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1 **Impaired mitochondrial microbicidal responses in chronic obstructive pulmonary**  
2 **disease macrophages.**

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

33  
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35 DHD and by the MRC COPD-MAP Consortium.

36

37 **Running title:** COPD impairs mROS mediated bacterial killing

38 **Descriptor Number:** 10.9 Pathogen/Host cell interactions

39 **Total word count:** 3442

40 **At a glance summary:**

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

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

4 **Footnote:**

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

1 **Abstract:**

2

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

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

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

13 **Measurements and Main Results:** COPD AM had elevated levels of Mcl-1, an anti-  
14 apoptotic Bcl-2 family member, with selective reduction of delayed intracellular  
15 bacterial killing. CD68.hMcl-1 AM phenocopied the microbicidal defect since  
16 transgenic mice demonstrated impaired clearance of pulmonary bacteria and increased  
17 neutrophilic inflammation. Murine bone marrow-derived macrophages (BMDM) and  
18 human monocyte-derived macrophages (MDM) generated mitochondrial reactive  
19 oxygen species (mROS) in response to pneumococci, which co-localized with bacteria  
20 and phagolysosomes to enhance bacterial killing. The Mcl-1 transgene increased oxygen  
21 consumption rates and mROS expression in mock-infected BMDM but reduced caspase-  
22 dependent mROS production after pneumococcal challenge. COPD AM also increased  
23 basal mROS expression, but failed to increase production after pneumococcal challenge,  
24 in keeping with reduced intracellular bacterial killing. The defect in COPD AM  
25 intracellular killing was associated with a reduced ratio of mROS /superoxide dismutase  
26 2.

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

4

5 Word count: 250

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

1 **Introduction**

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

8

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

23

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

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

11

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

19

## 20 **Materials and Methods.**

### 21 **Bacteria and Infection**

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

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

### 3 **Isolation and culture of macrophages**

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

12

### 13 **Western blot**

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

16

### 17 **Immunohistochemistry**

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

21

### 22 **Flow Cytometry**

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

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

3

#### 4 **Microscopy**

5 Nuclear fragmentation and condensation indicative of apoptosis were detected using  
6 4'6'-diamidino-2-phenylindole (DAPI) (11). To visualise mROS cells were stained with  
7 2 $\mu$ M MitoSOX-red (Invitrogen) and visualized on a Leica DMRB 1000, 40x objective.

8 For co-localisation experiments, lysosomes were stained with 0.50 $\mu$ M Cresyl violet  
9 (Sigma) or challenged with D39 labelled with Alexa Fluor 647 carboxylic acid

10 succinimidyl ester (Life Technologies) and co-stained with MitoSOX-red for 15 min,  
11 and visualised by confocal microscopy (Zeiss LSM 510, 63x1.4 oil objective). In other

12 experiments, MDM and BMDM were challenged with D39 labelled with Alexa Fluor  
13 647 and 16 h post-challenge cells were stained with MitoSOX and visualised by

14 structured illumination microscopy (SIM). Detailed information can be found in the  
15 online supplement.

16

#### 17 **Intracellular killing assay.**

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

20

#### 21 **Metabolic measurements**

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

25

1 ***In vivo* infections**

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

7

8 **Statistics**

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

15

16 **Results**

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

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

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

11

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

1

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

3 CD68.hMcl-1<sup>+</sup> transgenic mice were used to explore the functional consequence of Mcl-  
4 1 upregulation for bacterial clearance and the putative association of Mcl-1 upregulation  
5 with altered intracellular bacterial clearance in COPD AM. Expression of a human Mcl-  
6 1 transgene in myeloid cell populations extends macrophage survival while ensuring  
7 cells remain sensitive to physiological constraints on viability so that there is normal  
8 distribution of myeloid subsets and development (21). Using a low dose of  
9 pneumococci, which AM are able to contain (9), we demonstrate that the presence of the  
10 macrophage transgene results in impaired bacterial clearance from the lung and also  
11 increased bacteremia (Figure 3A-B). These changes were found in association with  
12 reduced AM apoptosis (Figure 3C) and increased numbers of neutrophils in the  
13 bronchoalveolar lavage fluid (BAL) (Figure 3D).

14

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

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

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

6

7 *Mcl-1 modulates mitochondrial oxidative phosphorylation in macrophages.*

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

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

6

7 *COPD AM fail to increase mROS production after pneumococcal challenge*

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

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

4

5

## 6 **Discussion**

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

19

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

1 impaired handling of respiratory pathogens is a central feature of COPD pathogenesis.  
2 In line with these observations several groups have demonstrated that COPD AM have  
3 altered activation states (32, 33) cytokine responses (34) and phagocytic capacity (15,  
4 35, 36). Despite this there has been little investigation of microbicidal responses in  
5 COPD AM. Moreover prior studies have varied in the extent to which they identify a  
6 systemic versus a local alveolar macrophage defect. Although confounding effects of  
7 smoking and corticosteroids are important considerations we had low rates of current  
8 smokers in the main patient group studied and although corticosteroid inhaler use was  
9 more frequent in the COPD group the patients studied by histochemistry contained very  
10 few who used corticosteroid inhalers, arguing against a major confounding effect of  
11 these on Mcl-1 expression.

12

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

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

3

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

20

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

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

14

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

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

5  
6 Although several defects in innate immune function have been identified in COPD the  
7 identification of a critical defect in the late phase of mitochondrial microbicidal killing  
8 in COPD AM represents a new therapeutic target. Manipulation of mitochondrial  
9 homeostasis, metabolism, or inhibition of Mcl-1, all represent potential approaches by  
10 which this critical defect could be modified.

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## 1   **References**

- 2
- 3
- 4   1.     Tuder RM, Petrache I. Pathogenesis of chronic obstructive pulmonary disease. *J Clin Invest* 2012;122:2749-2755.
- 5
- 6   2.     Willemse BW, ten Hacken NH, Rutgers B, Lesman-Leegte IG, Postma DS, Timens W. Effect of 1-year smoking cessation on airway inflammation in copd and asymptomatic smokers. *Eur Respir J* 2005;26:835-845.
- 7
- 8   3.     van Gemert F, Kirenga B, Chavannes N, Kanya M, Luzige S, Musinguzi P, Turyagaruka J, Jones R, Tsiligianni I, Williams S, de Jong C, van der Molen T. Prevalence of chronic obstructive pulmonary disease and associated risk factors in uganda (fresh air uganda): A prospective cross-sectional observational study. *Lancet Glob Health* 2015;3:e44-51.
- 9
- 10   4.     Donaldson GC, Seemungal TA, Bhowmik A, Wedzicha JA. Relationship between exacerbation frequency and lung function decline in chronic obstructive pulmonary disease. *Thorax* 2002;57:847-852.
- 11
- 12   5.     Wilkinson TM, Patel IS, Wilks M, Donaldson GC, Wedzicha JA. Airway bacterial load and fev1 decline in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2003;167:1090-1095.
- 13
- 14   6.     Sapey E, Stockley RA. Copd exacerbations . 2: Aetiology. *Thorax* 2006;61:250-258.
- 15
- 16   7.     Torres A, Dorca J, Zalacain R, Bello S, El-Ebiary M, Molinos L, Arevalo M, Blanquer J, Celis R, Iriberry M, Prats E, Fernandez R, Irigaray R, Serra J. Community-acquired pneumonia in chronic obstructive pulmonary disease: A spanish multicenter study. *Am J Respir Crit Care Med* 1996;154:1456-1461.
- 17
- 18   8.     Torres A, Blasi F, Dartois N, Akova M. Which individuals are at increased risk of pneumococcal disease and why? Impact of copd, asthma, smoking, diabetes, and/or chronic heart disease on community-acquired pneumonia and invasive pneumococcal disease. *Thorax* 2015;70:984-989.
- 19
- 20   9.     Dockrell DH, Marriott HM, Prince LR, Ridger VC, Ince PG, Hellewell PG, Whyte MK. Alveolar macrophage apoptosis contributes to pneumococcal clearance in a resolving model of pulmonary infection. *J Immunol* 2003;171:5380-5388.
- 21
- 22   10.    Aberdein JD, Cole J, Bewley MA, Marriott HM, Dockrell DH. Alveolar macrophages in pulmonary host defence the unrecognized role of apoptosis as a mechanism of intracellular bacterial killing. *Clin Exp Immunol* 2013;174:193-202.
- 23
- 24   11.    Dockrell DH, Lee M, Lynch DH, Read RC. Immune-mediated phagocytosis and killing of streptococcus pneumoniae are associated with direct and bystander macrophage apoptosis. *J Infect Dis* 2001;184:713-722.
- 25
- 26   12.    Marriott HM, Bingle CD, Read RC, Braley KE, Kroemer G, Hellewell PG, Craig RW, Whyte MK, Dockrell DH. Dynamic changes in mcl-1 expression regulate macrophage viability or commitment to apoptosis during bacterial clearance. *J Clin Invest* 2005;115:359-368.
- 27
- 28   13.    Bewley MA, Marriott HM, Tulone C, Francis SE, Mitchell TJ, Read RC, Chain B, Kroemer G, Whyte MK, Dockrell DH. A cardinal role for cathepsin d in coordinating the host-mediated apoptosis of macrophages and killing of pneumococci. *PLoS Pathog* 2011;7:e1001262.
- 29
- 30   14.    Taylor AE, Finney-Hayward TK, Quint JK, Thomas CM, Tudhope SJ, Wedzicha JA, Barnes PJ, Donnelly LE. Defective macrophage phagocytosis of bacteria in copd. *Eur Respir J* 2010;35:1039-1047.
- 31
- 32
- 33
- 34
- 35
- 36
- 37
- 38
- 39
- 40
- 41
- 42
- 43
- 44
- 45
- 46
- 47
- 48
- 49

- 1 15. Berenson CS, Garlipp MA, Grove LJ, Maloney J, Sethi S. Impaired phagocytosis  
2 of nontypeable haemophilus influenzae by human alveolar macrophages in chronic  
3 obstructive pulmonary disease. *J Infect Dis* 2006;194:1375-1384.
- 4 16. Gordon SB, Irving GR, Lawson RA, Lee ME, Read RC. Intracellular trafficking  
5 and killing of streptococcus pneumoniae by human alveolar macrophages are influenced  
6 by opsonins. *Infect Immun* 2000;68:2286-2293.
- 7 17. Marriott HM, Ali F, Read RC, Mitchell TJ, Whyte MK, Dockrell DH. Nitric  
8 oxide levels regulate macrophage commitment to apoptosis or necrosis during  
9 pneumococcal infection. *The FASEB journal : official publication of the Federation of*  
10 *American Societies for Experimental Biology* 2004;18:1126-1128.
- 11 18. Liu H, Perlman H, Pagliari LJ, Pope RM. Constitutively activated akt-1 is vital  
12 for the survival of human monocyte-differentiated macrophages. Role of mcl-1,  
13 independent of nuclear factor (nf)-kappab, bad, or caspase activation. *J Exp Med*  
14 2001;194:113-126.
- 15 19. Bogaert D, van der Valk P, Ramdin R, Sluijter M, Monninkhof E, Hendrix R, de  
16 Groot R, Hermans PW. Host-pathogen interaction during pneumococcal infection in  
17 patients with chronic obstructive pulmonary disease. *Infect Immun* 2004;72:818-823.
- 18 20. DeLeo FR, Allen LA, Apicella M, Nauseef WM. NADPH oxidase activation and  
19 assembly during phagocytosis. *J Immunol* 1999;163:6732-6740.
- 20 21. Zhou P, Qian L, Bieszczad CK, Noelle R, Binder M, Levy NB, Craig RW. Mcl-1  
21 in transgenic mice promotes survival in a spectrum of hematopoietic cell types and  
22 immortalization in the myeloid lineage. *Blood* 1998;92:3226-3239.
- 23 22. West AP, Brodsky IE, Rahner C, Woo DK, Erdjument-Bromage H, Tempst P,  
24 Walsh MC, Choi Y, Shadel GS, Ghosh S. TLR signalling augments macrophage  
25 bactericidal activity through mitochondrial ROS. *Nature* 2011;472:476-480.
- 26 23. Stowe DF, Camara AK. Mitochondrial reactive oxygen species production in  
27 excitable cells: Modulators of mitochondrial and cell function. *Antioxid Redox Signal*  
28 2009;11:1373-1414.
- 29 24. Kelly B, O'Neill LA. Metabolic reprogramming in macrophages and dendritic  
30 cells in innate immunity. *Cell Res* 2015;25:771-784.
- 31 25. Ricci JE, Gottlieb RA, Green DR. Caspase-mediated loss of mitochondrial  
32 function and generation of reactive oxygen species during apoptosis. *J Cell Biol*  
33 2003;160:65-75.
- 34 26. MacNee W. Oxidants/antioxidants and COPD. *Chest* 2000;117:303S-317S.
- 35 27. Kinnula VL. Focus on antioxidant enzymes and antioxidant strategies in  
36 smoking related airway diseases. *Thorax* 2005;60:693-700.
- 37 28. Li N, Ragheb K, Lawler G, Sturgis J, Rajwa B, Melendez JA, Robinson JP.  
38 Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing  
39 mitochondrial reactive oxygen species production. *J Biol Chem* 2003;278:8516-8525.
- 40 29. Jin M, Opalek JM, Marsh CB, Wu HM. Proteome comparison of alveolar  
41 macrophages with monocytes reveals distinct protein characteristics. *Am J Respir Cell*  
42 *Mol Biol* 2004;31:322-329.
- 43 30. Sethi S, Murphy TF. Bacterial infection in chronic obstructive pulmonary disease  
44 in 2000: A state-of-the-art review. *Clin Microbiol Rev* 2001;14:336-363.
- 45 31. Richmond BW, Brucker RM, Han W, Du RH, Zhang Y, Cheng DS, Gleaves L,  
46 Abdolrasulnia R, Polosukhina D, Clark PE, Bordenstein SR, Blackwell TS, Polosukhin  
47 VV. Airway bacteria drive a progressive COPD-like phenotype in mice with polymeric  
48 immunoglobulin receptor deficiency. *Nat Commun* 2016;7:11240.
- 49 32. Shaykhiiev R, Krause A, Salit J, Strulovici-Barel Y, Harvey BG, O'Connor TP,  
50 Crystal RG. Smoking-dependent reprogramming of alveolar macrophage polarization:

- 1 Implication for pathogenesis of chronic obstructive pulmonary disease. *J Immunol*  
2 2009;183:2867-2883.
- 3 33. Hodge S, Matthews G, Mukaro V, Ahern J, Shivam A, Hodge G, Holmes M,  
4 Jersmann H, Reynolds PN. Cigarette smoke-induced changes to alveolar macrophage  
5 phenotype and function are improved by treatment with procysteine. *Am J Respir Cell*  
6 *Mol Biol* 2011;44:673-681.
- 7 34. Berenson CS, Wrona CT, Grove LJ, Maloney J, Garlipp MA, Wallace PK,  
8 Stewart CC, Sethi S. Impaired alveolar macrophage response to haemophilus antigens in  
9 chronic obstructive lung disease. *Am J Respir Crit Care Med* 2006;174:31-40.
- 10 35. Hodge S, Hodge G, Ahern J, Jersmann H, Holmes M, Reynolds PN. Smoking  
11 alters alveolar macrophage recognition and phagocytic ability: Implications in chronic  
12 obstructive pulmonary disease. *Am J Respir Cell Mol Biol* 2007;37:748-755.
- 13 36. Taylor AE, Finney-Hayward TK, Quint JK, Thomas CM, Tudhope SJ, Wedzicha  
14 JA, Barnes PJ, Donnelly LE. Defective macrophage phagocytosis of bacteria in copd.  
15 *Eur Respir J* 2010;35:1039-1047.
- 16 37. Cohen AB, Cline MJ. The human alveolar macrophage: Isolation, cultivation in  
17 vitro, and studies of morphologic and functional characteristics. *J Clin Invest*  
18 1971;50:1390-1398.
- 19 38. Jubrail J, Morris P, Bewley MA, Stoneham S, Johnston SA, Foster SJ, Peden  
20 AA, Read RC, Marriott HM, Dockrell DH. Inability to sustain intraphagolysosomal  
21 killing of staphylococcus aureus predisposes to bacterial persistence in macrophages.  
22 *Cell Microbiol* 2016;18:80-96.
- 23 39. Keane J, Balcewicz-Sablinska MK, Remold HG, Chupp GL, Meek BB, Fenton  
24 MJ, Kornfeld H. Infection by mycobacterium tuberculosis promotes human alveolar  
25 macrophage apoptosis. *Infect Immun* 1997;65:298-304.
- 26 40. Wiegman CH, Michaeloudes C, Haji G, Narang P, Clarke CJ, Russell KE, Bao  
27 W, Pavlidis S, Barnes PJ, Kanerva J, Bittner A, Rao N, Murphy MP, Kirkham PA,  
28 Chung KF, Adcock IM. Oxidative stress-induced mitochondrial dysfunction drives  
29 inflammation and airway smooth muscle remodeling in patients with chronic obstructive  
30 pulmonary disease. *J Allergy Clin Immunol* 2015;136:769-780.
- 31 41. Rabinovich RA, Bastos R, Ardite E, Llinas L, Orozco-Levi M, Gea J, Vilaro J,  
32 Barbera JA, Rodriguez-Roisin R, Fernandez-Checa JC, Roca J. Mitochondrial  
33 dysfunction in copd patients with low body mass index. *Eur Respir J* 2007;29:643-650.
- 34 42. Tannahill GM, Curtis AM, Adamik J, Palsson-McDermott EM, McGettrick AF,  
35 Goel G, Frezza C, Bernard NJ, Kelly B, Foley NH, Zheng L, Gardet A, Tong Z, Jany  
36 SS, Corr SC, Haneklaus M, Caffrey BE, Pierce K, Walmsley S, Beasley FC, Cummins  
37 E, Nizet V, Whyte M, Taylor CT, Lin H, Masters SL, Gottlieb E, Kelly VP, Clish C,  
38 Auron PE, Xavier RJ, O'Neill LA. Succinate is an inflammatory signal that induces il-  
39 1beta through hif-1alpha. *Nature* 2013;496:238-242.
- 40 43. Chouchani ET, Pell VR, Gaude E, Aksentijevic D, Sundier SY, Robb EL, Logan  
41 A, Nadtochiy SM, Ord EN, Smith AC, Eyassu F, Shirley R, Hu CH, Dare AJ, James  
42 AM, Rogatti S, Hartley RC, Eaton S, Costa AS, Brookes PS, Davidson SM, Duchon  
43 MR, Saeb-Parsy K, Shattock MJ, Robinson AJ, Work LM, Frezza C, Krieg T, Murphy  
44 MP. Ischaemic accumulation of succinate controls reperfusion injury through  
45 mitochondrial ros. *Nature* 2014;515:431-435.
- 46 44. Zhang Y, Choksi S, Chen K, Pobeziinskaya Y, Linnoila I, Liu ZG. Ros play a  
47 critical role in the differentiation of alternatively activated macrophages and the  
48 occurrence of tumor-associated macrophages. *Cell Res* 2013;23:898-914.
- 49 45. Perciavalle RM, Stewart DP, Koss B, Lynch J, Milasta S, Bathina M, Temirov J,  
50 Cleland MM, Pelletier S, Schuetz JD, Youle RJ, Green DR, Opferman JT. Anti-

1 apoptotic mcl-1 localizes to the mitochondrial matrix and couples mitochondrial fusion  
2 to respiration. *Nat Cell Biol* 2012;14:575-583.

3 46. Patel IS, Seemungal TA, Wilks M, Lloyd-Owen SJ, Donaldson GC, Wedzicha  
4 JA. Relationship between bacterial colonisation and the frequency, character, and  
5 severity of copd exacerbations. *Thorax* 2002;57:759-764.

6 47. Bosch AA, Biesbroek G, Trzcinski K, Sanders EA, Bogaert D. Viral and  
7 bacterial interactions in the upper respiratory tract. *PLoS Pathog* 2013;9:e1003057.

8 48. Tikhomirova A, Kidd SP. Haemophilus influenzae and streptococcus  
9 pneumoniae: Living together in a biofilm. *Pathog Dis* 2013;69:114-126.

10 49. Ratner AJ, Aguilar JL, Shchepetov M, Lysenko ES, Weiser JN. Nod1 mediates  
11 cytoplasmic sensing of combinations of extracellular bacteria. *Cell Microbiol*  
12 2007;9:1343-1351.

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14  
15  
16  
17  
18  
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## 2 **Figure Legends**

### 3 **Figure 1: Mcl-1 upregulation occurs in chronic obstructive pulmonary disease**

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

16

### 17 **Figure 2: COPD alveolar macrophages have a deficiency in apoptosis-associated**

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

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

3

4 **Figure 3: Mcl-1 upregulation in AM impairs bacterial clearance in the lung.**

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

12

13 **Figure 4: Mcl-1 modulates generation of mitochondrial ROS and mROS-dependent**

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

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

11

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

1

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

22