ORIGINAL ARTICLE

Impaired Mitochondrial Microbicidal Responses in Chronic Obstructive Pulmonary Disease Macrophages

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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: AMs were obtained from bronchoalveolar lavage from healthy donors or patients with COPD 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 induced myeloid leukemia cell differentiation protein Mcl-1 (CD68.hMcl-1) was used to model the molecular aspects of COPD.

Measurements and Main Results: COPD AMs had elevated levels of Mcl-1, an antiapoptotic B-cell lymphoma 2 family member, with selective reduction of delayed intracellular bacterial killing. CD68.hMcl-1 AMs phenocopied the microbicidal defect because transgenic mice demonstrated impaired clearance of pulmonary bacteria and increased neutrophilic inflammation. Murine bone marrow–derived macrophages and human monocyte-derived macrophages generated mROS in response to pneumococci, which colocalized with bacteria and phagolysosomes to enhance bacterial killing. The Mcl-1 transgene increased oxygen consumption rates and mROS expression in mock-infected bone marrow–derived macrophages but reduced caspase-dependent mROS production after pneumococcal challenge. COPD AMs also increased basal mROS expression, but they 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: Up-regulation of Mcl-1 and chronic adaption to oxidative stress alter mitochondrial metabolism and microbicidal function, reducing the delayed phase of intracellular bacterial clearance in COPD.

Keywords: apoptosis; mitochondrial reactive oxygen species; *Streptococcus pneumoniae*

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At a Glance Commentary

Scientific Knowledge on the Subject: Patients with chronic obstructive pulmonary disease (COPD) are at increased risk for 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 increases 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 contribute to impaired clearance of pneumococci in the lung.

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 chemokine (C-X-C motif) ligand 8 levels (5). This suggests that a key driver of COPD pathogenesis is a defect in airway innate immune responses to pathogenic bacteria. Although *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 in smokers, another group at increased risk of IPD (8). This suggests that patients with COPD possess significant defects in their host defenses to pneumococcal disease in the lower airway. However, the basis of this increased susceptibility to pneumococcal pneumonia remains undefined.

Alveolar macrophages (AMs) are central to the organization of pulmonary innate immunity and are critical for clearance of pneumococci from the alveolar space (9). However, AMs 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 defense strategies, and induction of apoptosis is required for efficient clearance of intracellular bacteria after phagocytosis (9, 11). Apoptosis is controlled by expression of the antiapoptotic induced myeloid leukemia cell differentiation 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 of impaired bacterial phagocytosis of nontypeable *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 AMs have persistent up-regulation of Mcl-1, and we used patient AMs and a unique murine transgenic macrophage to test how Mcl-1 up-regulation alters pulmonary antibacterial host defense. Specifically, we addressed how Mcl-1 influenced AMs' 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 were previously reported in the form of an abstract (16).

Methods

Bacteria and Infection

Serotype 2 *S. pneumoniae* (D39 strain; NCTC 7466) and serotype 1 *S. pneumoniae* (World Health Organization reference laboratory strain SSISP 1/1; Statens Serum Institut, Copenhagen, Denmark), used in murine experiments (9), and serotype 14 *S. pneumoniae* (NCTC 11902), 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 (BMDMs) were obtained as described previously (9). Human monocyte-derived macrophages (MDMs) were isolated from whole blood donated by healthy volunteers with written informed consent, as approved by the South Sheffield Regional Ethics Committee (11). AMs from patients with COPD (enrolled through the Medical Research Council COPD MAP Consortium) or from healthy control subjects (*see* Table E1 in the online supplement) were isolated from bronchoalveolar lavage (BAL) as previously described (17), with written approved consent obtained 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 Analysis

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

Immunohistochemistry

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

Flow Cytometry

Caspase activity was measured using the CellEvent Caspase 3/7 Green Flow Cytometry Kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. mROS were measured by flow cytometry using the dye MitoSOX Red (Life Technologies). 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 stain (11).

To visualize mROS, cells were stained with 2 μM MitoSOX Red and visualized using a Leica DMRB 1000 microscope (Leica Microsystems, Buffalo Grove, IL) with a ×40 lens objective. For colocalization experiments, lysosomes were stained with 0.50 μM Cresyl violet (Sigma-Aldrich, St. Louis, MO) or challenged with D39 labeled with Alexa Fluor 647 carboxylic acid succinimidyl ester (Life Technologies) and costained with MitoSOX Red for 15 minutes, and then they were visualized by confocal microscopy (LSM 510, 63 × 1.4 oil immersion lens objective; Carl Zeiss Microscopy, Jena, Germany). In other experiments, MDMs and BMDMs were challenged with D39 labeled with Alexa Fluor 647, and 16 hours after challenge, cells were stained with MitoSOX Red and visualized by structured illumination microscopy. Detailed information can be found in the online supplement.

Intracellular Killing Assay

Assessment of intracellular bacterial viability was performed by gentamicin protection assay as previously described (18).

Metabolic Measurements

Measurements of oxygen consumption rate (OCR) and extracellular acidification rate were performed using the Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience/Agilent Technologies, Santa Clara, CA). 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 U.K. Home Office License 40/3251 with approval of the Sheffield Ethical Review Committee, Sheffield, United Kingdom.

Statistics

Data are presented as mean and SE unless otherwise indicated in the figure legends. Sample sizes were informed by SEs 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., La Jolla, CA), and significance was defined as $P < 0.05$. Decisions on use of parametric or nonparametric tests were based upon results of D'Agostino-Pearson normality tests.

Results

Mcl-1 Is Up-regulated and Is Associated with Reduced Intracellular Bacterial Killing in COPD AMs

The B-cell lymphoma 2 family member Mcl-1 regulates both macrophage viability (19) 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 AMs by COPD. These experiments were conducted with a strain of *S. pneumoniae* that frequently colonizes this patient group and that also can cause IPD (20). Because macrophages from patients with COPD have an impaired capacity to ingest bacteria (14, 15), we modified the inoculum presented to control human AMs to normalize intracellular bacterial numbers (Figure E1) and demonstrated significant reduction in Mcl-1 expression in healthy donor AMs but no reduction in expression in COPD AMs after bacterial challenge (Figure 1A). We also examined whether there was increased Mcl-1 expression in AMs in the COPD lung. As illustrated, quantification revealed enhanced Mcl-1 expression in lung biopsies of patients with COPD compared with control donors without COPD, which showed a focal distribution of high intensity (Figures 1B–1D). The level of expression in control subjects was not altered by whether they were current smokers or nonsmokers, and results were similar when expression was analyzed either by individual cell expression (Figure 1C) or by donor overall (Figure 1D).

We next addressed whether COPD AMs had any defects in early microbicidal responses. In keeping with prior reports on MDMs (14), we documented reduced phagocytosis of pneumococci by COPD AMs (Figure 2A). Of note, in contrast to AMs from healthy donors, there was no evidence of the normal opsonic uplift in phagocytosis of pneumococci in COPD AMs (17). 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 (21). To exclude confounding effects of differential bacterial internalization, we adjusted the multiplicity of infection used to challenge control AMs, ensuring normalization of initial internalization (Figure E1) because apoptosis is directly related to initial bacterial ingestion (11). Up-regulation of Mcl-1 was associated with reduced apoptosis in COPD AMs (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 AMs (12, 13).

Mcl-1 Up-regulation in AMs Impairs Bacterial Clearance in the Lung

CD68.hMcl-1$^+$ transgenic mice were used to explore the functional consequence of Mcl-1 up-regulation for bacterial clearance and the putative association of Mcl-1 up-regulation with altered intracellular bacterial clearance in COPD AMs. 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 (22). Using a low dose of pneumococci, which AMs are able to contain (9), we demonstrated that the presence of the macrophage transgene results in impaired bacterial clearance from the lung and also increased bacteremia (Figures 3A and 3B). These changes were found in association with reduced AM apoptosis (Figure 3C) and increased numbers of neutrophils in the BAL fluid (Figure 3D).

Mcl-1 Modulates Generation of mROS and mROS-Dependent Bacterial Killing

We next explored the links between induction of the Mcl-1–regulated apoptotic program and microbicidal responses, using BMDMs as a model of differentiated macrophages. Mcl-1 regulates apoptosis at the level of the mitochondrion (12, 13), and mROS has emerged as an important microbicidal strategy used by macrophages (23). mROS was significantly increased in CD68.hMcl-1$^-$ (but not CD68.hMcl-1$^+$) BMDMs 20 hours after bacterial challenge, with significantly lower levels in CD68.hMcl-1$^+$ compared with CD68.hMcl-1$^-$ BMDMs (Figures 4A and 4B). mROS colocalized with phagolysosomes and with bacteria, in contrast to the endoplasmic reticulum, used as a control, which did not colocalize with either bacteria or

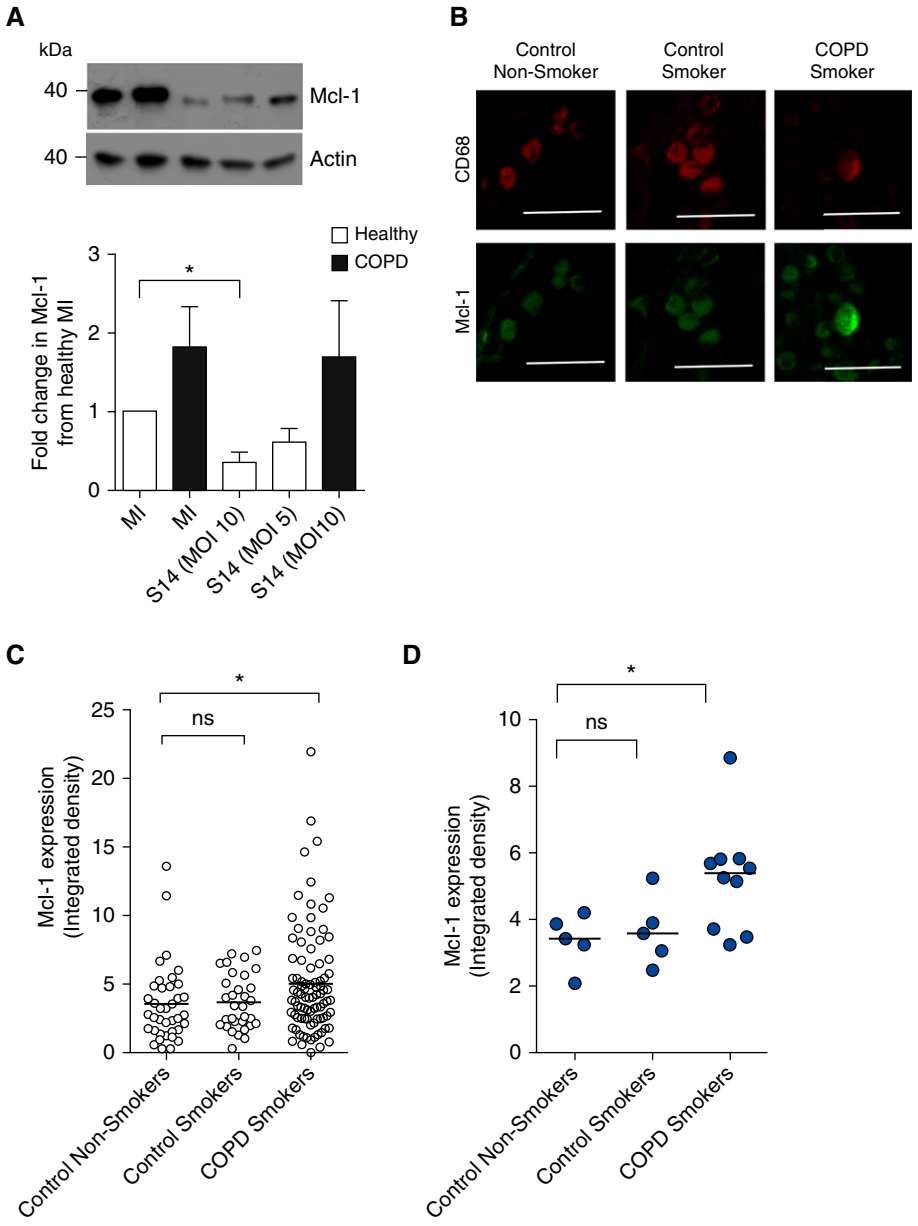

Figure 1. Induced myeloid leukemia cell differentiation protein Mcl-1 up-regulation occurs in chronic obstructive pulmonary disease (COPD). (*A*) Alveolar macrophages obtained from bronchoalveolar lavage of healthy control subjects or patients with COPD were mock infected (MI) or challenged with opsonized serotype 14 *Streptococcus pneumoniae* (S14) at the designated multiplicity of infection (MOI). Sixteen hours after challenge, the levels of Mcl-1 on alveolar macrophages were probed by western blotting. A representative blot and densitometric analysis are shown. n = 6; $^*P < 0.05$, repeated measures one-way analysis of variance. (*B* and *C*) Lung sections from patients with COPD or healthy control subjects were double-stained with CD68 and Mcl-1. Total corrected cellular fluorescence of Mcl-1 in $CD68^+$ cells was quantified. Representative images (*B*) and collated data (*C* and *D*) are shown (*scale bars* = 50 μM). In *C*, each point represents an individual cell (n = 74 healthy control, n = 90 COPD, from 10 donors), and in *D*, each point represents the median fluorescence of all cells analyzed from individual donors. For *C* and *D*, $^*P < 0.05$, Kruskal-Wallis test. ns = nonsignificant.

phagolysosomes (Figure 4C–4F, E2, and E3). mROS staining was inhibited by an inhibitor (mitoTEMPO; Enzo Life Sciences, Exeter, UK), and each stain provided minimal background signal (Figures E2–E4). mROS colocalization with bacteria was also visible in human MDMs (Figures 4E, E2F, and E2G). mitoTEMPO blocked the delayed phase of pneumococcal killing in CD68.hMcl-1^- (but not CD68.hMcl-1^+) BMDMs (Figure 4G) and also in MDMs (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 (24). 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 (25), 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 OCR in mock-infected cells, though it had no effect on the levels after pneumococcal challenge (Figures 5B–5F). In association with alterations in OCR parameters, the transgene was also associated with increased baseline mROS levels in mock-infected cells (Figure 5G). Because caspase activation enhances mROS production through interference with complex I of the electron transport chain (26), 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 Figures 5H and 5I, Mcl-1 overexpression inhibited the increase in mROS production after bacterial challenge. Mcl-1 also specifically reduced the mROS production due to caspase activation after bacterial challenge because treatment with the caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (zVAD) reduced MitoSOX Red levels to levels comparable to those of the Mcl-1–transgenic mice. In these experiments with zVAD and benzyl N-[1-[(4-fluoro-3-oxobutan-2-yl)amino]-1-oxo-3-phenylpropan-2-yl]carbamate (zFA), the baseline level of mROS was lower than in the experiments shown in Figure 5G, reflecting reduced sensitivity of detection in the presence of these chemicals, and the baseline

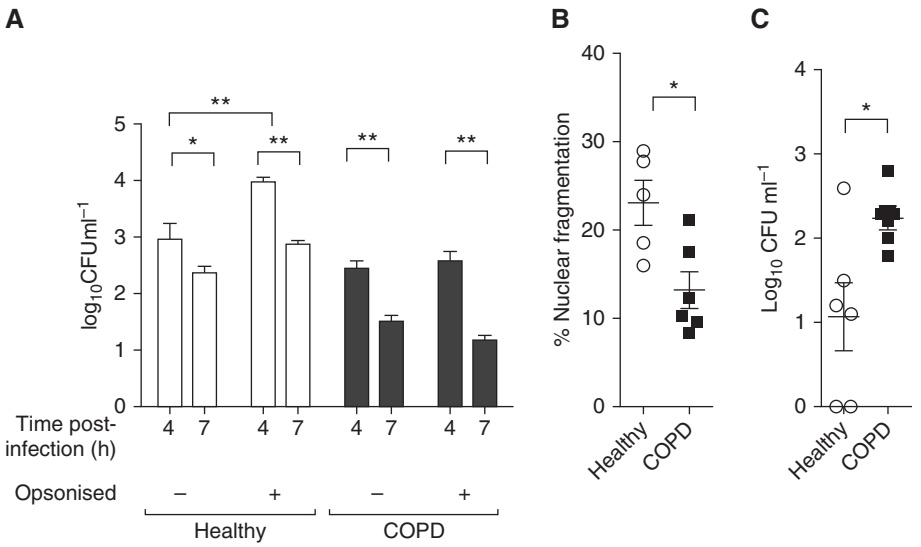

Figure 2. Chronic obstructive pulmonary disease (COPD) alveolar macrophages (AMs) have a deficiency in apoptosis-associated killing. (*A*) AMs were collected from healthy donors or patients with COPD and were challenged with nonopsonized (−) or opsonized (+) serotype 14 *Streptococcus pneumoniae* at a multiplicity of infection (MOI) of 10 for 4 hours before extracellular bacteria were killed and viable intracellular bacteria were measured. Viable bacteria in duplicate wells were measured again 3 hours later (7 h after infection). $^{*}P < 0.05$, $^{**}P < 0.01$ by two-way analysis of variance. (*B* and *C*) Healthy or COPD AMs were challenged with serotype 14 *Streptococcus pneumoniae* at an MOI of 10 for COPD cells or an MOI of 5 for healthy cells to normalize levels of bacterial internalization. Cells were analyzed for (*B*) nuclear fragmentation or condensation and (*C*) intracellular bacterial CFU at 20 hours after challenge. n = 5–6, $^{*}P < 0.05$ by Student's *t* test (for *B*) or Mann-Whitney *U* test (for *C*).

alteration in mROS production due to the transgene was no longer apparent.

COPD AMs Fail to Increase mROS Production after Pneumococcal Challenge

Because patients with COPD had enhanced expression of Mcl-1 in AMs (Figures 1A–1C), phenocopying the CD68.hMcl-1^{+} BMDMs, we next addressed whether they also had modulation of mROS generation in response to pneumococci and whether this influenced intracellular bacterial killing. AMs from patients with COPD had enhanced levels of mROS at baseline and no increase with infection (Figure 6A). Inhibition of mROS in COPD AMs did not increase intracellular bacterial numbers, suggesting that mROS plays little role in bacterial killing in COPD AMs (Figure 6B). COPD is associated with enhanced antioxidant expression as an adaptation to chronic production of ROS (27). The major antioxidant against superoxide in AMs that are localized to mitochondria is manganese superoxide dismutase 2 (MnSOD, also termed SOD2) (28). AMs from patients with COPD had enhanced expression of SOD2 at baseline and maintained expression after 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 after infection in healthy control but not COPD AMs (Figure 6D). To test whether increased mROS reconstituted bacterial killing in COPD AMs, we added the mitochondrial complex I inhibitor rotenone, which enhances mROS production (29), confirming it enhanced bacterial killing in both healthy control and COPD AMs (Figure 6E). In keeping with a limited role for mROS in induction of apoptosis under these circumstances, as well as with the well-developed resistance of AMs to oxidative stress (30), 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 AMs 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 antiapoptotic protein Mcl-1 (12, 13), and we provide evidence that Mcl-1 is up-regulated in COPD AMs. 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 OCR during oxidative phosphorylation and mROS production in mock-infected macrophages, but during infection, its major effect is to regulate caspase-dependent mROS production. COPD AMs 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, 31). Bacterial load in the airway correlates with progressive airway obstruction and maintenance of neutrophilic inflammation (5). Moreover, researchers using a recent murine model involving polymeric immunoglobulin-deficient mice demonstrated that persistent exposure to lung bacteria drive inflammatory changes and lung remodeling in the small airways (32). This suggests that innate immune dysfunction and impaired handling of respiratory pathogens are central features of COPD pathogenesis. In line with these observations, several groups have demonstrated that COPD AMs have altered activation states (33, 34), cytokine responses (35), and phagocytic capacity (14, 15, 36). Despite this, there has been little investigation of microbicidal responses in COPD AMs. 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 included very few who used corticosteroid inhalers,

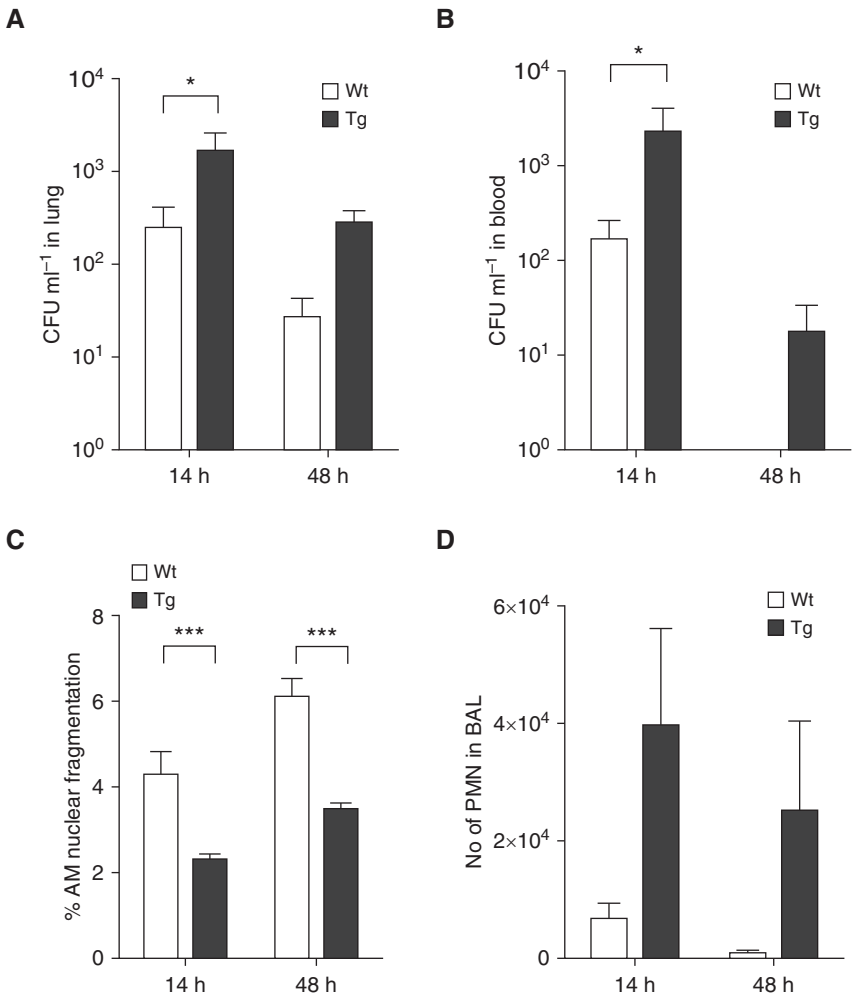

Figure 3. Induced myeloid leukemia cell differentiation protein Mcl-1 up-regulation in alveolar macrophages (AMs) impairs bacterial clearance in the lung. (*A–D*) Wild-type (Wt) or CD68.hMcl-1–transgenic (Tg) mice were challenged with 10^4 serotype 1 *Streptococcus pneumoniae*. At the designated time after instillation, (*A*) bacterial CFU in the lung homogenate, (*B*) bacterial CFU in the blood, (*C*) AM nuclear fragmentation or condensation in bronchoalveolar lavage (BAL), and (*D*) total polymorphonuclear leukocyte (PMN) numbers in BAL were measured. n = 4–11 mice per group from three independent experiments; $^*P < 0.05$, $^{***}P < 0.001$, two-way analysis of variance.

arguing against a major confounding effect of these on Mcl-1 expression.

AMs require additional microbicidal mechanisms to complement early phagolysosomal bacterial killing because they lack myeloperoxidase (37) and the granule-associated serine proteases found in neutrophils (30). 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). AMs respond by activating a delayed phase of intracellular killing in response to a diverse range of pathogens, extending from pneumococci to *Mycobacterium tuberculosis* (12, 13, 39). Generation of mROS has emerged as an important microbicidal strategy used by macrophages (23), and its production is increased by caspase 3–mediated inhibition of complex I (26). 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 AMs.

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 suggest 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 (33, 34). Failure to generate classical activation will reduce succinate generation, an important driver of acute mROS production required for microbicidal responses (42, 43), whereas the chronic production of mROS can favor alternative activation with consequences for innate immune responses (44). Up-regulation of antioxidant defenses, such as SOD2, in COPD (27) will further compromise mitochondrial microbicidal capacity. Our results suggest that 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 up-regulation could theoretically drive chronic mROS production in COPD AMs. The production of mROS is influenced by more than the basal and maximal OCRs, however, and how COPD influences hydrogen ion leak and the function of uncoupling proteins in AMs in COPD is unknown (24). Mcl-1 can exist as a form that localizes to the outer mitochondrial membrane and regulates apoptosis and as 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 AMs, either the expression of the

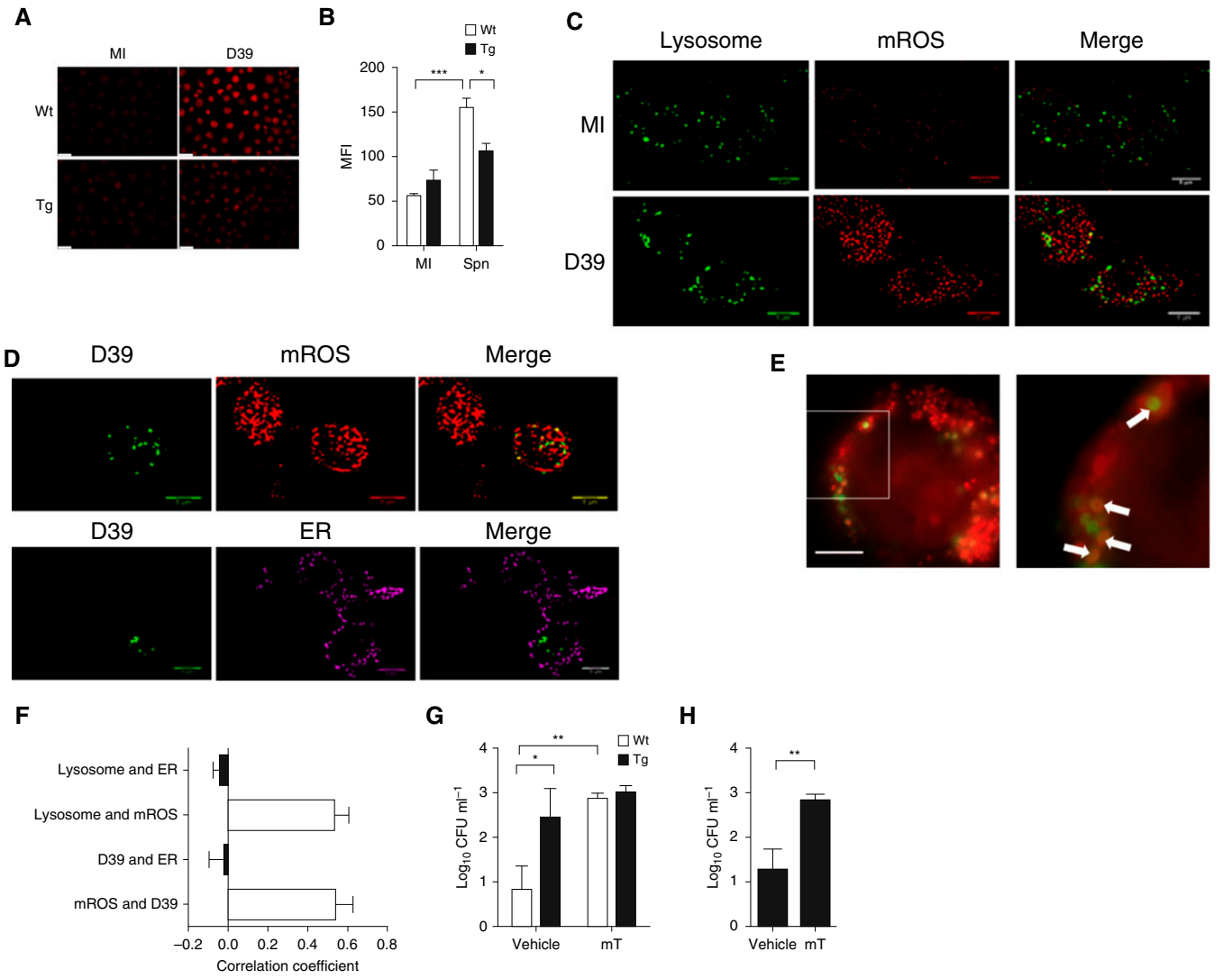

Figure 4. Induced myeloid leukemia cell differentiation protein Mcl-1 modulates generation of mitochondrial reactive oxygen species (mROS) and mROS-dependent bacterial killing. (*A*) Wild-type (Wt) or CD68.hMcl-1–transgenic (Tg) bone marrow–derived macrophages (BMDMs) were mock infected (MI) or challenged with opsonized serotype 2 (D39) *Streptococcus pneumoniae* (Spn). Twenty hours after challenge, cells were stained with MitoSOX Red and visualized by microscopy to assess mROS generation. Images are representative of three independent experiments; *scale bar* = 50 μm. (*B*) At the designated time after challenge, mROS were also assessed by flow cytometry. n = 3; $^{*}P < 0.05$ for D39 Wt versus D39 Tg and $^{***}P < 0.001$ for MI Wt versus Spn Wt by two-way analysis of variance. (*C*) MI or D39-infected Wt BMDMs were stained with Cresyl violet to detect lysosomes (*green*) and MitoSOX Red (*red*) at 20 hours and analyzed by confocal microscopy. Colocalized signals are shown in *yellow* (Merge); *scale bar* = 5 μm. (*D*) Confocal fluorescence microscopy of D39 BMDMs challenged with Alexa Fluor 647–labeled bacteria (*green*) and stained with MitoSOX Red (*red*; *upper panels*) or endoplasmic reticulum (ER) tracker (*purple*; *lower panels*) 20 hours after bacterial challenge. Colocalized signals are shown in *yellow* (Merge; *upper* and *lower panels*); *scale bar* = 5 μm. (*E*) Pseudocolored structured illumination microscopic image of a monocyte-derived macrophage 16 hours after bacterial challenge with *S. pneumoniae* (*green*) and stained with MitoSOX Red (*red*) for mROS. Enlarged region on *right* shows bacteria colocalized with mROS (*arrows*). *Scale bar* = 1 μm. (*F*) Pearson's correlation coefficients were calculated for the colocalization of mROS or ER with D39 or lysosomes. Data are shown as mean ± SEM (n = 4–8). (*G* and *H*) Wt or Tg BMDMs (*G*) or human monocyte-derived macrophages (*H*) were challenged with D39 in the presence or absence (vehicle) of mitoTEMPO (mT). Sixteen hours after challenge, intracellular CFU were assessed. n = 5 (for *G*) and n = 8 (for *H*); $^{*}P < 0.05$ and $^{**}P < 0.01$ by repeated measures two-way analysis of variance (for *G*) or Wilcoxon matched-pairs signed-rank test (for *H*). MFI = mean fluorescence intensity.

matrix-localized form must be altered or there must be additional factors modulating proton leak to result in greater mROS production. An additional implication of this is that the reduced induction of mROS we documented after pneumococcal challenge in association with maintenance of Mcl-1 expression may be a consequence of not just reduced caspase activation (and therefore inhibition of complex I [26]) but also preservation of the matrix-localized form of Mcl-1 (45). Regardless of these considerations, there is still potential to overwhelm Mcl-1 in COPD AMs and reengage both induction of mROS and microbicidal capacity, as evidenced by the capacity of a complex I inhibitor to enhance bacterial clearance.

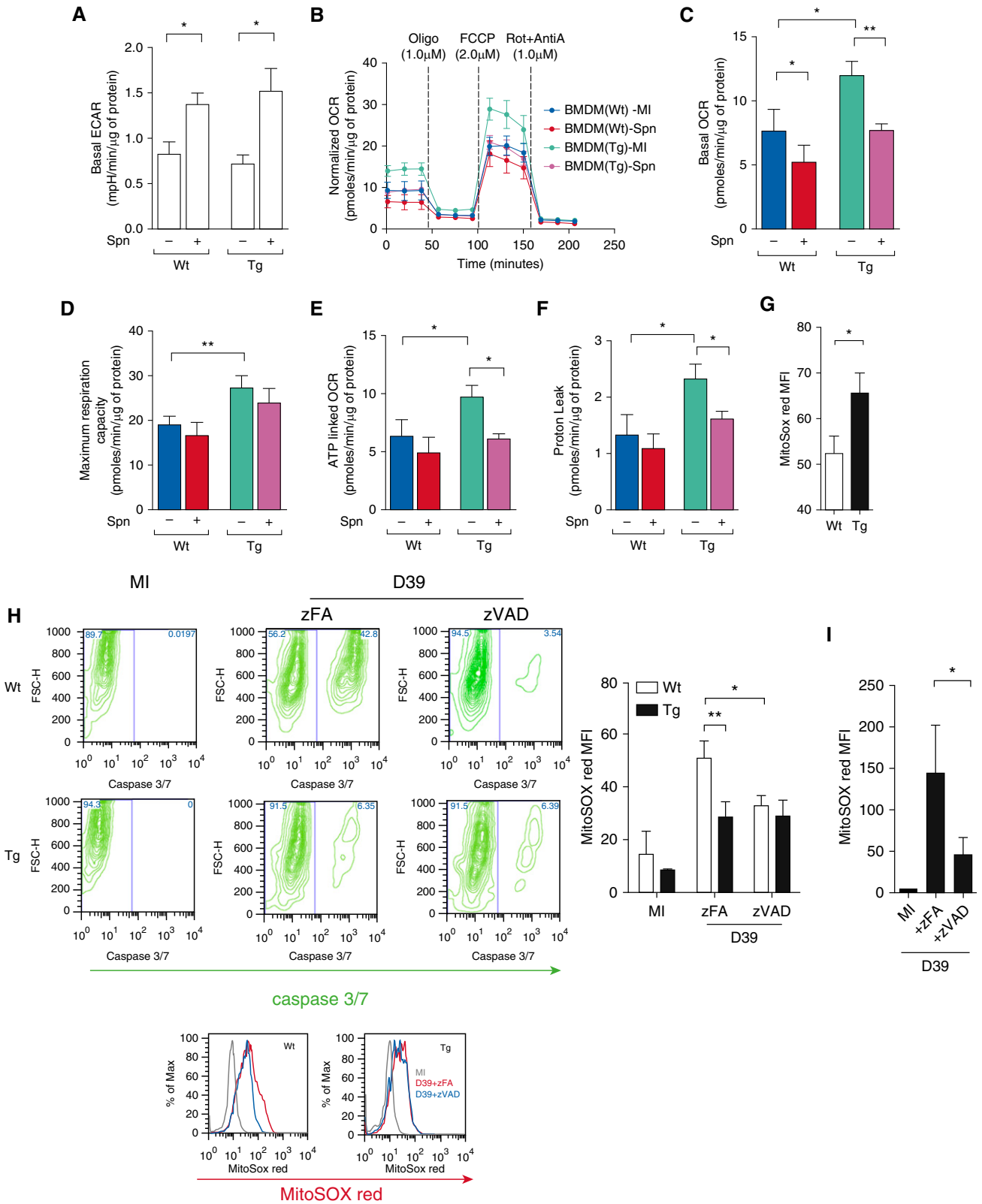

Figure 5. Induced myeloid leukemia cell differentiation protein Mcl-1 modulates mitochondrial responses leading to mitochondrial reactive oxygen species (mROS) generation. (*A–F*) Wild-type (Wt) or CD68.hMcl-1–transgenic (Tg) bone marrow–derived macrophages (BMDMs) were mock infected (MI) or challenged with opsonized serotype 2 (D39) *Streptococcus pneumoniae* (Spn) for 4 hours before extracellular acidification (ECAR) (*A*), and parameters related to oxidative phosphorylation were measured kinetically using oligomycin (Oligo), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP),

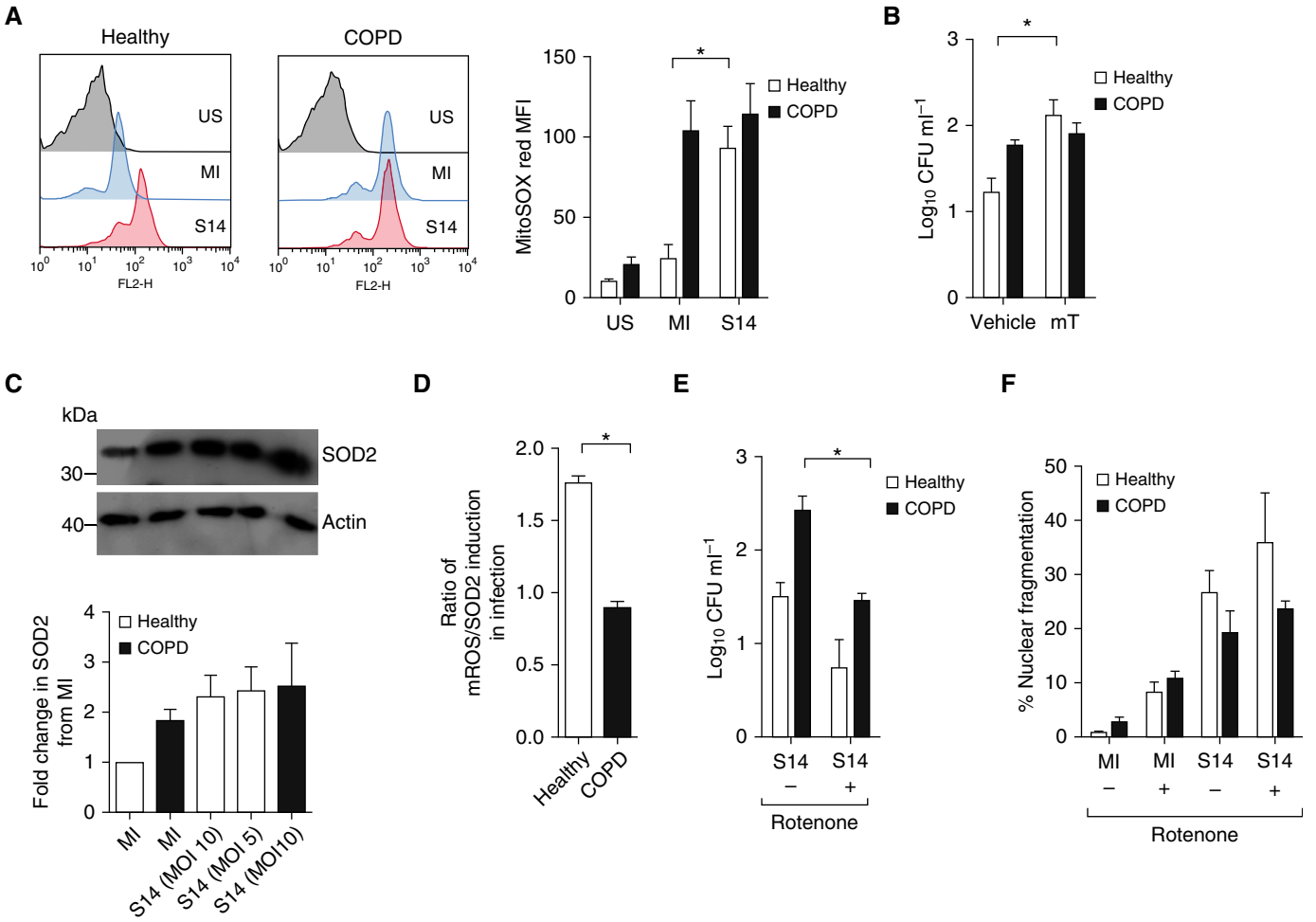

Figure 6. Chronic obstructive pulmonary disease (COPD) alveolar macrophages (AMs) fail to increase mitochondrial reactive oxygen species (mROS) production after pneumococcal challenge. (*A* and *B*) AMs obtained from bronchoalveolar lavage (BAL) of healthy control subjects or patients with COPD were mock infected (MI) or challenged with opsonized serotype 14 *Streptococcus pneumoniae* (S14), at a multiplicity of infection (MOI) of 10 for COPD cells or an MOI of 5 for healthy cells. AMs were left unstained (US) or stained with MitoSOX Red, and mean fluorescence intensity (MFI) was recorded at 16 hours as a measure of mROS, with representative plots shown and collated data graphed (*A*), and intracellular bacterial CFU were estimated in the presence or absence (vehicle) of mitoTEMPO (mT) (*B*) at 20 hours. Both n = 6; $^{*}P < 0.05$, paired Student's *t* test (*A*) or Wilcoxon signed-rank test (*B*). (*C*) AMs from BAL of healthy control subjects or patients with COPD were MI or challenged with S14 at the designated MOI. At 16 hours after challenge, the levels of superoxide dismutase 2 (SOD2) in AMs were probed by western blotting. Representative blot and densitometric analysis are shown. n = 4. (*D*) The ratio of mROS to SOD2 induced by bacterial challenge was calculated for healthy and COPD AMs using the samples in *C*. n = 4; $^{*}P < 0.05$, Student's *t* test. (*E* and *F*) Healthy or COPD AMs were MI or challenged with S14 in the presence (+) or absence (−) of rotenone to induce mROS. AMs were assessed for (*E*) intracellular bacterial CFU and (*F*) nuclear fragmentation or condensation 20 hours after challenge. n = 3; $^{*}P < 0.05$, Wilcoxon signed-rank test.

Our findings are based exclusively on experiments with the pneumococcus, but they are likely to have broad impact in COPD, even though other bacteria such as nontypeable *H. influenzae* are frequently implicated as colonizers of the COPD airway and drive inflammation (46). Pneumococci are the second most frequent 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

Figure 5. (Continued). rotenone (Rot), or antimycin A (AntiA) at the indicated concentrations. Using the kinetic data (*B*), basal oxygen consumption rate (OCR) (*C*), maximum respiration capacity (*D*), ATP-linked OCR (*E*), and proton leak (*F*) were calculated. n = 6 per group; $^{*}P < 0.05$, $^{**}P < 0.01$ by two-way analysis of variance (ANOVA). (*G*) MI Wt and Tg BMDMs were stained with MitoSOX Red to measure baseline mROS production. (*H* and *I*) Wt or Tg BMDMs (*H*) or human monocyte-derived macrophages (*I*) were MI or challenged with D39 in the presence of the pan-caspase inhibitor zVAD or control zFA. At 20 hours after challenge, cells were stained for mROS and caspase 3/7 activity. MitoSOX Red staining was assessed for the whole-cell populations (histograms) showing forward scatter (FSC-H) versus caspase 3/7. Representative plots are shown, with collated data graphed. n = 4; $^{*}P <$ 0.01 by two-way ANOVA (for *H*) or one-way ANOVA (for *I*). MFI = mean fluorescence intensity; zFA = benzyl N-[1-[(4-fluoro-3-oxobutan-2-yl)amino]-1-oxo-3-phenylpropan-2-yl]carbamate; zVAD = carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone.

and decline in FEV_1 (colonization with a monoculture of pneumococci specifically increased the risk of acute exacerbation in one study [20]) and indirect effects because 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). Pneumococci can also synergize with *H. influenzae* to promote proinflammatory 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 against a range of pathogens is important (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 AMs 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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