Impaired mitochondrial microbicidal responses in chronic obstructive pulmonary disease macrophages.

Martin A. Bewley[1†], Julie A. Preston[1], Mohammed Mohasin[1], Helen M. Marriott[1], Richard C Budd[1, 2], Julie Swales[1], Paul Collini[1,2], David R. Greaves[3], Ruth W. Craig[4], Christopher E. Brightling[5] Louise E. Donnelly[6], Peter J. Barnes[6], Dave Singh[7], Steven D. Shapiro[8], Moira K.B. Whyte[9], and David H. Dockrell[10]*

[1]The Florey Institute for Host-Pathogen Interactions and Department of Infection, Immunity and Cardiovascular Disease, University of Sheffield Medical School, S10 2RX, UK, [2]Sheffield Teaching Hospitals, S10 2RX, UK, [3]Sir William Dunn School of Pathology, University of Oxford, OX1 3RF, UK, [4]Department of Pharmacology and Toxicology, Geisel School of Medicine at Dartmouth, 03755, NH, USA, [5]Instiute for Lung Heath, University of Leicester, LE3 9QP, UK, [6]Airway Disease National Heart and Lung Institute, University of London Imperial College, SW3 6LY, UK, [7]Centre for Respiratory Medicine & Allergy, Medicines Evaluation Unit, University Hospital of South Manchester & University of Manchester, M23 9QZ UK, [8]Division of Pulmonary, Allergy and Critical Care Medicine, University of Pittsburgh Medical Center, USA, [9]Department of Respiratory Medicine and MRC Centre for Inflammation Research, University of Edinburgh, [10]Department of Infection Medicine and MRC Centre for Inflammation Research, University of Edinburgh. **Corresponding Author:** David H. Dockrell, The MRC/University of Edinburgh Centre for Inflammation Research, 47 Little France Crescent, Edinburgh, Edinburgh EH16 4TJ UK Phone: +44 (0) 131 242 658 Fax: +44 (0) 131 242 6578 email: david.dockrell@ed.ac.uk

Author contributions: JAP and MAB contributed equally to this work. JAP made and validated the transgenic mouse. MAB performed killing assays, flow cytometry and microscopy collected data and produced figures. MM performed seahorse experiments, and imaging, HMM performed *in vivo* experiments and JS contributed to design of imaging experiments. RCB and DS co-ordinated and performed bronchoscopies to obtain patient samples. DRG designed the CD68 construct. RWC designed the Mcl-1 construct. LED designed experiments measuring COPD associated phagocytic defects. PJB and CEB co-ordinated collection of the COPD patient cohort. MKBW, SDS and DHD designed and conceived the experiments. JAP, MAB and DHD wrote the manuscript with input from all other authors.

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At a glance summary:

Scientific Knowledge on the Subject: COPD patients are at increased risk from bacterial respiratory infections, which cause acute exacerbations adding to morbidity. Previous studies have identified potential defects in innate immunity but the effect of COPD on macrophage microbicidal responses has been little investigated. Host-mediated macrophage apoptosis in response to bacteria gives an increment to bacterial killing once canonical phagolysosomal killing has become exhausted. Defects in this pathway alter bacterial clearance.

What This Study Adds to the Field: We show that inhibition of macrophage apoptosis and a failure to induce mitochondrial reactive oxygen species generation in COPD macrophages contributes to impaired clearance of pneumococci in the lung.

Footnote:

Presented in part as "Alveolar macrophages during COPD demonstrate reduced apoptosis-associated bacterial killing and reduced mitochondrial reactive oxygen species-dependent killing". M. Bewley, R. Budd, D. Singh, P.J. Barnes, L.E. Donnelly, M.K.B. Whyte, D.H. Dockrell, Medical Research Council COPD MAP Consortium. American Thoracic Society International Conference San Diego May 19th, 2014. Am J. Respir Crit Care Med 18:2014; A2862

Abstract:

Rationale: Chronic obstructive pulmonary disease (COPD) is characterized by impaired clearance of pulmonary bacteria.

Objectives: The effect of COPD on alveolar macrophage (AM) microbicidal responses was investigated.

Methods: Alveolar macrophages (AMs) were obtained from bronchoalveolar lavage from healthy donors or COPD patients and challenged with opsonized serotype 14 *Streptococcus pneumoniae.* Cells were assessed for apoptosis, bactericidal activity and mitochondrial reactive oxygen species (mROS) production. A transgenic mouse line, in which the CD68 promoter ensures macrophage specific expression of human Mcl-1 (CD68.hMcl-1), was used to model the molecular aspects of COPD.

Measurements and Main Results: COPD AM had elevated levels of Mcl-1, an anti-apoptotic Bcl-2 family member, with selective reduction of delayed intracellular bacterial killing. CD68.hMcl-1 AM phenocopied the microbicidal defect since transgenic mice demonstrated impaired clearance of pulmonary bacteria and increased neutrophilic inflammation. Murine bone marrow-derived macrophages (BMDM) and human monocyte-derived macrophages (MDM) generated mitochondrial reactive oxygen species (mROS) in response to pneumococci, which co-localized with bacteria and phagolysosomes to enhance bacterial killing. The Mcl-1 transgene increased oxygen consumption rates and mROS expression in mock-infected BMDM but reduced caspase-dependent mROS production after pneumococcal challenge. COPD AM also increased basal mROS expression, but failed to increase production after pneumococcal challenge, in keeping with reduced intracellular bacterial killing. The defect in COPD AM intracellular killing was associated with a reduced ratio of mROS /superoxide dismutase 2.

Conclusions: Upregulation of Mcl-1 and chronic adaption to oxidative stress alters mitochondrial metabolism and microbicidal function, reducing the delayed phase of intracellular bacterial clearance in COPD.

Word count: 250

Key words: Apoptosis, mitochondrial reactive oxygen species, *Streptococcus pneumoniae*.

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by incompletely reversible airway obstruction. Neutrophilic inflammation drives airway narrowing and alveolar destruction (1). Cigarette smoke and biomass fuels are major factors initiating COPD pathogenesis and persistent neutrophilic inflammation in those who quit smoking emphasizes the importance of additional etiologic factors in maintaining progressive airway destruction (2, 3).

Clinical exacerbations of COPD, punctuate periods of relative stability in many patients, and contribute to the decline in respiratory function (4). Exacerbations are frequently associated with the presence of pathogenic bacteria in the lower airway and the lower airway bacterial load correlates with markers of neutrophilic inflammation such as CXCL8 levels (5). This suggests that a key driver of COPD pathogenesis is a defect in airway innate immune responses to pathogenic bacteria. While *Haemophilus influenzae, Streptococcus pneumoniae* and *Moraxella catarrhalis* are all associated with infective exacerbations, *S. pneumoniae* remains the major cause of community-acquired pneumonia (CAP) in COPD (6, 7). The relative risk of CAP, pneumococcal CAP and invasive pneumococcal disease (IPD) is elevated in patients with COPD to a greater extent than smokers another group at increased risk of IPD (8). This suggests that patients with COPD possess significant defects in their host defences to pneumococcal disease in the lower airway. However the basis of this increased susceptibility to pneumococcal pneumonia remains undefined.

Alveolar macrophages (AM) are central to the organization of pulmonary innate immunity and are critical for clearance of pneumococci from the alveolar space (9). However, AM do not possess several of the microbicidal molecules used by neutrophils

and pathogens have acquired adaptions to resist others (10), which challenges AM microbicidal capacity. Macrophages therefore employ additional host defence strategies and induction of apoptosis is required for efficient clearance of intracellular bacteria after phagocytosis (9, 11). Apoptosis is controlled by expression of the anti-apoptotic protein Mcl-1, which is dynamically regulated after bacteria are internalized (12, 13). COPD is associated with decreased macrophage innate competence as illustrated by evidence for impaired bacterial phagocytosis of non-typeable *H. influenzae* and *S. pneumoniae*, the pathogens that most frequently colonize the lower airway in COPD (14, 15). Little is known, however, concerning the effect of COPD on AM microbicidal responses.

We observed that COPD AM have persistent upregulation of Mcl-1 and have used patient AM and a unique murine transgenic macrophage to test how Mcl-1 upregulation alters pulmonary antibacterial host defense. Specifically, we addressed how Mcl-1 influenced macrophages ability to generate a mitochondrial microbicidal response involving generation of mitochondrial reactive oxygen species (mROS) in response to *S. pneumoniae* and how this influenced intracellular bacterial killing. Some of the results of these studies have been previously reported in the form of an abstract (16).

Materials and Methods.

Bacteria and Infection

Serotype 2 *S. pneumoniae* (D39 strain, NCTC 7466) , serotype 1 *S. pneumoniae* (WHO reference laboratory strain SSISP 1/1: Statens Seruminstitut), used in murine experiments (9) and serotype 14 *S. pneumoniae* (NCTC11902) used in COPD

experiments were cultured and opsonized in human (11) or murine serum before infection of cells as previously described (13).

Isolation and culture of macrophages

Bone marrow-derived macrophages (BMDM) were obtained as described (9). Human monocyte-derived macrophages (MDM) were isolated from whole blood donated by healthy volunteers with written informed consent, as approved by the South Sheffield Regional Ethics Committee (11). AM from patients with COPD (enrolled through MRC COPD-MAP) or from healthy controls (online data supplement Table E1) were isolated from BAL as previously described (16), with written approved consent prior to inclusion in the study as approved by the National Research Ethics Service Committee for Yorkshire and the Humber. Further information can be found in the online supplement.

Western blot

Whole cell extracts were isolated using SDS-lysis buffer and separated by SDS gel electrophoresis. Detailed information can be found in the online supplement.

Immunohistochemistry

Preparation of healthy and COPD lung sections (online data supplement Table E2), immunostaining and semi-quantitative evaluation, are described in the online supplement.

Flow Cytometry

Caspase activity was measured using the CellEvent caspase 3/7 green flow kit (Life Technologies), according to the manufacturers instructions. Mitochondrial reactive

oxygen species (mROS) were measured by flow cytometry using the dye MitoSOX-Red (Invitrogen). Detailed information can be found in the online supplement.

Microscopy

Nuclear fragmentation and condensation indicative of apoptosis were detected using 4′6′-diamidino-2-phenylindole (DAPI) (11). To visualise mROS cells were stained with 2μM MitoSOX-red (Invitrogen) and visualized on a Leica DMRB 1000, 40x objective. For co-localisation experiments, lysosomes were stained with 0.50μM Cresyl violet (Sigma) or challenged with D39 labelled with Alexa Fluor 647 carboxylic acid succinimidyl ester (Life Technologies) and co-stained with MitoSOX-red for 15 min, and visualised by confocal microscopy (Zeiss LSM 510, 63x1.4 oil objective). In other experiments, MDM and BMDM were challenged with D39 labelled with Alexa Fluor 647 and 16 h post-challenge cells were stained with MitoSOX and visualised by structured illumination microscopy (SIM). Detailed information can be found in the online supplement.

Intracellular killing assay.

Assessment of intracellular bacterial viability was carried out by gentamicin protection assay as previously described (17).

Metabolic measurements

Measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were performed using the XF24 extracellular flux analyser (Seahorse, Bioscience). Additional information can be found in the online supplement.

In vivo infections

Mcl-1 transgenic mice and wild-type littermates were infected and analyzed as outlined in the online supplement. Animal experiments were conducted in accordance with the Home Office Animals (Scientific Procedures) Act of 1986, authorized under UK Home Office License 40/3251 with approval of the Sheffield Ethical Review Committee, Sheffield, United Kingdom.

Statistics

Data are represented as mean and standard error unless otherwise indicated in the figure legends. Sample sizes were informed by standard errors obtained from similar assays in prior publications (12, 13). Analysis was performed with tests, as outlined in the figure legends, using Prism 6.0 software (GraphPad Inc.) and significance defined as $p < 0.05$. Decisions on the use of parametric (for normally distributed data) or non-parametric tests (for non-normally distributed data) were informed by the distribution of data.

Results

Mcl-1 is upregulated and is associated with reduced intracellular bacterial killing in COPD AM.

The Bcl-2 family member Mcl-1 regulates both macrophage viability (18) and delayed bacterial killing through induction of apoptosis during exposure to bacteria such as the pneumococcus (12, 13). We therefore investigated whether Mcl-1 expression was altered in AM by COPD. These experiments were conducted with a strain of *S. pneumoniae* that frequently colonizes this patient group and which also can cause IPD (19). Since macrophages from patients with COPD have an impaired capacity to ingest bacteria (14, 15), we modified the inoculum presented to control human AM to

normalize intracellular bacterial numbers (supplemental Figure E1) and demonstrated significant reduction in Mcl-1 expression in healthy donor's AM but no reduction in expression in COPD AM after bacterial challenge (Figure 1A). We also examined whether there was increased Mcl-1 expression in AM in the COPD lung. As illustrated quantification revealed enhanced Mcl-1 expression in lung biopsies of patients with COPD compared to control donors without COPD, which showed a focal distribution of high intensity (Figure 1B-D). The level of expression in controls was not altered by whether these were current smokers or non-smokers and results were similar when expression was analysed either by individual cell expression (Figure 1C) or by donor overall (Figure 1D).

We next addressed whether COPD AM had any defects in early microbicidal responses. In keeping with prior reports with MDM (14), we documented reduced phagocytosis of pneumococci by COPD AM (Figure 2A). Of note there was no evidence of the normal opsonic uplift in phagocytosis of pneumococci in COPD AM (16) in contrast to those from healthy donors. Despite differences in initial internalization of opsonized bacteria and COPD there was no evidence of significant reduction in early intracellular bacterial killing, which is linked to initial bacterial phagocytosis in myeloid cells and reactive oxygen species (ROS) generation via nicotinamide adenine dinucleotide phosphate-oxidase (20). To exclude confounding effects of differential bacterial internalization we adjusted the MOI used to challenge control AM, ensuring normalization of initial internalization (supplemental Figure E1), since apoptosis is directly related to initial bacterial ingestion (11). Upregulation of Mcl-1 was associated with reduced apoptosis in COPD AM (Figure 2B) and with enhanced survival of intracellular bacteria at a later time point (Figure 2C), supporting a defect in delayed apoptosis-associated pneumococcal killing by AM (12, 13).

Mcl-1 upregulation in AM impairs bacterial clearance in the lung.

CD68.hMcl-1^{+} transgenic mice were used to explore the functional consequence of Mcl-1 upregulation for bacterial clearance and the putative association of Mcl-1 upregulation with altered intracellular bacterial clearance in COPD AM. Expression of a human Mcl-1 transgene in myeloid cell populations extends macrophage survival while ensuring cells remain sensitive to physiological constraints on viability so that there is normal distribution of myeloid subsets and development (21). Using a low dose of pneumococci, which AM are able to contain (9), we demonstrate that the presence of the macrophage transgene results in impaired bacterial clearance from the lung and also increased bacteremia (Figure 3A-B). These changes were found in association with reduced AM apoptosis (Figure 3C) and increased numbers of neutrophils in the broncholalveolar lavage fluid (BAL) (Figure 3D).

Mcl-1 modulates generation of mitochondrial ROS and mROS-dependent bacterial killing.

We next explored the links between induction of the Mcl-1 regulated apoptotic program and microbicidal responses, using BMDM as a model of differentiated macrophages. Mcl-1 regulates apoptosis at the level of the mitochondrion (12, 13) and mitochondrial ROS (mROS) has emerged as an important microbicidal strategy used by macrophages (22). mROS was significantly increased in CD68.hMcl-1^{-} BMDM (but not CD68.hMcl-1^{+}) 20h after bacterial challenge, with significantly lower levels in CD68.hMcl-1^{+} compared to CD68.hMcl-1^{-} BMDM (Figure 4A-B). mROS co-localized with phagolysosomes and with bacteria, in contrast to endoplasmic reticulum, used as a control, which did not co-localize with either bacteria or phagolysosomes (Figure 4C-F and supplemental Figure E2-3). mROS staining was inhibited by an inhibitor

mitoTEMPO and each stain provided minimal background signal (supplemental Figure E2-4). mROS co-localization with bacteria was also visible in human MDM (Figure 4E and supplemental Figure E2F-G). mitoTEMPO, blocked the delayed phase of pneumococcal killing in CD68.hMcl-1^{-} (but not CD68.hMcl-1^{+}) BMDM (Figure 4G) and also in MDM (Figure 4H).

Mcl-1 modulates mitochondrial oxidative phosphorylation in macrophages.

Generation of mROS occurs during oxidative phosphorylation when electron leak predominantly from complex I results in generation of superoxide (23). We examined whether Mcl-1 modulates oxidative metabolism. As anticipated pneumococcal infection enhanced glycolytic metabolism (Figure 5A), in keeping with the known enhancement of glycolytic metabolism during macrophage responses to bacteria (24), but the CD68.hMcl-1 transgene did not alter glycolytic metabolism after infection. Also as expected infection was associated with a reduction in several parameters associated with oxidative phosphorylation, but the transgene itself resulted in increased baseline and maximal oxygen consumption rate (OCR) in mock infected cells, though it had no effect on the levels after pneumococcal challenge (Figure 5B-F). In association with alterations in OCR parameters the transgene was also associated with increased baseline mROS levels in mock-infected cells (Figure 5G). Since caspase activation enhances mROS production through interference with complex I of the electron transport chain (25), we next tested if Mcl-1 inhibited the inducible mROS expression observed after pneumococcal challenge in a caspase-dependent process and whether this overwhelmed Mcl-1's baseline effects on oxidative phosphorylation. As shown in Figure 5H-I, we show that Mcl-1 overexpression inhibits the increase in mROS production following bacterial challenge. Mcl-1 also specifically reduced the mROS production due to caspase activation after bacterial challenge, as treatment with the casapse inhibitor

zVAD reduced MitoSOX levels to comparable levels to those of the Mcl-1 transgenic. In these experiments with zVAD and zFA the baseline level of mROS was lower than in the experiments in Figure 5G, reflecting reduced sensitivity of detection in the presence of these chemicals, and the baseline alteration in mROS production due to the transgene was no longer apparent.

COPD AM fail to increase mROS production after pneumococcal challenge

Since COPD patients had enhanced expression of Mcl-1 in AM (Figure 1 A-C), phenocopying the CD68.hMcl-1^{+} BMDM we next addressed whether they also had modulation of mROS generation in response to pneumococci and whether this influenced intracellular bacterial killing. AM from patients with COPD had enhanced levels of mROS at baseline and no increment with infection (Figure 6A). Inhibition of mROS in COPD AM did not increase intracellular bacterial numbers, suggesting mROS plays little role in bacterial killing in COPD AM (Figure 6B). COPD is associated with enhanced antioxidant expression as an adaptation to chronic production of ROS (26). The major antioxidant against superoxide in AM that is localized to mitochondria is manganese superoxide dismutase (MnSOD/SOD2) (27). AM from COPD patients had enhanced expression of SOD2 at baseline and maintained expression following bacterial challenge (Figure 6C). Thus, when we calculated a ratio of the change in mROS to SOD2 as a marker of mitochondrial oxidant/antioxidant balance, there was a significant increase in this ratio following infection in healthy but not COPD AM (Figure 6D). To test whether increased mROS reconstituted bacterial killing in COPD AM, we added the mitochondrial complex I inhibitor rotenone, which enhances mROS production (28), confirming it enhanced bacterial killing in both healthy and COPD AM (Figure 6E). In keeping with a limited role for mROS in induction of apoptosis under these circumstances, and the well developed resistance of AM to oxidative stress (29), we

found that rotenone resulted in only a limited increase in AM apoptosis. Once again this suggested mROS was an effector of bacterial killing downstream of apoptosis rather than a stimulus for apoptosis induction (Figure 6F).

Discussion

We demonstrate that COPD AM possess a specific defect in the delayed phase of intracellular bacterial killing in association with impairment of mROS generation. This phase of bacterial killing is regulated by the anti-apoptotic protein Mcl-1 (12, 13) and we provide evidence that Mcl-1 is upregulated in COPD AM. Using a novel transgenic mouse line in which human Mcl-1 is governed by the CD68 promoter we show that overexpression of Mcl-1 results in a reduction of bacterial clearance from the murine lung and that mROS is both required for the delayed phase of clearance and regulated via Mcl-1 expression. Mcl-1 enhances the oxygen consumption rate during oxidative phosphorylation and mROS production in mock-infected macrophages but during infection its major effect is to regulate caspase dependent mROS production. COPD AM have both high basal mROS generation and a failure to enhance mROS production after pneumococcal challenge, which results in decreased bacterial killing.

COPD is characterized by bacterial persistence in the airway and by enhanced rates of CAP and IPD (8, 30). Bacterial load in the airway correlates with progressive airway obstruction and maintenance of neutrophilic inflammation (5). Moreover, a recent murine model, involving polymeric immunoglobulin deficient mice, has demonstrated that persistent exposure to lung bacteria drive inflammatory changes and lung remodelling in the small airways (31). This suggests that innate immune dysfunction and

impaired handling of respiratory pathogens is a central feature of COPD pathogenesis. In line with these observations several groups have demonstrated that COPD AM have altered activation states (32, 33) cytokine responses (34) and phagocytic capacity (15, 35, 36). Despite this there has been little investigation of microbicidal responses in COPD AM. Moreover prior studies have varied in the extent to which they identify a systemic versus a local alveolar macrophage defect. Although confounding effects of smoking and corticosteroids are important considerations we had low rates of current smokers in the main patient group studied and although corticosteroid inhaler use was more frequent in the COPD group the patients studied by histochemistry contained very few who used corticosteroid inhalers, arguing against a major confounding effect of these on Mcl-1 expression.

AM require additional microbicidal mechanisms to compliment early phagolysosomal bacterial killing since they lack myeloperoxidase (37) and the granule-associated serine proteases found in neutrophils (29). Moreover differentiated macrophages continue to phagocytose bacteria after conventional phagolysosomal microbicidal strategies are exhausted (38). Respiratory pathogens also express genes enabling their resistance to microbicidals (10). AM respond by activating a delayed phase of intracellular killing to diverse pathogens ranging from pneumococci to *Mycobacterium tuberculosis* (12, 13, 39). Generation of mROS has emerged as an important microbicidal strategy used by macrophages (22) and its production is increased by caspase 3 mediated inhibition of complex I (25). Therefore it is well positioned to link induction of apoptosis to bacterial killing. In light of observations that bacteria such as pneumococci have adaptions to withstand oxidative stress, it is likely that mROS reacts to form other more potent microbicidals, such as reactive nitrogen species, to mediate bacterial killing (10). Our

results suggest that this critical microbicidal strategy functions ineffectively in COPD AM.

Mitochondrial function emerges as a key determinant of the COPD AM microbicidal response. In COPD there is increasing evidence of mitochondrial dysfunction involving airway smooth muscle cells and skeletal muscle (40, 41). Enhanced mROS production is well described and is believed to contribute to COPD pathogenesis by contributing to the overall oxidative stress, promoting senescence and inflammation. The impact of mitochondrial dysfunction on macrophage innate immune responses is less appreciated but our data suggests that an additional consequence is impaired macrophage microbicidal responses. These are likely to be compounded further by impaired generation of classical macrophage activation in COPD (32, 33). Failure to generate classical activation, will reduce succinate generation an important driver of acute mROS production, required for microbicidal responses (42, 43), while the chronic production of mROS can favour alternative activation with consequences to innate immune responses (44). Upregulation of anti-oxidant defences, such as SOD2, in COPD (26), will further compromise mitochondrial microbicidal capacity. Our results suggest AM adaptions to chronic mROS generation will compromise the ability to generate an acute microbicidal response with mROS in the phagolysosome.

The molecular regulation of mROS production involves Mcl-1. CD68.hMcl-1^{+} transgene expression increased markers of oxidative phosphorylation and mROS generation in mock-infected macrophages so its upregulation could theoretically drive chronic mROS production in COPD AM. The production of mROS is, however, influenced by more than the basal and maximal oxygen consumption rate and we do not know how COPD influences hydrogen ion leak and the function of uncoupling proteins in AM in COPD

(23). Mcl-1 can exist as a form that localizes to the outer mitochondrial membrane and regulates apoptosis and another form that localizes to the mitochondrial matrix and enhances oxidative phosphorylation, while limiting mROS generation (45). This suggests that if Mcl-1 is to contribute to enhanced mROS expression in COPD AM either the expression of the matrix localized form must be altered or there are additional factors modulating proton leak to result in greater mROS production. An additional implication of this is that the reduced induction of mROS we document following pneumococcal challenge in association with maintenance of Mcl-1 expression may not just be a consequence of reduced caspase activation (and therefore inhibition of complex I (25)) but may also result from preservation of the matrix localized form of Mcl-1 (45). Regardless of these considerations there is still potential to overwhelm Mcl-1 in COPD AM and re-engage both induction of mROS and microbicidal capacity, as evidenced by the capacity of a complex I inhibitor to enhance bacterial clearance.

Our findings have been based exclusively on experiments with the pneumococcus but are likely to have broad impact in COPD even though other bacteria such as non-typeable *H. influenzae* are frequently implicated as colonizers of the COPD airway and drive inflammation (46). Pneumococci are the second most frequently bacterial colonizer in the lower airway in patients with COPD (46). They are likely to exert both direct effects on the frequency of COPD exacerbations and decline in FEV1; colonization with a monoculture of pneumococci specifically increased the risk of acute exacerbation in one study (19), but also indirect effects since there is evidence that pneumococcal colonization can promote *H. influenzae* or *M. catarrhalis* growth in the upper airway or promote mixed *H. influenzae* and *S. pneumoniae* biofilms (47, 48). They can also synergize with *H. influenzae* to promote pro-inflammatory cytokine responses in epithelial cells (49). In addition they are the leading cause of CAP in

patients with COPD (7). However the induction of apoptosis-associated bacterial killing is important against a range of pathogens (10), and the specific defect in mitochondrial microbicidal responses is therefore likely to have consequences for other pathogens in COPD beyond its effect on pneumococci.

Although several defects in innate immune function have been identified in COPD the identification of a critical defect in the late phase of mitochondrial microbicidal killing in COPD AM represents a new therapeutic target. Manipulation of mitochondrial homeostasis, metabolism, or inhibition of Mcl-1, all represent potential approaches by which this critical defect could be modified.

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Figure Legends

Figure 1: Mcl-1 upregulation occurs in chronic obstructive pulmonary disease (COPD). (A) Alveolar macrophages (AM) obtained from bronchoalveolar lavage of healthy controls or COPD patients were mock-infected (MI) or challenged with opsonized serotype 14 *Streptococcus pneumoniae* (S14) at the designated multiplicity of infection (MOI). 16h post-challenge the levels of Mcl-1 on AM were probed by western blot. A representative blot and densitometry are shown, n=6, *= $p<0.05$, repeated measures 1-way ANOVA. **(B-C)** Lung sections from COPD patients or healthy controls were dual stained with CD68 and Mcl-1. Total corrected cellular fluorescence (TCCF) of Mcl-1 in CD68 +ve cells was quantified. Representative images (B) and collated data (C and D) are shown. In C each point represents an individual cell (n=74 healthy, n=90 COPD, from 10 donors) and in D, each point represents the median fluorescence of all cells analysed from individual donors. For C and D, *= $p<0.05$, Kruskal-Wallis test.

Figure 2: COPD alveolar macrophages have a deficiency in apoptosis-associated killing. (A) Alveolar macrophages (AM) were collected from healthy donors or patients with COPD and were challenged with non opsonized (-) or opsonized (+) serotype 14 *S. pneumoniae* at an MOI of 10 for 4h, before extracellular bacteria were killed and viable intracellular bacteria measured. Viable bacteria in duplicate wells were measured again three hours later (7h post-infection), *= $p<0.05$. **= $p<0.01$, 2-way ANOVA. **(B-C)** Healthy or COPD AM were challenged with S14, at an MOI of 10 for COPD cells, or MOI 5 for healthy cells, so as to normalize levels of bacterial internalization. Cells were analysed for (B) nuclear fragmentation or condensation and (C) intracellular bacterial

colony forming units (CFU) at 20h post-challenge n=5-6, *= $p<0.05$, Students t-test (for B) or Mann-Whitney U test (for C).

Figure 3: Mcl-1 upregulation in AM impairs bacterial clearance in the lung. **(A-D)** Wild-type (Wt) or CD68.hMcl-1 transgenic (Tg) mice were challenged with 10^4 serotype 1 *Streptococcus pneumoniae*. At the designated time after instillation, bacterial colony forming units (CFU) in the lung homogenate (A), CFU in the blood (B), alveolar macrophage (AM) nuclear fragmentation or condensation in bronchoalveolar lavage (BAL) (C) and total polymorphonuclear leukocyte (PMN) numbers in BAL (D) were measured. n=4-11 mice per group from three independent experiments, *= $p<0.05$ **= $p<0.01$, 2-way ANOVA.

Figure 4: Mcl-1 modulates generation of mitochondrial ROS and mROS-dependent bacterial killing. **(A)** Wild-type (Wt) or CD68.hMcl-1 transgenic (Tg) bone marrow-derived (BMDM) were mock-infected (MI) or challenged with opsonized serotype 2 (D39) *S. pneumoniae*. 20 h post-challenge cells were stained with MitoSOX and visualised by microscopy to assess mitochondrial reactive oxygen species (mROS) generation. Images representative of three independent experiments, scale bar 50 μM. **(B)** At the designated time post-challenge, mROS were also assessed by flow cytometry, n=3 *= $p<0.05$ D39 Wt vs. D39 Tg, 2-way ANOVA. **(C)** MI or D39 infected Wt BMDM were stained with Cresyl violet to detect lysosomes (green) and MitoSOX (red) at 20 h and analysed by confocal microscopy. Co-localized signals are yellow (Merge), scale bar 5 μm. **(D)** Confocal fluorescence microscopy of D39 BMDM challenged with Alexa Fluor 647 labelled bacteria (green) and stained with MitoSOX (red, 4D upper panels) or endoplasmic reticulum (ER) tracker (purple, 4D, lower panel) 20h after bacterial challenge. Co-localized signals are yellow (Merge, 4D upper and lower panels,

Scale bar 5 μm**). (E)** Pseudo-coloured structured illumination microscopy (SIM) image of a monocyte-derived macrophage 16 h after bacterial challenge with *S. pneumoniae* (green) and stained with MitoSOX for mROS (red). Enlarged region on right shows bacteria co-localized with mROS (arrows). Scale bar 10 μm. **(F)** Pearson's correlation coefficients were calculated for the co-localization of mROS or ER with D39 or lysosomes, mean ± SEM (n=4-8). **(G-H)** Wt or Tg BMDM (G) or human MDM (H) were challenged with D39, in the presence or absence (vehicle) of mitoTEMPO (mT). 16 h post-challenge intracellular colony forming units (CFU) were assessed, n=5 (for G) and n=8 (for H), **= $p<0.01$, repeated measures 2-way ANOVA (for G), or Wilcoxon matched-pairs signed rank test (for H).

Figure 5: Mcl-1 modulates mitochondrial responses leading to mitochondrial reactive oxygen species generation. **(A-F)** Wild-type (Wt) or CD68.hMcl-1 transgenic (Tg) bone marrow-derived (BMDM) were mock-infected (MI) or challenged with opsonized serotype 2 (D39) *S. pneumoniae* for 4h before extracellular acidification (ECAR) (A) and parameters related to oxidative phosphorylation were measured kinetically. From the kinetic data (B), basal rates of oxygen consumption (OCR) (C), maximum respiration capacity (D), ATP linked OCR (E), and proton leak (F) were calculated, n=6 per group, *= $p<0.05$ **= $p<0.01$, 2-way ANOVA. **(G)** Mock-infected Wt and Tg BMDM were stained with MitoSox to measure baseline mROS production. **(H-I)** Wt or Tg BMDM (H) or human monocyte-derived macrophages (MDM) (I) were MI or challenged with D39, in the presence of the pan-caspase inhibitor zVAD or control zFA. At 20h post-challenge, cells were stained for mROS and caspase 3/7 activity. MitoSox staining was assessed for the whole cell populations (histograms). Representative plots are shown, with collated data graphed, n=4 *= $p<0.01$, 2-way ANOVA (for H), or 1-way ANOVA (for I).

Figure 6. COPD AM fail to increase mROS production after pneumococcal challenge. (A-B) Alveolar macrophages (AM) obtained from bronchoalveolar lavage (BAL) of healthy controls (Healthy) or COPD patients (COPD) were mock-infected (MI) or challenged with opsonized serotype 14 *S. pneumoniae* (S14), at an MOI of 10 for COPD cells, or MOI 5 for healthy cells. AM were left unstained (US) or stained with MitoSOX and mean fluorescence intensity (MFI) recorded at 16h, as a measure of mitochondrial reactive oxygen species (mROS), with representative plots shown and collated data graphed, (A) and intracellular bacterial colony forming units (CFU) were estimated in the presence or absence (vehicle) of mitoTEMPO (mT) (B) at 20h. Both n= 6, *= $p<0.05$, paired Students t-test (A) or Wilcoxon signed rank test (B). **(C)** AM from BAL of healthy controls or COPD patients were mock-infected (MI) or challenged with S14 at the designated multiplicity of infection (MOI). At 16h post-challenge the levels of superoxide dismutase (SOD)2 in AM were probed by western blot. Representative blot and densitometry are shown, n=4. **(D)** The ratio of mROS to SOD2 induced by bacterial challenge was calculated for healthy and COPD AM, using the samples in (C), n=4, *= $p<0.05$, Students t-test. **(E-F)** Healthy or COPD AM were MI or challenged with S14, in the presence (+) or absence (-) of rotenone to induce mROS. AM were assessed for intracellular bacterial CFU (E) and nuclear fragmentation or condensation (F) 20h post-challenge, n=3, *= $p<0.05$, Wilcoxon signed rank test.