Functional and metabolic impairment in cigarette smoke-exposed macrophages is tied to oxidative stress

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Cigarette smoke inhalation exposes the respiratory system to thousands of potentially toxic substances and causes chronic obstructive pulmonary disease (COPD). COPD is characterized by cycles of inflammation and infection with a dysregulated immune response contributing to disease progression. While smoking cessation can slow the damage in COPD, lung immunity remains impaired. Alveolar macrophages (AMΦ) are innate immune cells strategically poised at the interface between lungs, respiratory pathogens, and environmental toxins including cigarette smoke. We studied the effects of cigarette smoke on model THP-1 and peripheral blood monocyte derived macrophages, and discovered a marked inhibition of bacterial phagocytosis which was replicated in primary human AMΦ. Cigarette smoke decreased AMΦ cystic fibrosis transmembrane conductance regulator (CFTR) expression, previously shown to be integral to phagocytosis. In contrast to cystic fibrosis macrophages, smoke-exposed THP-1 and AMΦ failed to augment phagocytosis in the presence of CFtR modulators. Cigarette smoke also inhibited THP-1 and AMΦ mitochondrial respiration while inducing glycolysis and reactive oxygen species. These effects were mitigated by the free radical scavenger N-acetylcysteine, which also reverted phagocytosis to baseline levels. Collectively these results implicate metabolic dysfunction as a key factor in the toxicity of cigarette smoke to AMΦ, and illuminate avenues of potential intervention.

Cigarette smoking infuses the respiratory tract with toxins, causing abundant, long-lasting consequences even after smoking cessation1-3. Among those consequences is chronic obstructive pulmonary disease (COPD), which is projected to soon be the third leading cause of death worldwide4,5. As the principal innate immune cell in the healthy lung, alveolar macrophages (AMΦ) are implicated in dysregulated inflammation and cycles of recurrent and chronic infections of COPD, yet the direct toxicity of cigarette smoke to AMΦ remains incompletely understood6. AMΦ are responsible for clearing pathogens, particulate matter, and cellular debris from the lung, and any impairment in this process is likely to have deleterious physiologic consequences7. For example, poor phagocytosis is known to negatively correlate with lung function in COPD8. Conversely, rescuing diseased AMΦ function may prove of therapeutic benefit; azithromycin has been shown to augment phagocytosis of apoptotic bronchial epithelial cells (also known as efferocytosis) in COPD AMΦ9,10. In addition to impaired efferocytosis9-12, COPD AMΦ are known to be defective in phagocytosis of bacteria13-16, however data on the acute effects of cigarette smoke on AMΦ are lacking.

An emerging area of research into COPD pathophysiology relates to the role of the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride and bicarbonate channel which is mutated in cystic fibrosis (CF)17. CFTR dysfunction has been demonstrated in bronchial epithelial cells from COPD patients, presumably contributing to mucous hyperviscosity and ciliary stasis with subsequent mucus plugging, impaction, and chronic infections18-22. The CFTR potentiator ivacaftor (also known as VX-770) is FDA approved as monotherapy for CF patients with CFTR gating mutations23,24, and is a component of combination therapies for patients with trafficking mutations25-29. As such its use has significantly improved outcomes for CF patients30. It has also shown...
promise in reversing cigarette smoke-induced CFTR dysfunction \textit{in vitro} and is currently in clinical trials for patients with chronic bronchitis\textsuperscript{31,32}.

CFTR dysfunction has been shown to impair phagocytosis in CF M\Phi\textsuperscript{33,34} in a manner that is reversible by the CFTR corrector lumacaftor\textsuperscript{35}, raising the possibility that CFTR activity is required for optimal function of COPD M\Phi as well. Herein we investigate the effects of cigarette smoke on AM\Phi phagocytosis of \textit{Pseudomonas aeruginosa}, CFTR expression, and the potential salutary effects of CFTR modulating drugs.

M\Phi phagocytosis is also understood to be intertwined with metabolism and reactive oxygen species\textsuperscript{36–39}. Inflammatory activation of M\Phi causes them to shunt glucose towards glycolysis in lieu of mitochondrial oxidation, and conversely, modulating metabolism impacts inflammatory state\textsuperscript{36,40}. Availability of glucose has been shown to modulate phagocytosis as well as glycolytic rates in an \textit{in vitro} diabetes model\textsuperscript{41}. Here we investigate how cigarette smoke impacts M\Phi metabolism and reactive oxygen species (ROS) generation, and probe the activity of the free-radical scavenger N-acetylcysteine (NAC) in rescuing smoke-induced dysfunction.

\textbf{Results}

\textbf{Cigarette smoke extract inhibits phagocytosis of \textit{Pseudomonas aeruginosa} by immortalized and primary macrophages.} We first determined the effects of cigarette smoke extract (CSE) on phagocytosis of \textit{P. aeruginosa} utilizing a gentamicin protection assay. We chose a 20 min incubation with bacteria, which was previously demonstrated to be within the linear phase of bacterial ingestion\textsuperscript{35}. Using THP-1 cells differentiated into M\Phi by addition of the protein kinase C agonist phorbol 12-myristate 13-acetate (PMA), we found a large decrease in \textit{P. aeruginosa} uptake after a 20 minute pretreatment with 10% CSE (Fig. 1a), a concentration determined in preliminary experiments to show effects without impacting cell viability as determined by lactate dehydrogenase (LDH) release (Supplementary Fig. S1). In primary human peripheral blood monocyte-derived M\Phi (MDM) we saw a smaller trend towards decreased phagocytosis that was not statistically significant (Fig. 1b).

While there are reports describing decreased phagocytosis in AM\Phi purified from patients with COPD and “healthy” smokers relative to non-smokers\textsuperscript{6,11,13,15}, there are no published reports on the acute effects of CSE on bacterial phagocytosis in healthy human AM\Phi. Here we found that CSE induces a dose-dependent decrement in phagocytosis in M\Phi from all three right lung lobes as well as pooled M\Phi with no significant differences in CSE effect between lobes (Fig. 1c). While there were some differences in the basal rates between experiments on AM\Phi from three separate individuals, these likely represented day-to-day experimental variability, as similar variations were seen with immortalized THP-1 cells (Fig. 1a) and MDM (Fig. 1b). Analyzing the AM\Phi data with linear models to control for batch effects, we found a highly significant effect of CSE concentration (p < 10\textsuperscript{-15}) on phagocytosis. RLL AM\Phi had lower baseline phagocytosis levels when compared with RUL (p < 0.05) or RML (p < 0.001) however there was no significant difference in their respective sensitivities to CSE.

\textbf{CFTR is diminished by CSE but CFTR modulators fail to impact phagocytosis.} Given the importance of CFTR for phagocytosis\textsuperscript{34,35,42} as well as reported decrements in CFTR expression and function in smoke-exposed epithelial cells\textsuperscript{39,43,45}, we determined the effects of CSE on CFTR expression in primary AM\Phi. We exposed AM\Phi to 5% CSE as this induced an intermediate inhibition of phagocytosis and was therefore felt to be an appropriate concentration for downstream experiments. We reasoned that if CFTR was relevant to phagocytosis, we should see a decrement in maximal respiration to THP-1 cells. The P. aeruginosa demonstrated a comparable decrease in maximal respiration to THP-1 cells. The

As lumacaftor was recently shown to rescue phagocytosis efficiency in monocyte-derived macrophages from CF donors\textsuperscript{35}, we sought to determine the effects of CFTR modulators on CSE-induced defects. Using previously published concentrations of ivacaftor (30 nM) and lumacaftor (3 \mu M) which were in turn derived from serum levels found in CF subjects\textsuperscript{35}, we pre-treated THP-1 derived M\Phi (Fig. 2b) or primary human AM\Phi (Fig. 2c) with ivacaftor, lumacaftor, or their combination (as in the FDA approved CF drug formulation Orkambi\textsuperscript{36}) for 48 hours. This extended pre-incubation was to allow for the slower effects of the CFTR corrector lumacaftor. Under these conditions we found that lumacaftor significantly reduces THP-1 phagocytosis (p < 0.01), and a marginally significant trend (p = 0.099) suggesting that ivacaftor may also reduce phagocytosis. As expected, there was a highly significant (p < 0.001) reduction in phagocytosis by 5% CSE. We confirmed that over a wide range of concentrations from 10 nM through 10 \mu M there was no dose-dependent effect of either compound on THP-1 phagocytosis in the presence or absence of CSE (Fig. S2). In the presence of CSE, CFTR modulators showed no effect on AM\Phi phagocytosis in any of individuals tested (Fig. 2c).

\textbf{CSE shifts M\Phi metabolism from oxidative phosphorylation to glycolysis.} Given the lack of effect of ivacaftor and lumacaftor on the phagocytosis defect, we sought to understand what other pathways besides CFTR dysfunction were involved. M\Phi metabolism has been demonstrated to be integral to function\textsuperscript{36,44,47}, therefore we measured oxygen consumption and proton production (as proxies for oxidative phosphorylation and glycolysis, respectively) with an extracellular flux analyzer after CSE injection. We performed a mitochondrial stress assay which allows for the quantitation of basal respiration, ATP-linked respiration, maximal respiration, proton leak and non-mitochondrial respiration\textsuperscript{48}. Figure 3a presents a schematic of the parameters measured with the assay. As seen in Fig. 3b, addition of CSE to THP-1 M\Phi immediately inhibited ATP-linked respiration with a compensatory increase in glycolysis. There were no differences in the proton leak or non-mitochondrial respiration, however there was a small decrease in the maximal respiratory rate. Primary AM\Phi meanwhile, exhibited a smaller decrement in ATP-linked respiration with CSE, with similar to increased relative glycolysis induction (Fig. 3c). AM\Phi demonstrated a comparable decrease in maximal respiration to THP-1 cells. The
discordant effects on spare respiratory capacity between the cell types are attributable to the stronger inhibition of respiration in THP-1 cells (acute injection) relative to maximal respiratory capacity. Pre-treatment of either THP-1 or AMΦ with ivacaftor, lumacaftor or their combination had no effect on any of the parameters studied here (Supplementary Fig. S3).

Cigarette smoke induces reactive oxygen species. As mitochondrial dysfunction can result in increased production of ROS due to incomplete reduction of oxygen, we tested the presence of free radicals using the probe 2',7'-dichlorofluorescin diacetate (DCF-DA), which is a ROS-activated fluorophore. There was a dose-dependent increase in the production of ROS by THP-1 MΦ in the presence of CSE (Fig. 4a) which was inhibited by the free radical scavenger NAC (Fig. 4b) and reproduced by the exogenous addition of hydrogen peroxide as a positive control (Fig. 4c). Figure 4a–f each represent data from one experiment which was graphed onto three separate plots for clarity due to overlap of the data. When cultured in reducing medium, primary AMΦ exhibited a similar pattern of ROS production to THP-1 MΦ however with approximately an order of magnitude greater signal (Fig. 4d–f).

N-acetylcysteine improves metabolic dysfunction in primary AMΦ. Mitochondrial dysfunction and ROS can form a feedback loop whereby mitochondrial ROS can further inhibit mitochondrial function, therefore we tested the ability of NAC to interrupt this cycle by pre-treating AMΦ prior to CSE injection. Preliminary experiments with the mitochondrial stress assay demonstrated significant artefact in the presence of CSE.
of NAC, potentially due to direct buffering effects in the otherwise unbuffered media; we therefore chose to use a glycolytic rate assay (buffered with 5 mM HEPES) for further investigation. Figures 5c,d illustrate how NAC decreases CSE-induced glycolysis in a dose-dependent manner. AM\(\Phi\) from upper and lower lobes are known to exhibit differential inflammatory responses to hypoxia\(^5\) which is a known inducer of oxidative stress, however we saw similar effects in right upper lobe and right lower lobe AM\(\Phi\).

CSE phagocytosis inhibition is replicated by hydrogen peroxide and reversed by NAC. The data presented thus far suggest a connection between the metabolic and functional defects induced by CSE in THP-1 and alveolar M\(\Phi\), therefore we tested the hypothesis that CSE-induced ROS are directly interfering with phagocytosis. Hydrogen peroxide replicated the inhibitory effect of CSE on phagocytosis in THP-1 cells, causing a dose-responsive decrement in the phagocytosis efficiency (Fig. 6a), with a comparable effect to 5% CSE at 250\(\mu\)M. Linear models revealed a highly significant (\(p < 10^{-9}\)) dose-dependent effect of hydrogen peroxide. Further implicating ROS in this process, NAC reversed the phagocytosis defect of either hydrogen peroxide or CSE (Fig. 6b). In THP-1, NAC had a greater effect when it was added to the medium after the hydrogen peroxide or CSE (NAC PostTx) rather than before (NAC preTx), reaching statistical significance in both cases by paired t-tests, whereas pre-treatment with NAC only produced a trend towards significance. Linear models demonstrate that both of NAC, potentially due to direct buffering effects in the otherwise unbuffered media; we therefore chose to use a glycolytic rate assay (buffered with 5 mM HEPES) for further investigation. Figures 5c,d illustrate how NAC decreases CSE-induced glycolysis in a dose-dependent manner. AM\(\Phi\) from upper and lower lobes are known to exhibit differential inflammatory responses to hypoxia\(^5\) which is a known inducer of oxidative stress, however we saw similar effects in right upper lobe and right lower lobe AM\(\Phi\).
hydrogen peroxide (p < 0.0001) and 5% CSE (p < 0.01) decrease phagocytosis, whereas NAC post-treatment has a positive effect (p < 0.0001). Experiments using primary AMΦ from three donors revealed broadly similar results with subtle differences. In this model, NAC post-treatment continued to reverse the effects of hydrogen peroxide, however in CSE-treated cells, NAC pre-treatment reached statistical significance whereas NAC post-treatment fell just short of this threshold (p = 0.055).

Discussion
While there is little doubt that AMΦ dysfunction plays a key role in COPD, there is less known about the mechanisms thereof, making it challenging to target therapeutically. We originally hypothesized that CFTR may play a role, as it has been previously shown to be important for CF MΦ. In epithelial cells CFTR is known to be inhibited by cigarette smoke, both at the channel function and protein expression levels. It has been previously reported in RAW 264.7 murine MΦ that P. aeruginosa phagocytosis is blocked by CSE in a partially
CFTR-dependent manner\textsuperscript{53}, however there were methodological differences in their CSE preparation and phagocytosis assays including MOI and length of infection, in addition to the cell type studied. In the presence of CSE, these authors saw increased clearance of bacteria from cell culture media with the preclinical CFTR potentiator and corrector VRT-532, which occurred without an appreciable increase in CFTR protein levels. Unfortunately, two clinically available CFTR modulators in our hands were ineffective at improving phagocytosis. The same group showed that oxidative stress is connected to impaired CFTR trafficking in smoke-exposed epithelial-like cells, leading to aggresome formation and degradation of misfolded proteins\textsuperscript{54}. Whether or not similar CFTR trafficking pathways are involved in smoke-exposed M\(\Phi\) remains to be seen, as well as whether they are amenable to targeting by CFTR modulators. It is important to note that our data do not demonstrate that CFTR is irrelevant to phagocytosis and/or metabolism in smoke-exposed macrophages, only that two CFTR modulators were unable to reverse the acute insult of CSE exposure on macrophage function. Complementary approaches using CFTR mutants and knockouts are currently underway in our laboratory to better address this question. A second CFTR corrector, tezacaftor, has recently been approved, and other CFTR modulators with different mechanisms are in the pipeline which will increase the pharmacologic arsenal and could have different results\textsuperscript{25,26}.

Regarding the relationship between CFTR and phagocytosis, we postulate that the CFTR decrement seen here is a separate downstream effect of CSE, and therefore targeting more proximal events such as oxidative stress is likely to be of higher yield than targeting CFTR itself. Indeed NAC proved more effective at reversing the phagocytosis defect in our system, as well as metabolic consequences of CSE. NAC was even able to improve phagocytosis when added after smoke or hydrogen peroxide exposure (Fig. 6b,c), increasing its therapeutic potential. NAC has had mixed results as a treatment for COPD; while there are some data that it can decrease exacerbations at high doses in a cohort of patients who are subject to frequent exacerbations\textsuperscript{55,56}, the effects are modest at best, while other studies have shown no benefit\textsuperscript{57}. This may be due to the high (mM) concentrations needed to produce effects \textit{in vitro}, which could be difficult to attain \textit{in vivo}.

While the experimental \textit{ex vivo} system presented herein has strengths in that it allows us to evaluate the acute effects of CSE, it is also limited by that acuity. Administration of CSE is quite different from the chronic exposure to which smokers’ lungs are subject, and it will be interesting to see the relative contributions of CFTR and oxidative stress in smoker and COPD M\(\Phi\). These two pathways are not mutually exclusive as CFTR itself is known to be a glutathione transporter\textsuperscript{58,59}, and oxidative stress is exacerbated by CFTR dysfunction impairing the normal glutathione response\textsuperscript{60}. Oxidative stress can then further decrease CFTR levels\textsuperscript{61}, forming a vicious

Figure 4. CSE induction of ROS is mitigated by NAC. THP-1 derived M\(\Phi\) were plated in 96-well plates, then loaded with 10\(\mu\)M DCF-DA for 30 min. Cells were washed once with PBS and treated with vehicle (a) or NAC 4\(\text{mM}\) (b,c) as indicated for 30 min, followed by the addition of vehicle, CSE or \(\text{H}_2\text{O}_2\) (250\(\mu\)M). Fluorescence readings with excitation at 488 nm and emission 523 nm were taken every 5 min for one hour then every 30 min for 23 hrs. Readings were normalized to those of identically treated wells without fluorescent dye. Plots from (a–c) are derived from a single experiment, data were separated onto three graphs for clarity. Four wells per condition were run and mean \(\pm\) s.d. graphed, data are representative of three independent experiments. (d–f) Experiments were performed as above using primary M\(\Phi\) from a healthy donor. Data are representative of unique experiments on cells from three separate donors with six technical replicates each.
cycle. Experiments to illuminate these possibilities are ongoing in our laboratory. The source and identity of the elevated ROS will likewise be informative.

In summary, we find that cigarette smoke acutely inhibits bacterial phagocytosis as well as mitochondrial function in human AM\(\Phi\). While CSE also decreases CFTR levels, CFTR modulators ivacaftor and lumacaftor are unable to rescue CSE defects. Meanwhile, NAC partially reverses the dysfunction, consistent with increased oxidative stress seen in the presence of CSE. This provides further basic mechanistic rationale for the use of antioxidants in COPD, somewhat tempered by the high concentrations needed to see an effect in vitro. Further studies will extend this work into primary AM\(\Phi\) isolated from current smokers.

Methods

Human subjects. This study was approved by the Committee for the Protection of Human Subjects at the Geisel School of Medicine at Dartmouth (CPHS protocol #22781), and all procedures were performed in accordance with relevant guidelines and regulations. Healthy subjects (n = 24 total, 3–5 per experiment) were enrolled if they were between 18 and 55 years of age, non-smokers, and had no underlying pulmonary disorder or significant comorbidity. Informed consent was obtained from all participants for participation in research, phlebotomy, and for bronchoscopy individually. Phlebotomy (100 mL whole blood for monocyte isolation) was performed at the time of peripheral IV insertion. Next, after local anesthesia to the posterior oropharynx, with or without systemic sedation per patient preference, a flexible bronchoscope was passed through the mouth and vocal cords until wedged into sub-segmental bronchi of the right lung. Five lavages each of 20 cc 0.9% saline and 10 cc air were performed in the right upper lobe, right lower lobe, and right middle lobe sequentially. Bronchoalveolar lavage (BAL) samples from individual lobes were obtained and alveolar macrophages purified as previously described51.
Materials
Sources of reagents are available in the online Supplementary Information.

Generation of cigarette smoke extract. CSE was purified according to protocol of Blue and Janoff as described by others. One cigarette was lit and attached to 5 cm plastic tubing and placed on the end of a 60 mL syringe containing 10 mL of media. Smoke was drawn into the chamber of the syringe until 50 mL total volume was obtained, then agitated until the smoke cleared. This process was repeated until the cigarette was consumed, which was defined as 100% CSE.

Phagocytosis assay. A modified gentamicin protection assay was used to quantify phagocytosis. Specific details are available in the Supplementary Information.

Immunofluorescence. Primary alveolar macrophages were plated overnight, then treated with 5% CSE for one hour and stained for CFTR, followed by counterstaining with Hoechst to reveal nuclei. Specific details are available in the Supplementary Information.

Extracellular flux assay. Macrophages were plated onto Seahorse assay plates obtained from the manufacturer at 50,000/well. Assay was run according to manufacturer’s protocol for mitostress assay and glycolytic rate assay, respectively. CSE was added to port A to be injected prior to kit compounds in ports B through D respectively. Reagent concentrations and buffer composition are in the Supplementary Information.
Reactive oxygen species assay. Macrophages were loaded with 10μM 2’,7’-dichlorofluorescein diacetate (DCF-DA) for 30 min, washed once with PBS and treated with vehicle or NAC 4 mM for 30 min, followed by the addition of CSE or H2O2 (250 μM). AMΦ were cultured in the presence of 50 μM β-mercaptoethanol to reduce basal ROS. Cells were incubated in a Tecan plate reader at 37°C. Fluorescence readings (excitation 488 nm, emission 523 nm) were taken every 5 min for one hour then every 30 min for 23 hrs. Readings of identically treated wells without dye were subtracted to normalize for fluorescence from media.

Statistics. All graphs were prepared with GraphPad Prism 7.0 (San Diego, CA). Linear models were run in R as indicated in text and figure legends. ANOVA and Mann-Whitney tests were performed in Prism. All p values are two-tailed and p < 0.05 was interpreted to be statistically significant. All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

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**Author Contributions**

D.S.A., D.L.M. and A.A. conceptualized research and planned experiments; D.S.A., D.L.M., D.A.A., H.F.H., J.A.D., G.T.A. and J.L.C. conducted experiments; D.S.A., T.H.H. and A.A. analyzed data and prepared manuscript; All authors edited and approved final manuscript.

**Additional Information**

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