lung function which can be worsened by exacerbations leading to hospital admissions, morbidity and mortality [2-4]. In more developed nations, cigarette smoking induces inflammation and increased oxidative stress in the lungs, and is a major contributor to disease pathophysiology [5].

The lung microbiome in COPD patients is altered compared to healthy individuals, with colonisation of the lower respiratory tract with disease specific microorganisms, including *Haemophilus influenzae* and *Streptococcus pneumoniae* [6]. These bacteria, together with viruses and other microorganisms, may drive exacerbations of COPD, which are associated with accelerated disease progression and worse clinical prognosis [2].

Alveolar macrophages contribute 90–95% of cells found in bronchoalveolar lavage fluid and are highly phagocytic. Their main function is the removal of pathogens including microorganisms and apoptotic cells via phagocytosis and the regulation of inflammation [7]. However, in COPD, alveolar macrophages are defective at phagocytosis of bacteria, fungal spores, and apoptotic epithelial cells [8-10]. Monocyte-derived macrophages (MDM) are used as a model of alveolar macrophages, and also carry the defect in phagocytosis observed in COPD [11]. Different macrophage phenotypes can be generated by incubating monocytes with specific growth factors, to represent the heterogeneous populations of macrophages found in tissue including the lungs. Granulocyte macrophage-colony stimulating factor (GM-CSF) and macrophage-colony stimulating factor (M-CSF) have been used to generate macrophage phenotypes with differing inflammatory profiles and as such provide a useful model for the study of macrophages phenotypes in COPD [11, 12]. There is increasing evidence that from in vivo models, while tissue resident macrophages play a key role in lung immunity, during inflammation monocytes are recruited to the lungs, which differentiate into macrophages to increase the macrophage pool [13, 14]. There is a 20-fold increase in macrophage numbers in the lungs of COPD patients with severe emphysema [15], and increased numbers in bronchoalveolar lavage and sputum of patients with less severe disease [16] indicating that monocyte recruitment is a potential source of macrophages in the lung during disease and that the study of MDM is relevant in COPD.

Mitochondria function as cellular powerhouses, producing ATP via the electron transport chain, and are also important in intracellular calcium regulation, apoptosis, and controlling the innate immune system [17]. Exposure of macrophages to cellular stress, such a cigarette smoke, biomass fuels or pollution, alters their mitochondrial function [18]. In COPD, mitochondria have been shown to be dysfunctional in airway smooth muscle cells [19, 20] and skeletal muscle [21, 22]. The mitochondria in these tissues showed excessive mROS production, which are normally counteracted by antioxidants and enzymes [23, 24].

It is possible that the defects seen in macrophage phagocytosis in COPD could be exacerbated by exogenous oxidative stress such as cigarette smoke and reactive oxygen species, and lead to defective mitochondrial function, as observed in airway smooth muscle cells from these patients [19]. We therefore studied the effects of exogenous oxidative stress on phagocytosis and mitochondrial function, using both GM-CSF and M-CSF differentiated MDM and lung derived macrophages from COPD patients in comparison with cells from age-matched healthy non-smokers and smokers without COPD, to determine whether mitochondrial dysfunction is associated with defective phagocytosis.

Methods

Subject selection

COPD patients were recruited from the UK Medical Research Council (MRC) COPD-MAP consortium of GOLD II/III patients. Control subjects included healthy non-smokers and smokers without COPD after screening. All subjects gave written informed consent prior to the start of the study and the study was approved by London-Hampstead National Research Ethics Committee (13/LO/1403) and the London-Chelsea National Research Ethics Committee (09/H0801/85). Subject demographics are presented in Tables 1-3. There was no difference in smoking history between smokers and COPD patients. Data on whether patients were current or ex-smokers was not available for this study. COPD patients had significantly worse lung function compared to controls. Patients continued to take their maintenance therapy including long-acting anti-muscarinic agents, long-acting β_2-adrenergic agonists and inhaled corticosteroids.

Macrophage isolation

Monocyte-derived macrophages (MDM) were generated from monocytes isolated from peripheral blood mononuclear cells using a Percoll gradient and adherence technique, followed by culture in 2 ng/ml GM-CSF (Cat No. 7954-GM-059/CF, Bio-Techne, Abingdon, UK) to generate G-Mφ, or 100ng/ml M-CSF to generate M-Mφ (Cat No. 216-MC-500, Bio-Techne, Abingdon, UK) for 12 days to generate MDM as described previously [11]. All the MDM used in this study were derived from freshly isolated monocytes. Macrophages were seeded at $0.5x10^6$ cells/well in a 24 well plate. Alveolar macrophages were isolated from bronchoalveolar lavage, as described previously [25]. Lung tissue macrophages were isolated from lung parenchyma as described previously [26]. Lung tissue used in this study was assessed as being non-cancerous by the hpathologist and obtained from samples during tissue resection for lung cancer or emphysema.

Bacteria

Serotype 14 *S. pneumoniae* (NCTC 11902, National Collection of Type Cultures) was grown as previously described [27]. Non-typeable *H. influenzae* (NCTC 1269, National Collection of Type Cultures) was cultured on chocolate agar overnight and then grown to OD 0.6 in brain heart infusion (BHI) (Cat No. CM1135, Oxoid Ltd, Basingstoke, UK) supplemented with 20%

(v/v) foetal calf serum (FCS) (Cat No. FB-1001/500, Biosera, Heathfield, UK), 20 μg/ml NAD (Cat No. NAD-100RO, Sigma-Aldrich Company Ltd, Dorset, UK) and 10 μg/ml heme (Cat No. H9039, Sigma-Aldrich Company Ltd, Dorset, UK). Bacteria were not opsonized. Heat-killed bacteria were generated by incubation at 65 °C for 10 min as described previously [25].

Fluorescent labelling of heated killed bacteria

Bacterial cultures were fluorescently labelled using AlexaFluor 405 NHS ester (Cat No. A30000, Life Technologies, Paisley, UK), or AlexaFluor 488 NHS ester (1mg/ml in DMSO, Cat No. A20100, Life Technologies, Paisley, UK) and incubated overnight. Labelled bacteria were washed repeatedly in PBS to remove unbound label, resuspended in PBS and stored at -20°C.

Bacterial phagocytosis

Fluorescent bacterial stocks were sonicated and added to macrophages at $1.5x10^{10}$ CFU/ml for *H. influenzae*, and $1.7x10^{8}$ CFU/ml for *S. pneumoniae*, and incubated at 37°C for 4 h. For flow cytometry, cells were washed with PBS to remove free bacteria, and cells dissociated from the plate using cell dissociation media and vigorous pipetting. Cells were transferred to FACS tubes, centrifuged, resuspended in 200μl PBS, and analysed by flow cytometry using a BD FACS Canto II, in the pacific blue channel. Data was acquired using FACS DIVA software and subsequently analysed using Flowjo software (Treestar, USA). For plate reader assays, cells were washed with PBS and fluorescence of extracellular particles was quenched by adding Trypan blue (2% w/v) for 1 min. Excess fluid was removed and fluorescence determined using an excitation at λ 480 nm and emission λ 520 nm. The relative amount of bacteria phagocytosed is reported as relative fluorescent units (RFU).

Measurement of mROS using Mitosox assay

Post-phagocytosis, wells were washed with Hanks' balanced salt solution (HBSS (calcium free)), and 5μM mitosox dye (Cat No. M36008, Thermo Fisher, Loughborough, UK) added at as per manufacturer's instructions. Cells were incubated for 30 min at 37°C, then washed with HBSS containing calcium (0.185g/L). Cells were dissociated from the plate using cell dissociation media and vigorous pipetting. Cells were transferred to FACS tubes,

centrifuged, and resuspended in 200µl HBSS, and analysed by flow cytometry using a BD FACS Canto II, in the PE channel, and bacteria were measured in the pacific blue channel.

Measurement of mitochondrial membrane potential ($\Delta\Psi m$) using JC-1

Post phagocytosis, JC-1 assay was performed as per manufacturer's instructions (Cat No. M43152, Thermo Fisher, Loughborough, UK). Briefly, cells in a 24 well plate were washed with PBS and replaced with 500µl warm cell culture media. 50µM carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) was added to the positive control well, and plate incubated for 5 min at 37°C. 2µM JC-1 dye was then added to all wells for a further 25 min at 37°C, after which the cells were washed with PBS. Cells were dissociated from the plate using cell dissociation media and vigorous pipetting. Cells were transferred to FACS tubes, centrifuged, and resuspended in 200µl PBS, and analysed by flow cytometry using a BD FACS Canto II, in the PE and FITC channel, and bacteria were measured in the pacific blue channel.

Confocal Microscopy

MDM were cultured on Ibidi µ chamber slides, and phagocytosis performed. Cells were fixed with 4% v/v paraformaldehyde. Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole) and cytoplasm with cell tracker red. Slides were imaged on a Zeiss LSM-510 inverted confocal microscope, and analysed using FIJI software.

Cell viability

Cell viability was measured by incubating MDM in 0.1% (w/v) 3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) at 37°C for 30 min before removing MTT and adding of DMSO to lyse the cells. Absorbance was measured at λ570nm using a Spectramax photometer to quantify cell viability. Each treated well was normalised to the average of the non-treated control which was set to 100% viability.

Statistical analysis

Data are presented as mean ± SEM or as individual data points, and significance analysed using either a Wilcoxon matched pairs signed rank test for comparison of two variables, or Kruskal Wallis test for comparison of multiple variables with post hoc Dunn's test using GraphPad Prism 5 software (GraphPad, USA).

Results

Correlation between phagocytosis of alveolar macrophages and MDM

In order to further validate the use of MDM to assess phagocytic capacitiy of macropahges, alveolar macrophages and MDM were isolated from the same patient, and phagocytic assays performed. Significant correlations were observed in the phagocytic capacity of alveolar macrophages and either M-Mφ or G-Mφ for both *H. influenzae* (r=0.63 and 0.71 respectivly, Figure 1A, B), and *S. pneumoniae* (r=0.40 and 0.60 respectivly, Figure 1C, D). Internalisation of bacteria was confirmed by confocal microscopy (Fig. 1E-G). From these data, the MDM model was used in future experiments, where alveolar macrophages were not available.

Defective macrophage phagocytosis in COPD

Experiments were performed in order to confirm previous data showing defective phagocytosis by COPD macrophages. Regardless whether MDM were differentiated with GM-CSF or M-CSF, there was a significant reduction in uptake of both *H. influenzae* and *S. pneumoniae* by COPD macrophages in comparison to healthy volunteer derived cells ($p<0.05$), measured by MFI (Fig. 2B and 2D). However, irrespective of subject group, 60-80% of cells were capable of phagocytosis, measured by % macrophage phagocytosis (Fig. 2A and 2C). In addition, there were no differences between the ability of G-Mφ and M-Mφ to phagocytose, although M-Mφ displayed a trend for increased phagocytosis compared with G-Mφ from all subject groups.

Exogenous oxidative stress on macrophage phagocytosis

Alveolar macrophages and MDM were exposed to 200μM H_2O_2 for 24h, and phagocytosis measured by median fluorescence intensity (MFI). Pilot studies showed that 200μM H_2O_2 was the maximal concentration to have no effect on cell viability (Supplementary Figure 1). Exposure of alveolar macrophages to H_2O_2 decreased phagocytosis of *H. influenzae* (NS $p<0.05$, COPD $p<0.05$, Fig 3A) and *S. pneumoniae* (NS $p<0.05$, COPD $p<0.05$, Fig. 3B) in cells from non-smokers and COPD patients. Exposure of non-smoker G-Mφ and M-Mφ to H_2O_2 decreased phagocytosis of *H. influenzae* ($p<0.05$, Fig 3C, E) and *S. pneumoniae* ($p<0.05$, Fig. 3D, F), while exposure of COPD M-Mφ and G-Mφ decreased phagocytosis of *S. pneumoniae* ($p<0.05$, Fig. 3D, F), but not *H. influenzae* ($p>0.05$, Fig 3C, E, F).

Macrophage mitochondrial reactive oxygen species in response to phagocytosis

At baseline, mROS levels were similar in all cells from all subject groups irrespective of the differentiation process (Table 4). Similarly phagocytosis of either *H. influenzae or S. pneumoniae* had no effect on mROS levels in either G-Mφ and M-Mφ from non-smokers (Fig. 4A and B) or smokers without COPD (Fig. 4C and D), However, in COPD M-Mφ, phagocytosis of both *H. influenzae* and *S. pneumoniae* led to a significant increase in mROS levels of 58% ($p<0.01$) and 59% ($p<0.05$) respectively (Fig 4E). In contrast, only phagocytosis of *H. influenzae* (50%, $p<0.05$) but not *S. pneumoniae* increased mROS levels in COPD G-Mφ (Fig. 4F).

We next measured $\Delta\Psi m$ to determine whether this observation could be due to changes in mitochondrial function. At baseline, there was no difference in $\Delta\Psi m$ between patient groups, or macrophage phenotypes (Table 4). After phagocytosis, there was no change in $\Delta\Psi m$ in both G-Mφ and M-Mφ from non-smokers and smokers without COPD (Fig. 5A-D). In contrast, phagocytosis of *H. influenzae*, but not *S. pneumoniae* caused a significant decrease in $\Delta\Psi m$ in both G-Mφ ($p<0.05$, Fig. 5D) and M-Mφ ($p<0.05$, Fig 5D) from COPD patients. The positive control CCCP reduced $\Delta\Psi m$ in all cell types ($p<0.001$, Fig 5).

Exogenous oxidative stress and mitochondrial function

Having shown that exogenous oxidative stress impairs phagocytosis in cells from non-smokers and COPD patients, the effect on mitochondrial function was also investigated. At baseline, H_2O_2 had no effect on mROS or $\Delta\Psi m$ in any cell type from any of the subjects used in this study (Table 4). However, after phagocytosis, changes were observed. In M-Mφ, non-smoker cells that had been exposed to H_2O_2 showed a decrease in mROS after phagocytosis of both *H. influenzae* ($p<0.05$, Fig 6 A) and *S. pneumoniae* ($p<0.05$, Fig 6B), while in COPD a decrease was also seen with both bacterial species ($p<0.01$, Fig 6A and B). In G-Mφ, non-smoker cells that had been exposed to H_2O_2 showed a decrease in mROS after phagocytosis of both *H. influenzae* ($p<0.05$, Fig 6C) and *S. pneumoniae* ($p<0.05$, Fig 6D), while in smokers without COPD a decrease was also seen with both bacterial species ($p<0.05$, Fig 6C, D). In COPD G-Mφ, decrease was seen with *S. pneumoniae* ($p<0.05$, Fig 6D) but did not reach

significance in cells exposed to *H. influenzae* (p=0.054). No effect of H_2O_2 and phagocytosis was seen on $\Delta\Psi m$ in any cell type studied (p>0.05, Fig 7).

Discussion

Alveolar macrophages are phagocytic cells that patrol the airways and removed inhaled particulates and microorganisms [28]. However, COPD macrophages phagocytose bacteria poorly, which could contribute to bacterial colonisation of the lungs, and lead to worsening of symptoms and lung function decline [8, 11]. MDM have been shown to be an effective model of alveolar macrophages, as they share the functional defect in phagocytosis and are more easily obtainable than alveolar macrophages [11]. In this study, we generated MDM by differentiating monocytes in GM-CSF (G-Mφ) or M-CSF (M-Mφ) to investigate two macrophage phenotypes that have been characterised by others previously [29-31], and confirmed key data using alveolar macrophages isolated from lung tissue [32].

In the present study, we have demonstrated that in both G-Mφ and M-Mφ phenotypes, there was a significant decrease in phagocytosis of the respiratory pathogens, *H. influenzae* ($p<0.05$) and *S. pneumoniae* ($p<0.05$), by COPD macrophages compared to non-smoker macrophages. However, there was no difference in the percentage of macrophages from healthy subjects or COPD patients that were able to phagocytose. These data suggest that while all macrophages are able to undergo the process of phagocytosis, it is the amount of bacteria, as measured by MFI, that can be consumed that is reduced in COPD. Importantly, there was no significant decrease in phagocytosis in smoker macrophages compared to non-smokers, although there was a trend to decrease phagocytosis of *H. influenzae* with G-Mφ ($p=0.08$), indicating that it is the disease rather then smoking history that determines this defect. These data confirm our previous observations [11], but we now show that differentiation of macrophages in M-CSF also leads to an aberrant macrophage phenotype in COPD suggesting that this effect is not due to culture conditions alone but is potentially inherent in monocyte precursors. Therefore, this systemic defect further contributes to the reduced clearance of bacterial pathogens by macrophages in the lungs and may account for increased bacterial colonisation and the concomitant decline in lung function observed in COPD. In addition, the increased rates of pneumococcal bacteraemia in COPD may also be influenced by impaired function of blood monocytes and splenic macrophages [33]. Potential roles for defective circulating monocyte function in COPD co-morbidities could be an area for future study.

In more developed nations, most COPD cases are due to cigarette smoking, which introduces high levels of oxidative stress into the lungs [34] could be a contributing factor to the decreased function of COPD macrophages. To test this, we applied exogenous oxidative stress in the form of H_2O_2, to macrophages at a physiologically relevant concentration for 24h [35]. H_2O_2 is released from activated inflammatory and structural cells, and is a component of cigarette smoke and air pollution [36]. We showed that exogenous oxidative stress caused a reduction in phagocytosis in both healthy and COPD alveolar macrophages and in both G-Mφ and M-Mφ from non-smokers, indicating that exposure to high levels of oxidative stress in the lungs could be a factor that influences defective macrophage phagocytosis in COPD. Interestingly, MDM from smokers without COPD did not respond to exogenous oxidative stress, which could indicate a protective mechanism within smoker cells, such as increased expression of anti-oxidants. This is important as we have already demonstrated the role of Nrf2 in COPD [37].

We studied this further, and showed that macrophages cultured in the absence of exogenous oxidative stress showed no baseline differences in mROS or $\Delta\Psi$m between patient groups at the early time point studied. However, when the cells were analysed after phagocytosis of *H.influenzae*, both COPD MDM phenotypes showed increased levels of mROS and reduced $\Delta\Psi$m, whereas macrophages from control subjects had no change compared to baseline. After phagocytosis of *S.pneumoniae,* there was a similar increase in mROS levels in M-Mφ, but not in G-Mφ from COPD patients, and there was no change in $\Delta\Psi$m, indicating a differential response to bacterial species. One caveat with these data, is that extracellular fluorescence could not be quenched and therefore there maybe some contribution of extracellular bacteria to the response. Nevertheless, the differential responses from the different macrophage phenotypes may reflect alternative mechanisms for deriving ATP. It is known that M1 macrophages ulitize a glycolytic pathway whereas M2 macrophages utilise oxidative phosphorylation [38], an effect mirrored by G-Mφ and M- Mφ respectively [39].

ROS induces damage by oxidising proteins and DNA, including mitochondrial DNA [40]. mROS released from mitochondria that are adjacent to phagolysosomes containing bacteria has been shown to be bactericidal in macrophages [41] and, so increasing the early production of mROS post phagocytosis, may be a mechanism by which macrophages clear bacteria [42]. However, this increase in mROS will then be scavenged by intracellular

enzymes including superoxide dismutase, to prevent damage to the cell [17]. The lack of a difference in mROS levels post-phagocytosis in macrophages from healthy subjects suggests that if mROS are induced to assist in bacterial clearance, these oxidative species are rapidly quenched. The continued elevation in mROS in COPD macrophages suggests that the cells are unable to regulate mROS production, or that excessive levels of mROS are being generated which may overwhelm the endogenous anti-oxidant enzymes. It has been shown that levels of antioxidants, such as hemeoxygenase (HO)-1 that are regulated by the transcription factor Nrf-2, and superoxide dismutase, are decreased in COPD macrophages, which could contribute to the dysregulation of mROS seen here [43, 44]. Recently, we demonstrated that the Nrf-2 activator, sulforophane, was able to restore both non-opsonic and opsonic phagocytosis of *S. pneumoniae* in both alveolar macrophages and MDM from COPD patients, further supporting the pivotal role of ROS in driving the defects in macrophage responsiveness to pathogens [37]. We have also identified chronic oxidative stress in alveolar macrophages from COPD patients [45] and our observations of intrinsic alterations in mitochondrial function and mROS generation in COPD MDM could result in the chronic oxidative stress observed in COPD alveolar macrophages *in vivo*. The data presented in the current study complements these findings in a separate cohort of subjects and, suggests that intrinsic mitochondrial function could drive both phagocytic defects and the oxidative stress that leads to this.

Excess levels of oxidants within the cell can cause damage to cellular components and induce apoptosis [17]. Therefore, following phagocytosis, increased mROS in COPD macrophages, may alter the capacity of the cell to phagocytose subsequently, and contribute to the defective phagocytic pattern of '*early satiety*' seen in COPD. Exactly why the mitochondria are producing excessive ROS is unclear, however this study shows a decreased $\Delta\Psi$m indicative of unhealthy mitochondria (20). Excessive elevated mROS generated following phagocytosis in COPD macrophages may lead to generation of pro-inflammatory cytokines due to activation of the inflammasome, transcription factors, and further to cell damage in COPD (18).

It has previously been shown that mROS is elevated in alveolar macrophages from COPD patients at baseline, but not following *in vitro* infection with *S. pneumoniae* [45]. This differs from our findings in MDM, but may suggest that macrophages from the lungs, which will have already been exposed to lung bacteria, are primed to have elevated mROS,

whereas blood macrophages require priming by exposure to bacteria to unmask the intrinsic mitochondrial defect we describe, and subsequently display increased oxidative stress. Further analysis of mitochondrial function in alveolar and MDM is warrented to understand this complex processes.

Having established that mitochondrial function is abnormal in COPD macrophages, it was of interest to study whether exogenous oxidative stress was acting by altering mitochondrial function within macrophages. mROS and $\Delta\Psi$m were measured in MDM pre-treated with H_2O_2. At baseline H_2O_2 had no affect on both aspects of mitochondrial function, but after phagocytosis, H_2O_2 caused a decrease in mROS in both G-Mφ and M-Mφ from non-smokers, and COPD patients. This response may be due to exogenous oxidative stress reducing endogenous oxidative stress through alteration of the mROS/anti-oxidant balance within the cell, in order to limit the damage caused, or due to damage to the mitochondria itself, reducing cellular function. Alterations in mitochondrial function might lead to changes in metabolic balance of the cell and energy generation. One possible outcome from this, could be changes in the phagocytic process possibly via alterations in dynamics of the cytoskeleton. Further, mitochondrial ROS is utilised in the killing of bacteria inside the cell, and so dysfunctional ROS production may alter the cells ability to function appropriately to a bacterial pathogen. However, targeting mitochondrial defects is difficult but is a potential therapeutic approach [46].

In summary, both alveolar macrophages and G-Mφ and M-Mφ macrophage phenotypes from COPD patients demonstrate reduced phagocytosis of airway pathogens, which provides further support for the theory that there is an inherent circulating phagocytic defect in monocytes in disease. The reduction in phagocytosis is associated with dysregulation of mitochondrial function and intracellular mROS generation. Targeting mitochondria represents a treatment option for COPD, which may reverse the defective phagocytosis and lead to reduced exacerbations in this disease.

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Figure Legends

Figure 1. Correlations of phagocytosis of respiratory pathogens between alveolar macroohages and MDM from the same subjects.

Panels A-D - Non-smoker (▲n=7), smoker (■ n=7) and COPD (● n=6) alveolar macrophages, M-Mφ and G-Mφ were isolated from the same subject, co-cultured with fluorescently-labelled *H. influenzae* (HI, panels A and B) or *S. pneumoniae* (SP, panels C and D) bacteria for 4 hours and phagocytosis measured by relative fluorescence units (RFU). Data are individual data points and correlation (r) is reported as Spearman's rank coefficient.

Panels E-F - Confocal microscopic images of samples confirm internalisation of bacteria in G-Mφ (Panel E) and M-Mφ (Panel F), and in orthogonal images of a Z-stack of G-Mφ macrophages (Panel G). Images show cytoplasm (cell tracker red), nucleus (blue - DAPI) and *H. influenzae* (green, Alexa-488).

Figure 2 – Internalisation of bacteria by macrophages from non-smokers, smokers and COPD patients

M-Mφ and G-Mφ from non-smokers (solid bars), smokers without COPD (vertical hatched bars) and COPD patients (horizontal hatched bars) were co-cultured with fluorescently-labelled *H. influenzae* (HI, panels A and B) or *S. pneumoniae* (SP, panels C and D) bacteria for 4 hours, after which the percentage of cells that had undergone phagocytosis (panels B and D) and median fluorescence intensity (MFI, panel A and C) were measured by flow cytometry. Data are represented as mean ± SEM of 9-12 independent experiments. *$p<0.05$ non-smoker *vs* COPD.

Figure 3 – Effect of exogenous oxidative stress decreases macrophage phagocytosis.

Alveolar macrophages (panels A and B, healthy=13, COPD=7), M-Mφ (panels C and D) or G-Mφ (panels E and F) from non-smokers (N=12), smokers without COPD (N=8-9) and COPD patients (N=10-11) were left untreated, or treated with 200μM hydrogen peroxide (H_20_2) for 24 hours, then co-cultured with fluorescently-labelled *H. influenzae* (HI, panels A, C, E) or *S. pneumoniae* (SP, panels B, D, F) bacteria for 4 hours, after which median fluorescence intensity (MFI) of phagocytosis was measured by flow cytometry. Data are represented as individual data points and as mean±SEM of untreated (■) or H_20_2 treated cells (▲). *$p<0.05$, **$p<0.01$, ***$p<0.001$ untreated *vs* H_20_2.

Figure 4 – Increased production of mitochondrial reactive oxygen species (mROS) in COPD macrophages in response to phagocytosis

M-Mφ (panels A, C, E) or G-Mφ (panels B, D, F) from non-smokers (N=10, panels A-B), smokers without COPD (N=9-10, panels C-D) and COPD patients (N=13, panels E-F) were analysed for mROS levels after 4 hour co-culture with fluorescently-labelled *H. influenzae* (HI) or *S. pneumoniae* (SP). Data are normalised to un-treated control (UT). *p<0.01, **p<0.01 non-stimulated *vs* bacteria.

Figure 5 – Decreased mitochondrial membrane potential ($\Delta\Psi$m) in COPD macrophages in response to phagocytosis. M-Mφ (panels A, C, E) or G-Mφ (panels B, D, F) from non-smokers (N=10, panels A-B), smokers without COPD (N=10, Panels C-D) and COPD patients (N=10, panels E-F) were analysed for $\Delta\Psi$m after 4 hour co-culture with fluorescently-labelled *H. influenzae* (HI) or *S. pneumoniae* (SP), with carbonyl cyanide m-chlorophenyl hydrazine (CCCP) acting as a positive control. Data show fold change from un-treated control (UT). *p<0.05, *p<0.01, ***p<0.001 *vs*. non-stimulated.

Figure 6 – Exogenous oxidative stress decreases mitochondrial reactive oxygen species (mROS) production in macrophages.

M-Mφ (panels A-B) or G-Mφ (panels C-D) from non-smokers (N=12), smokers without COPD (N=7-9) and COPD patients (N=9-11) were treated with 200μM hydrogen peroxide (H_20_2) for 24 hours, then co-cultured with fluorescently-labelled *H. influenzae* (HI, panels A and C) or *S. pneumoniae* (SP, panels B and D) bacteria for 4 hours, after which mROS was measured by flow cytometry. Data are represented as individual data points and as mean±SEM of untreated (■) or H_20_2 treated cells (▲). *p<0.05 untreated *vs* H_2O_2.

Figure 7 – Exogenous oxidative stress does not affect mitochondrial membrane potential in macrophages.

M-Mφ (panels A-B) or G-Mφ (panels C-D) from non-smokers (N=8), smoker (N=8-9) and COPD patients (N=8-11) were treated with 200μM hydrogen peroxide (H_20_2) for 24 hours, then co-cultured with fluorescently-labelled *H. influenzae* (HI, panels A and C) or *S. pneumoniae* (SP, panels B and D) bacteria for 4 hours, after which $\Delta\Psi$m was measured by

flow cytometry. Data are represented as individual data points and as mean±SEM of untreated (■) or H_20_2 treated cells (▲).

Supplementary Figure 1 – Exogenous oxidative stress does not alter macrophage viability

M-Mφ (black circles, N=7) or G-Mφ (grey squares, N=10) were treated with 1-200μM hydrogen peroxide (H_20_2) for 24 hours then co-cultured with fluorescently-labelled *H. influenzae* (HI, panel A) or *S. pneumoniae* (SP, panel B) bacteria for 4 hours and cell viability measured by MTT assay. Data are pooled from non-smoker, smoker and COPD subjects, and show mean ± SEM.

Table 1

	Non-smoker n=17	Smoker n=16	COPD n=17
Age	61±3	57±3	60±2
Pack years	0	32±10*	30±3**
FEV1(L)	3.2±0.3	2.9±0.4	1.7±0.2**
FEV1 % predicted	109±6	92±4	68±3***
FVC (L)	3.7±0.3	3.7±0.4	2.7±0.3
FEV1:FVC	0.80±0.02	0.76±0.03	0.69±0.04*

Table 1 – Subject demographics for patients analysed in figure 1 . Data represented as mean ± SEM. *p<0.05, **p<0.01 Non-smoker vs. smoker/COPD. Chonric obstructive pulmonary disease (COPD), Male (M), Female (F), Forced experiatory volume in one second (FEV1), Litres (L), Forced vital capacity (FVC).

Table 2.

	Healthy n=13	COPD n=7
Age	62±3	68±4
Pack years	20±5	32±4
FEV1(L)	2.7±0.2	1.7±0.2***
FEV1 % predicted	94±4	70±8*
FVC (L)	3.7±0.2	3.1±0.2
FEV1:FVC	0.73±0.03	0.56±0.05**

Table 2 – Subject demographics for lung macrophages used in Figure 3. Macrophages were isolated from tissue resection (n=12) or bronchialveolar lavage (n=8). Data represented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 Healthy vs. COPD. Chronic obstructive pulmonary disease (COPD), Male (M), Female (F), Forced experiatory volume in one second (FEV1), Litres (L), Forced vital capacity (FVC).

Table 3

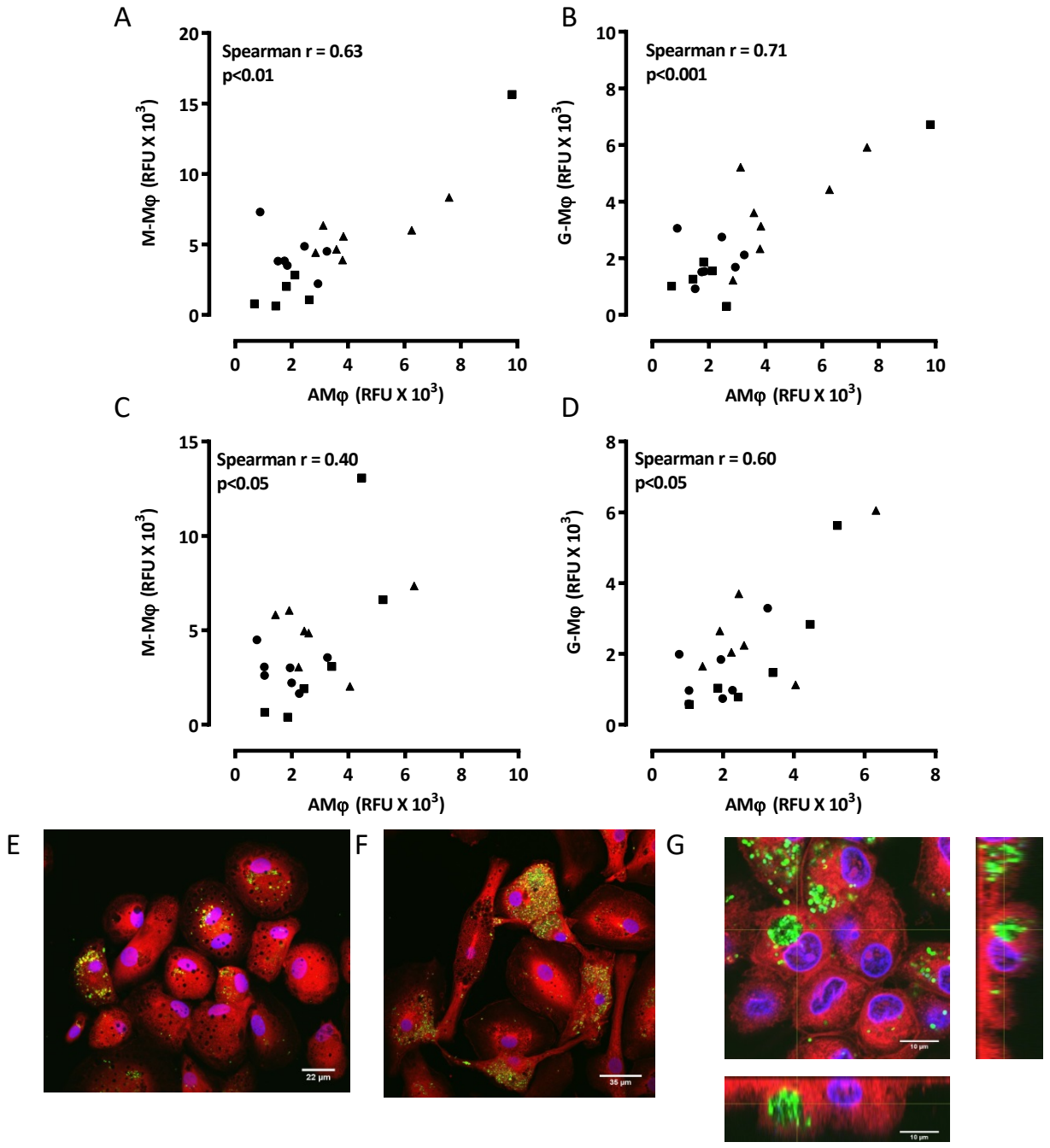
	Non-smoker	Smoker	COPD
Sex (M:F)	10:7	13:3	10:7
Age	61±2	63±2	71±3
Pack years	0	29±4***	47±7***
FEV1(L)	2.9±0.3	3.0±0.2###	1.5±0.2***
FEV1 % predicted	100±5	94±3###	55±5***
FVC (L)	3.9±0.3	4.2±0.3#	2.8±0.3*
FEV1:FVC	0.76±0.02	0.73±0.02###	0.52±0.04***

Table 3 – Subject demographics for monocyte-derived macrophages. Data represented as mean ± SEM. *p<0.05, ***p<0.001 Non-smoker vs. smoker/COPD. #p<0.05, ##p<0.01 ###p<0.001, smoker vs. COPD. Chonric obstructive pulmonary disease (COPD), Male (M), Female (F), Forced experiatory volume in one second (FEV1), Litres (L), Forced vital capacity (FVC).

Table 4

	mROS				ΔΨm			
	M-Mφ		G-Mφ		M-Mφ		G-Mφ	
	UT	H_2O_2	UT	H_2O_2	UT	H_2O_2	UT	H_2O_2
Non-smoker	2.06 (0.4)	1.92 (0.4)	2.34 (0.4)	2.3 (0.4)	1.09 (0.06)	1.05 (0.09)	1.39 (0.09)	1.43 (0.15)
Smoker	2.13 (0.6)	2.37 (0.7)	2.27 (0.5)	2.20 (0.5)	1.08 (0.07)	1.09 (0.06)	1.15 (0.07)	1.14 (0.09)
COPD	2.24 (0.4)	1.81 (0.4)	2.55 (0.5)	2.42 (0.5)	1.06 (0.11)	1.02 (0.10)	1.28 (0.15)	1.29 (0.12)

Table 4 – Baseline values for mROS and ΔΨm. Data shows baseline values for untreated (UT) cells or cells treated with 200Mm H_2O_2 for 24 h. Data represented as mean±SEM. Chonric obstructive pulmonary disease (COPD), mitochondrial reactive oxygen species (mROS), mitochondrial membrane potential (ΔΨm), M-CSF derived macrophages (M-Mφ), GM-CSF derived macrophage (G-Mφ).


A
Spearman r = 0.63
p<0.01
M-Mφ (RFU X 10^3)
AMφ (RFU X 10^3)
B
Spearman r = 0.71
p<0.001
G-Mφ (RFU X 10^3)
AMφ (RFU X 10^3)
C
Spearman r = 0.40
p<0.05
M-Mφ (RFU X 10^3)
AMφ (RFU X 10^3)
D
Spearman r = 0.60
p<0.05
G-Mφ (RFU X 10^3)
AMφ (RFU X 10^3)
E
F
G

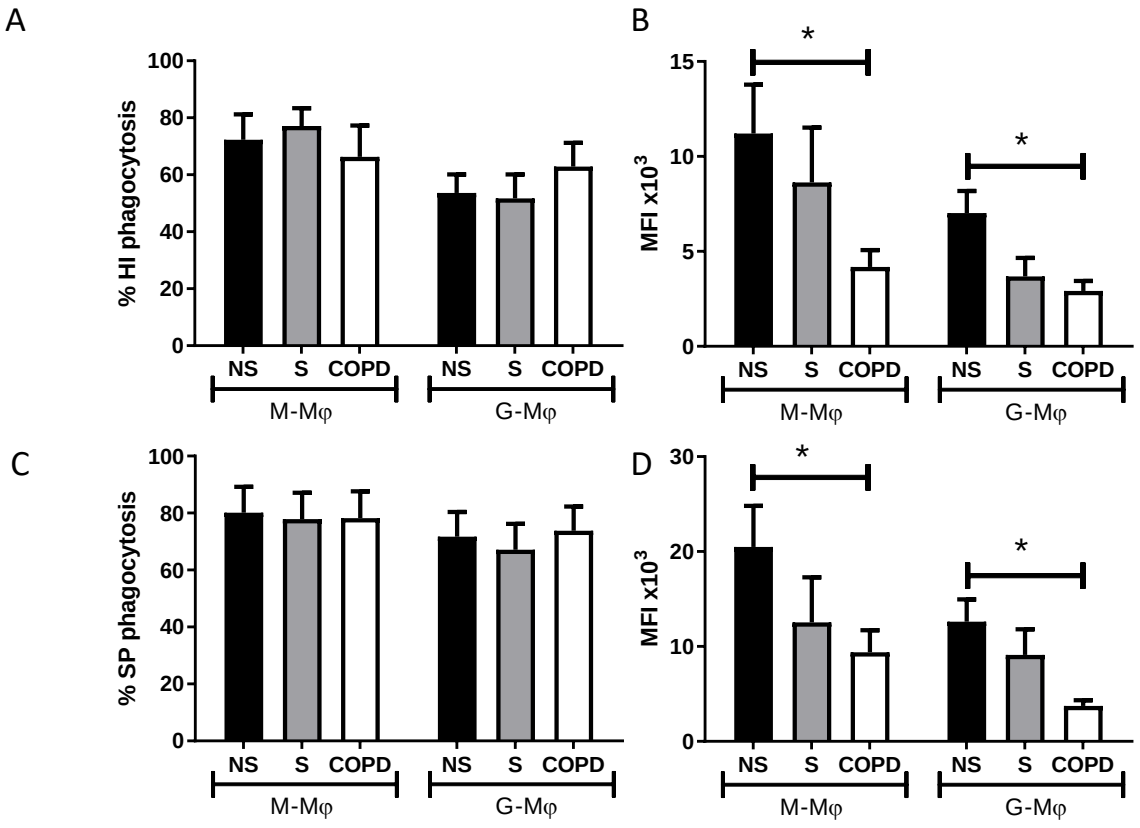
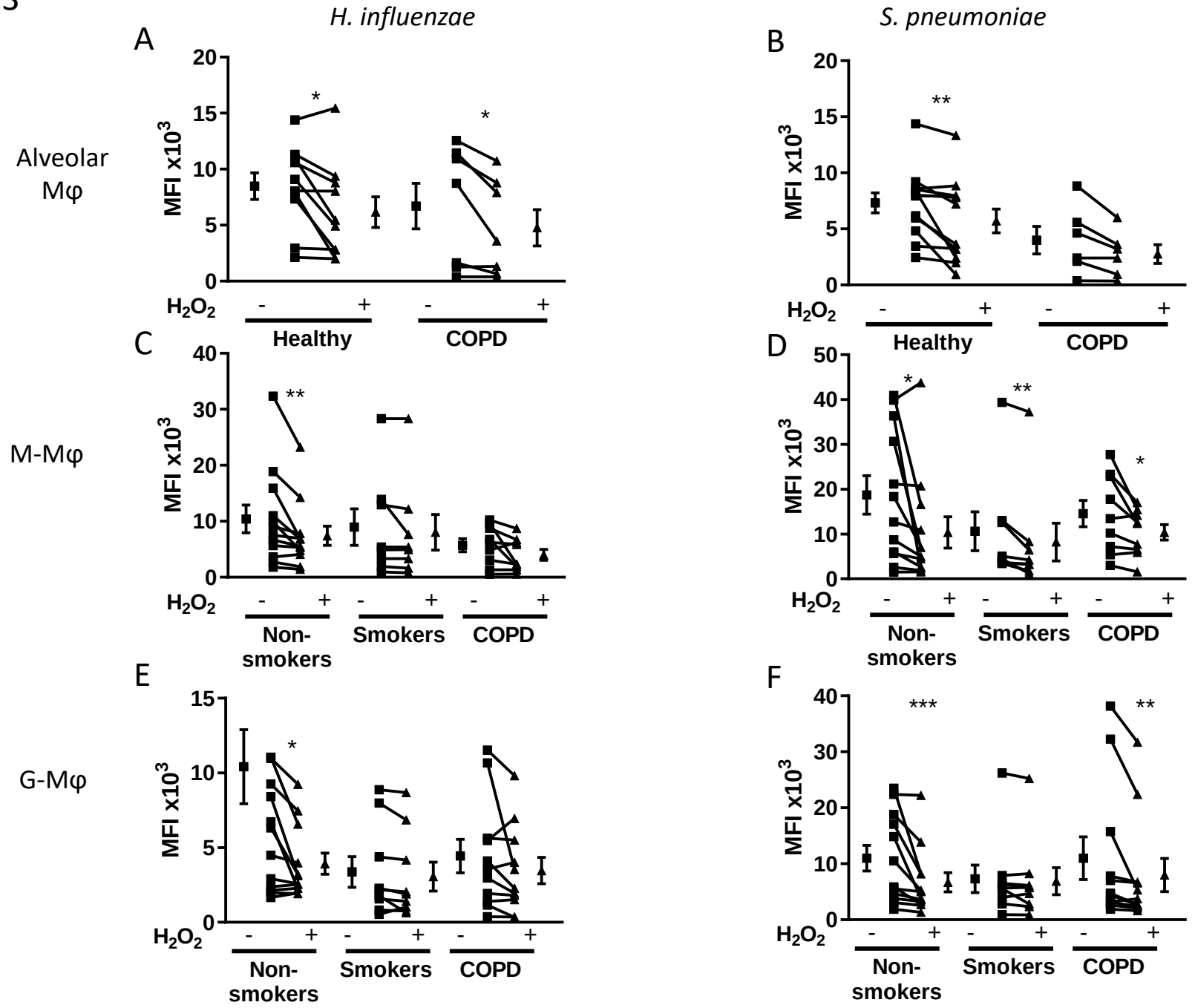

A
B
C
D
% HI phagocytosis
MFI x10^3
% SP phagocytosis
MFI x10^3
NS
S
COPD
M-Mφ
G-Mφ
*

Figure 2

Figure 3


H. influenzae
S. pneumoniae
A
B
C
D
E
F
Alveolar Mφ
M-Mφ
G-Mφ
MFI x10^3
H2O2
Healthy
COPD
Non-smokers
Smokers
COPD

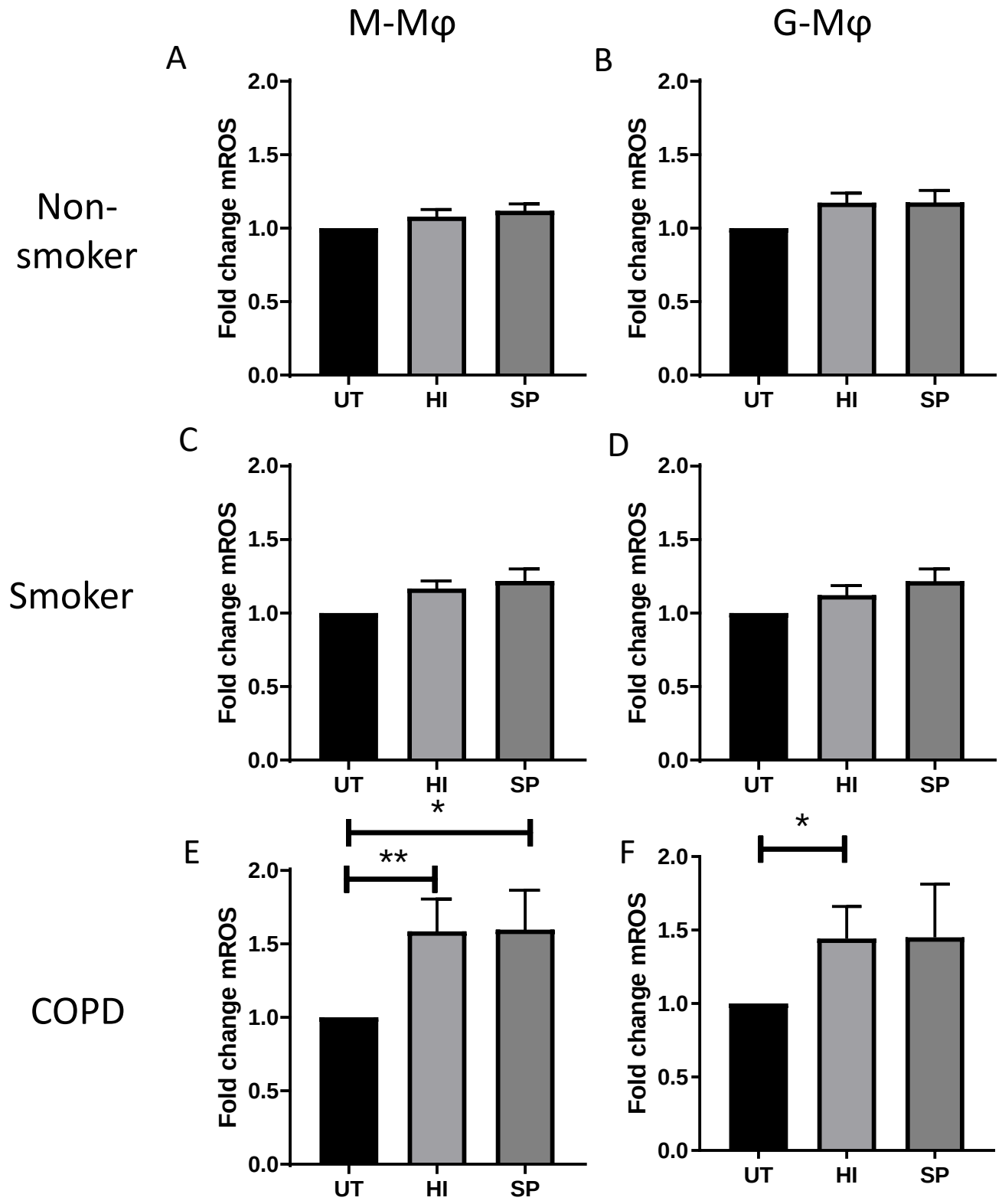

M-Mφ
G-Mφ
A
B
C
D
E
F
Non-smoker
Smoker
COPD
Fold change mROS
2.0
1.5
1.0
0.5
0.0
UT
HI
SP
*
**

Figure 4

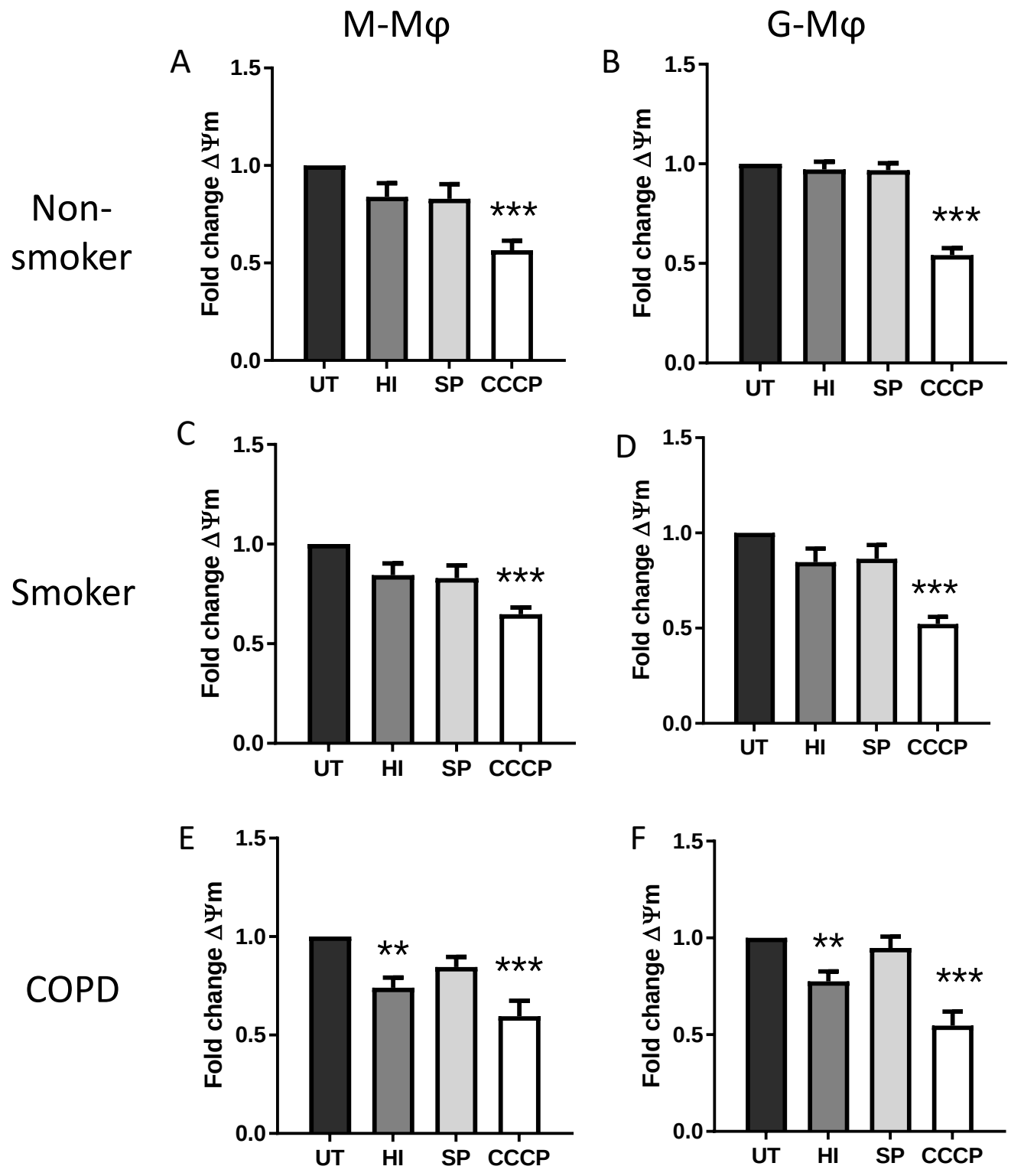

M-Mφ
G-Mφ
Non-smoker
Smoker
COPD
A
B
C
D
E
F
Fold change ΔΨm
1.5
1.0
0.5
0.0
UT
HI
SP
CCCP
***
**

Figure 5

Figure 6

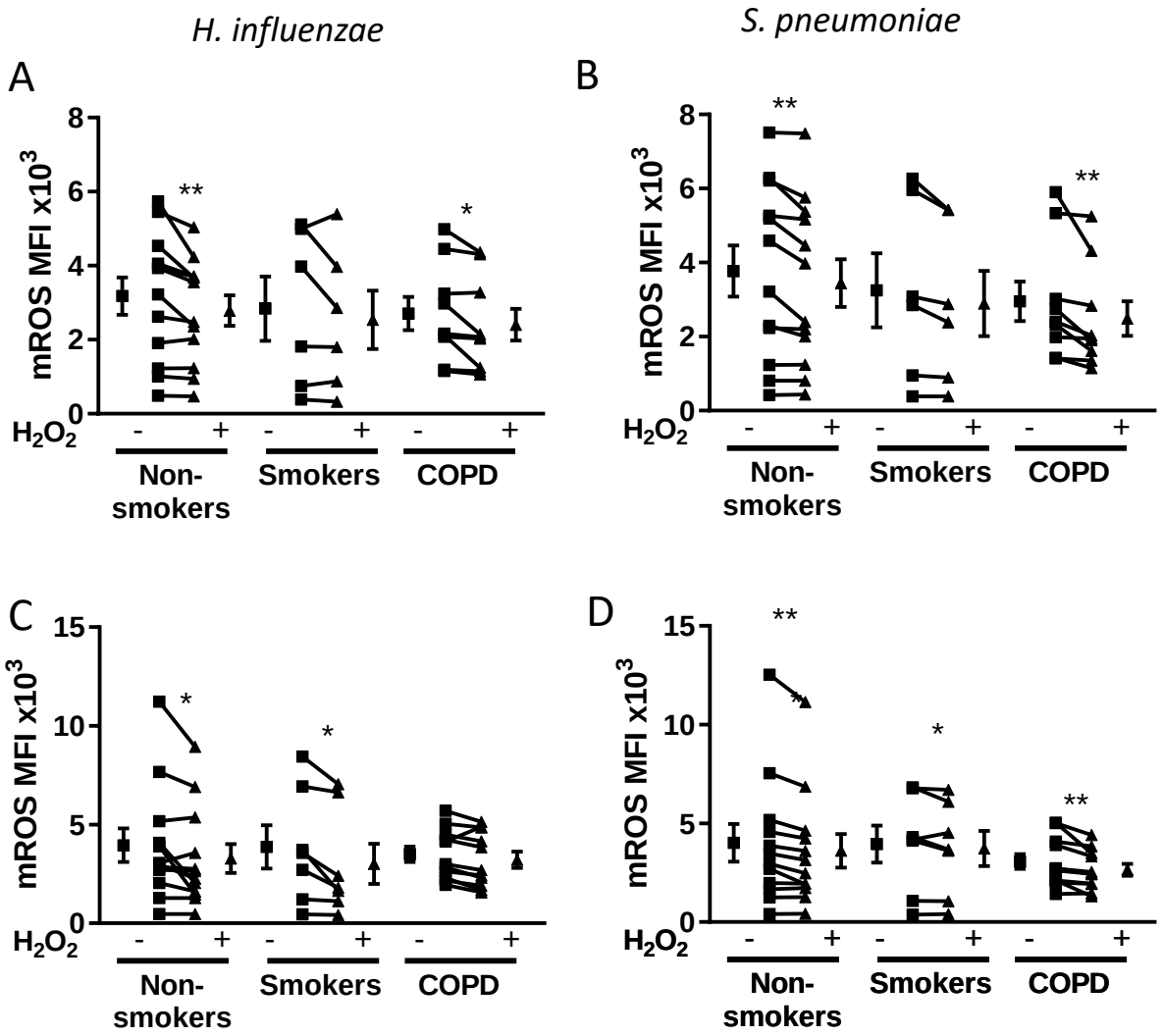
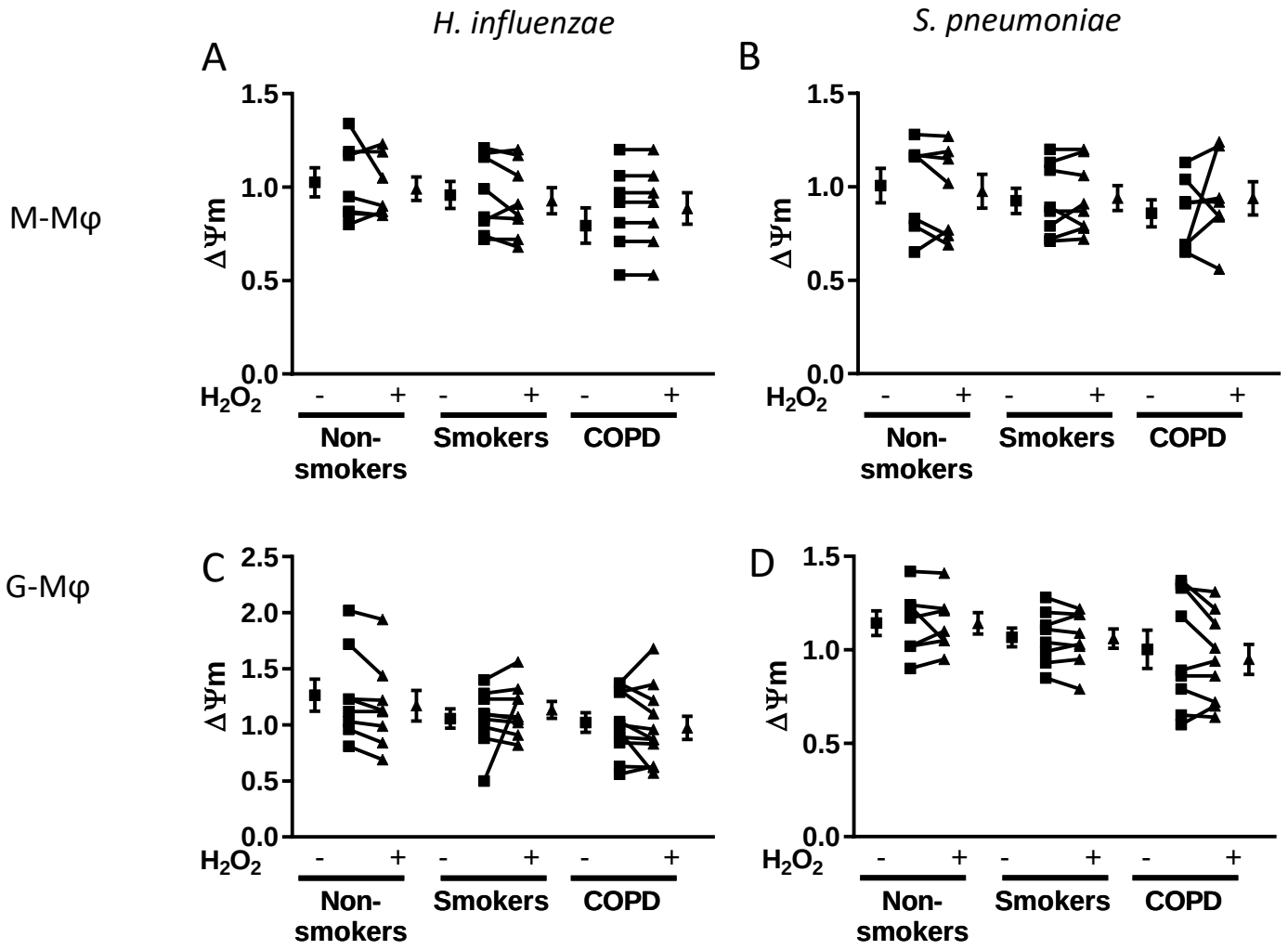
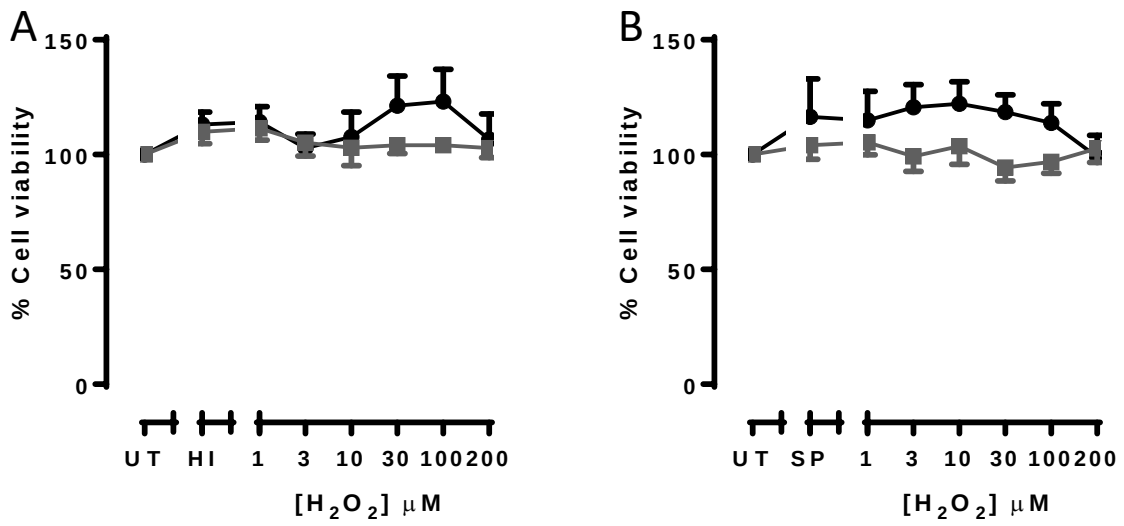

H. influenzae
S. pneumoniae
A
B
C
D
M-Mφ
G-Mφ
mROS MFI x10^3
H2O2
Non-smokers
Smokers
COPD

Figure 7


H. influenzae
S. pneumoniae
M-Mφ
G-Mφ
A
B
C
D
ΔΨm
H2O2
-
+
Non-smokers
Smokers
COPD



Supplementary figure 1