Opsonic Phagocytosis in Chronic Obstructive Pulmonary Disease Is Enhanced by Nrf2 Agonists

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Abstract

Rationale: Previous studies have identified defects in bacterial phagocytosis by alveolar macrophages (AMs) in patients with chronic obstructive pulmonary disease (COPD), but the mechanisms and clinical consequences remain incompletely defined.

Objectives: To examine the effect of COPD on AM phagocytic responses and identify the mechanisms, clinical consequences, and potential for therapeutic manipulation of these defects.

Methods: We isolated AMs and monocyte-derived macrophages (MDMs) from a cohort of patients with COPD and control subjects within the Medical Research Council COPD MAP consortium and measured phagocytosis of bacteria in relation to opsonic conditions and clinical features.

Measurements and Main Results: COPD AMs and MDMs have impaired phagocytosis of *Streptococcus pneumoniae*. COPD AMs have a selective defect in uptake of opsonized bacteria, despite the presence of antipneumococcal antibodies in BAL, not observed in

MDMs or healthy donor AMs. AM defects in phagocytosis in COPD are significantly associated with exacerbation frequency, isolation of pathogenic bacteria, and health-related quality-of-life scores. Bacterial binding and initial intracellular killing of opsonized bacteria in COPD AMs was not reduced. COPD AMs have reduced transcriptional responses to opsonized bacteria, such as cellular stress responses that include transcriptional modules involving antioxidant defenses and Nrf2 (nuclear factor erythroid 2–related factor 2)–regulated genes. Agonists of the cytoprotective transcription factor Nrf2 (sulforaphane and compound 7) reverse defects in phagocytosis of *S. pneumoniae* and nontypeable *Haemophilus influenzae* by COPD AMs.

Conclusions: Patients with COPD have clinically relevant defects in opsonic phagocytosis by AMs, associated with impaired transcriptional responses to cellular stress, which are reversed by therapeutic targeting with Nrf2 agonists.

Keywords: chronic obstructive pulmonary disease; macrophage; phagocytosis; antioxidant; nuclear factor erythroid 2–related factor 2 (Nrf2)

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At a Glance Summary

Scientific Knowledge on the

Subject: Chronic obstructive pulmonary disease (COPD) macrophages have defective phagocytosis, but the mechanism and clinical relevance remain unknown.

What This Study Adds to the

Field: COPD alveolar macrophages have a specific defect in opsonic phagocytosis that correlates with clinical phenotype. COPD alveolar macrophages fail to engage an antioxidant transcriptional module after exposure to opsonized bacteria. Agonists of a key transcriptional regulator of antioxidant host defense, Nrf2 (nuclear factor erythroid 2-related factor 2), reverse the opsonic phagocytosis defect in COPD and offer a potential therapeutic approach to correct the defect.

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory lung condition characterized by progressive airflow limitation (1, 2). COPD is associated with increased susceptibility to bacterial airway infection. Exacerbations cause acute worsening of symptoms, leading to hospitalization (3) and to disease progression (4). Approximately 50% of exacerbations are due to bacterial infection (5), and, in a long-term cohort study, the lower airways were chronically colonized with *Streptococcus pneumoniae* in one-third of patients (6). Individuals living with COPD are also at increased risk of community-acquired pneumonia (CAP) with increased mortality, most often caused by *S. pneumoniae* (7). This suggests that COPD leads to an innate immune defect against *S. pneumoniae* and other bacteria.

Alveolar macrophages (AMs) are the resident phagocytes enabling bacterial clearance from the lung, but COPD AMs have demonstrated reduced phagocytosis of *Haemophilus influenzae* and *Pseudomonas aeruginosa* (8, 9), and COPD monocyte-derived macrophages (MDMs) have also shown impaired phagocytosis of *S. pneumoniae* in previous studies (10). Bacterial phagocytosis by macrophages involves both nonopsonic and opsonic pathways (11, 12). Previous studies of COPD

macrophages have examined nonopsonic or complement-mediated phagocytosis, but phagocytosis in the presence of opsonizing antibody has not been studied in detail. A specific defect in opsonic phagocytosis would be particularly relevant to capsulated microorganisms, such as *S. pneumoniae*, which require opsonization for efficient phagocytosis (13), involving both IgG and complement present in alveolar fluid (14).

We investigated mechanisms underlying phagocytic defects in the COPD lung. In patients with COPD, opsonization fails to enhance AM phagocytosis, although it enhances MDM phagocytosis. The degree of AM opsonic phagocytosis was strongly associated with clinical and microbiological phenotype. AM responses to opsonized *S. pneumoniae* activated cellular stress transcriptional responses to antioxidant responses, but these were abrogated in COPD AMs. Agonists of the antioxidant transcription factor, nuclear factor erythroid 2-related factor 2 (NFE2L2) or Nrf2, a prominent component of antioxidant transcriptional responses, corrected the defect in AM opsonic phagocytosis in COPD. Some of the results of these studies were previously reported in the form of an abstract (15).

Methods

Macrophage Donors

Patients with COPD, free from exacerbation, were recruited from the U.K. Medical Research Council (MRC) COPDMap consortium with written approved consent, as outlined in the online supplement.

Cells and Infection

AMs were isolated from BAL as previously described (13). Cells were more than 95% AMs as assessed by DIFF-QUIK staining (Dade Behring) visualized by light microscopy (Leica DMRB 1000; Leica Microsystems). Human MDMs were differentiated for 14 days from peripheral blood mononuclear cells isolated from donors (with written informed consent) by using Percoll gradient (Sigma-Aldrich). Cells were cultured in RPMI 1640 medium (Lonza) supplemented with 10% fetal calf serum with low LPS (Lonza). Some cells were incubated with 10 μ M sulforaphane, 0.065 μ M Compound 7, a selective inhibitor of the KEAP1 (Kelch-like ECH-

associated protein 1)–Nrf2 interaction (16), or with vehicle control, for 16 hours before challenge with bacteria.

Bacteria

Serotype 14 *S. pneumoniae* (NCTC 11902; National Collection of Type Cultures) is a serotype commonly causing infection in COPD (17). Stocks were grown as previously described (18). Nontypeable *H. influenzae* (NTHi; NCTC 1269; National Collection of Type Cultures) was cultured as outlined in the online supplement. Macrophages were infected at a multiplicity of infection of 10:1. *S. pneumoniae* were opsonized for 15 minutes with immune serum obtained from volunteers vaccinated with pneumococcal polysaccharide vaccine, and with detectable antibody concentrations against *S. pneumoniae*, before macrophage challenge (13). Viable intracellular bacteria were measured at 4 hours after challenge as a measure of bacterial internalization using a gentamicin protection assay (GPA) as previously described (19). For assessment of early *S. pneumoniae* killing, macrophages were challenged for 4 hours before GPA, and additional wells were placed in media containing 0.75 μ g/ml vancomycin before GPA at the designated time points.

Bacterial Binding

Bacterial binding and internalization were assessed by fluorescence microscopy (Leica DMRB 1000) (13). Detailed information can be found in the online supplement.

Cell Surface Marker Expression

Cell surface marker expression was measured by flow cytometry, as described in the online supplement.

Transcriptomic Analysis

RNA was extracted and hybridized onto the Affymetrix GeneChip Human Genome U133 Plus 2.0 Array (Thermo Fisher Scientific). Data were analyzed in R using affyPML and limma (R Foundation for Statistical Computing). Enrichment analysis of Gene Ontology (GO) terms using a hypergeometric model and the GOstats package in R was performed for differentially expressed genes. False discovery rates were corrected with the Benjamini-Hochberg procedure. More detailed information is included 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, as described in the online supplement.

Statistics

Results are recorded as mean and SEM. Sample sizes were informed by SEs obtained from similar assays in prior publications (13, 18). Decisions on use of parametric or nonparametric tests were based on results of D'Agostino-Pearson normality tests. Comparisons were made by paired Student's *t* tests, and correlations were determined by Spearman's test using Prism 6.0 software (GraphPad Software Inc.). Significance was defined as $P < 0.05$.

Results

Demographic Data for Macrophage Donors

The demographic features for the COPD MAP macrophage donors are listed in Table 1. The patients with COPD had a significantly greater number of pack-years of cigarette exposure. Sixteen of 42 patients

with COPD (38.1%) had a history of frequent exacerbations (≥ 2 /yr). Vaccine history was available in 69%, and of these, 83% of patients with COPD had received a pneumococcal vaccine.

COPD AMs Have Selective Defects in Phagocytosis of Opsonized *S. pneumoniae*

Both COPD AMs and MDMs demonstrated reduced intracellular numbers of *S. pneumoniae* compared with healthy control subjects, regardless of opsonic conditions (Figures 1A–1D). COPD AMs (but not MDMs) from frequent exacerbators had reduced intracellular bacteria, regardless of opsonic conditions (Figures 1A–1D). Frequent exacerbations was set at two or more exacerbations per year, and, as shown in Figure E1 in the online supplement, patients with only one exacerbation did not have a reduction in bacteria uptake, whereas those with two or more exacerbations per year did. Paired analysis of intracellular bacteria numbers, comparing MDMs with AMs from the same donor, showed that intracellular *S. pneumoniae* was lower in AMs than in MDMs in COPD (but not in healthy groups), regardless of opsonic condition or exacerbation frequency (Figures

1E and 1F). Opsonization significantly increased numbers of intracellular bacteria in all MDM groups, but it significantly increased numbers only in healthy, not COPD, AMs (Figures 1G and 1H).

The number of viable intracellular *S. pneumoniae* is influenced by both phagocytosis and the rate of early intracellular killing (13). To establish that lower intracellular viable bacteria in COPD AMs were not due to alterations in bactericidal activity, we measured the kinetics of intracellular killing. Opsonization did not alter the rate of bacterial killing in any macrophages (Figures E2A–E2D). Opsonization appropriately increased both the percentage of healthy AM-binding bacteria and the number of internalized bacteria binding per macrophage, but it did not enhance uptake in COPD (Figure E3). Binding of nonopsonized or opsonized *S. pneumoniae* was not altered by COPD or exacerbation frequency. Surface expression of the Fcγ receptors CD16, CD32, and CD64 was similar in AMs/MDMs of patients with COPD and control subjects (Figures E4A and E4B). Studies have demonstrated a central role for the exchange protein activated by cAMP-1 (Epac-1) in the

Table 1. Demographics of Macrophage Donors

	Healthy Nonsmokers	Healthy Ex-smokers	Subjects with COPD
No. of subjects	12	6	42
Age, yr	56 (43–65)	58 (48–69)	66 (53–77)
Sex, female/male	6/6	2/4	7/35
FEV ₁ , L	3.19 (2.25–4.77)	2.99 (2.50–3.70)	1.88 (1.00–2.72)
FEV ₁ , % predicted	110 (74–127)	108 (84–121)	50.8 (32–67)
FVC, L	3.76 (2.25–5.6)	4.19 (3.45–5.2)	3.49 (1.86–5.24)
GOLD stage*	N/A	N/A	9 GOLD A 14 GOLD B 4 GOLD C 10 GOLD D
Nonfrequent/frequent	N/A	N/A	NF, <i>n</i> = 26 (0 exacerbations, <i>n</i> = 19; 1 exacerbation, <i>n</i> = 7) F, <i>n</i> = 16 (2 exacerbations, <i>n</i> = 3; 3 exacerbations, <i>n</i> = 7; >3 exacerbations, <i>n</i> = 6)
Pack-years	N/A	18 (10–35)	50 (32–67)
Smoking status: current/ex/never	0/0/12	0/6/0	7/35/0
Inhaled corticosteroid use	0	0	35
Vaccine history	N/A	N/A	24 yes, 5 no, 13 N/A
SGRQ total score	N/A	N/A	39.8 (6–83)
CAT	N/A	N/A	16.3 (4–33)
6-minute-walk distance, m	N/A	N/A	400 (264–496)

Definition of abbreviations: CAT = COPD Assessment Test; COPD = chronic obstructive pulmonary disease; F = frequent; GOLD = Global Initiative for Chronic Obstructive Lung Disease; MRC = Medical Research Council; N/A = not applicable; NF = nonfrequent; SGRQ = St. George's Respiratory Questionnaire. *Five donors did not have CAT or MRC data and therefore could not be classified A–D.

Figure 1. Chronic obstructive pulmonary disease (COPD) alveolar macrophages (AMs) show deficient opsonic bacterial phagocytosis that correlates with exacerbation frequency. (A–D) AMs (A and B) or monocyte-derived macrophages (MDMs) (C and D) from healthy or COPD (nonfrequent [NF] and frequent [F] exacerbators) were challenged with either nonopsonized (A and C) or opsonized (B and D) serotype 14 *Streptococcus pneumoniae*. At 4 hours after challenge, viable intracellular bacteria were assessed. *n* values for healthy/COPD-NF/COPD-F, respectively: (A) *n* = 18/27/15; (B) *n* = 10/19/13; (C) *n* = 14/18/12; (D) *n* = 14/15/12. ****P* < 0.001, one-way ANOVA. (E and F) A pairwise comparison of phagocytosis of nonopsonized (E) and opsonized (F) bacteria in MDMs and AMs from matched donors. ns = not significant. *n* values for healthy/COPD-NF/COPD-F, respectively: (E) *n* = 11/12/12; (F) *n* = 10/11/12. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, paired *t* test. (G and H) A pairwise comparison of phagocytosis of nonopsonized and opsonized *S. pneumoniae* in matched AM (G) or MDM (H) donors. *n* values for healthy/COPD-NF/COPD-F, respectively: (G) *n* = 10/20/11; (H) *n* = 7/8/5. **P* < 0.05, paired *t* test.

inhibition of Fc γ receptor-mediated phagocytosis (20). However, AM expression of Epac-1, or of its primary target Rap-1 (21), was unaltered by *S. pneumoniae* challenge or by COPD (Figures E4C and E4D). Similarly, there was no difference in expression of Rac1, a Rho-family GTP-binding protein that regulates lamellipodia formation and membrane ruffling in Fc γ receptor-mediated phagocytosis in AMs (22).

Because COPD AMs demonstrate a specific defect in opsonic phagocytosis, we confirmed if patient BAL fluid samples had significant concentrations of antipneumococcal antibodies. We measured antibodies against the 13 serotypes included in PREVNAR 13 (Pfizer Limited), a licensed protein conjugate vaccine, using a sensitive multiplex immunoassay. Unconcentrated BAL samples had detectable pneumococcal antibodies to 2.9 ± 0.5 serotypes, and 72% of samples had antibodies against at least one serotype, with a range of 0–9 serotypes. Antibodies were most common to serotype 3 (38%), serotype 14 (45%), and serotype 19A (45%) (see Figure E5). More specifically, for the COPD sample, 73% had detectable antibodies to one or more serotypes.

Decreased Opsonic Phagocytosis in COPD Is Associated with Bacterial Colonization and Correlates with Clinical Features

COPD lungs are often colonized with bacteria, most often *H. influenzae* and *S. pneumoniae* (23), and colonization is associated with increased exacerbation frequency (6). Because AMs are essential mediators of pulmonary innate immunity (24), we established whether AM phagocytic defects were associated with bacterial colonization. We found that patients with COPD who were culture positive for pathogenic microorganisms in their sputum had significantly lower degrees of AM phagocytosis for opsonized, but not nonopsonized, *S. pneumoniae* compared with culture-negative patients (Figures 2A and 2B). In contrast, using quantitative PCR to identify pathogenic microorganisms in BAL, we determined that PCR-positive samples were not associated with lesser degrees of opsonic or nonopsonic AM phagocytosis of *S. pneumoniae* (Figures 2C and 2D).

Correlation analysis of nonopsonized and opsonized phagocytosis of *S. pneumoniae* against FEV₁ showed that there was a

Figure 2. Defects in phagocytosis in chronic obstructive pulmonary disease alveolar macrophages are associated with bacterial colonization in the lung. (A–D) Nonopsonic (A and C) and opsonic (B and D) phagocytosis was stratified into groups dependent on whether the donor had negative (–ve) or positive (+ve) sputum culture (A and B, $n = 15$ and $n = 14$, respectively) or –ve or +ve (defined as $>10^4$ copies/ml) quantitative PCR of BAL (C and D, $n = 27$ and $n = 26$, respectively) results indicative of bacterial colonization, $*P < 0.05$, Student's t test. Open circles indicate frequent exacerbations; solid circles indicate nonfrequent exacerbations.

significant relationship between FEV₁ and degrees of opsonic phagocytosis but not of nonopsonic phagocytosis (Figures 3A and 3B). However, because FEV₁ correlates poorly with symptoms in COPD (25), we also looked to see if AM phagocytosis degrees were related to scores derived from health-related quality-of-life instruments: the St. George's Respiratory Questionnaire (SGRQ), the COPD Assessment Test (CAT), or the 6-minute-walk distance. For the SGRQ and CAT scores (although not for the 6-minute-walk distance), there was a significant correlation between impaired opsonic phagocytosis and scores representative of increased symptom severity, but, in contrast, nonopsonic uptake was not correlated with any health-related quality-of-life score,

suggesting that it was less tightly associated with COPD symptoms (Figures 4A–4F).

COPD AMs Have Reduced Transcription of Antioxidant Genes Induced in Response to Opsonized Bacteria

To provide further insights into the mechanisms influencing the selective defect in opsonic phagocytosis in AMs, we next looked at the transcriptional response of AMs to opsonized *S. pneumoniae*. There were significantly fewer differentially expressed genes in the COPD AMs in response to infection than in healthy AMs (Figure 5A). Tables E1 and E2 show the top 10 upregulated and downregulated gene probes in healthy and COPD AMs, respectively. We reviewed the enriched GO

terms and noted fewer terms differentially regulated in COPD and lesser degrees of induction (Figure 5B). We also observed that, within the biological processes differentially regulated, although the GO term relating to the cellular response to stress was prominently enriched in healthy AMs, it comprised significantly fewer components in the COPD AMs (Table E3). Included in this response are a series of genes regulating antioxidant defense, which were prominent in the genes altered in healthy AMs (Figure 5C, Figure E6, and Table E4), but these showed comparatively less differential regulation in COPD. Although these responses are not recognized as a major feature of innate host responses to bacteria, antioxidant responses modulate inflammatory responses. These antioxidant responses are activated by a variety of sources of oxidative stress, including microbicidal responses to bacteria, and baseline reductions in antioxidant responses were previously described in COPD (26).

Activation of Nrf2 Increases Phagocytosis of Nonopsonized and Opsonized *S. pneumoniae* in AMs but Not MDMs

Increased oxidative stress in the COPD lung has been associated with impairment of phagocytosis of nonopsonized unencapsulated bacteria and apoptotic bodies (27, 28). The transcription factor Nrf2 is a key regulator of cytoprotective proteins, including antioxidants (29, 30), and treatment of macrophages with a pharmacological activator of Nrf2, sulforaphane, increases phagocytosis of NTHi and *P. aeruginosa* in COPD AMs (8). Within the differentially expressed genes in AM after pneumococcal challenge, we identified multiple Nrf2-regulated genes in healthy AMs, but these were not differentially regulated in COPD AMs (Figure 5C and Table E5).

Because we identified impairment of an antioxidant transcriptional module, we next tested whether sulforaphane modulated phagocytosis of *S. pneumoniae*. We confirmed that sulforaphane activated heme oxygenase (HO)-1, an Nrf2 target gene, in COPD macrophages (Figures 6A and 6B) and did not induce either apoptosis or necrosis in macrophages (Figure E7). Sulforaphane significantly increased numbers of intracellular bacteria after challenge with nonopsonized *S. pneumoniae*

Figure 3. Opsonic phagocytosis correlates with FEV₁. (A) Nonopsonic and (B) opsonic phagocytosis rates were correlated against patient FEV₁ score. Pearson's correlation coefficients (r) and P values are shown, with correlation deemed significant if $P < 0.05$. Nonopsonic, $n = 36$; opsonic, $n = 32$. ns = not significant.

Figure 4. Opsonic phagocytosis correlates with markers of chronic obstructive pulmonary disease (COPD) severity. (A, C, and E) Nonopsonic and (B, D, and F) opsonic rates of phagocytosis were correlated against patient scores for a variety of markers of COPD severity: (A and B) the St. George's Respiratory Questionnaire (SGRQ) (nonopsonic, $n = 29$; opsonic, $n = 27$), (C and D) the COPD Assessment Test (CAT) (nonopsonic, $n = 34$; opsonic, $n = 30$), or (E and F) the 6-minute-walk distance (6MW) (nonopsonic, $n = 14$; opsonic, $n = 10$). Values for Pearson's (r) correlation coefficient and P values are shown, with correlation deemed significant if $P < 0.05$. ns = not significant.

in both healthy and COPD AMs (Figure 6C), but only in COPD (not healthy) AMs after challenge with opsonized *S. pneumoniae* (Figure 6D). In contrast, we failed to demonstrate an uplift in MDM ingestion under any of the conditions studied (Figures 6E and 6F). To determine if this pattern occurred with other bacteria, we confirmed that sulforaphane also increased intracellular numbers of NTHi in COPD AMs but not in

healthy AMs/MDMs or COPD MDMs (Figures 6G and 6H). We also confirmed that sulforaphane did not alter the rate of early intracellular killing of *S. pneumoniae* in COPD AMs (Figure 6I). Moreover, sulforaphane did not significantly induce Fcγ receptor expression (CD16, CD32, or CD64) in either AMs or MDMs (Figure E8). To determine if the uplift in phagocytosis was sulforaphane specific, cells were also treated with a more specific

Nrf2 agonist, Compound 7. This is a recently described potent and selective inhibitor of the KEAP1–Nrf2 protein–protein interaction (16). Treatment with Compound 7 also induced expression of HO-1 in COPD MDMs in a concentration-dependent manner (Figure 7A) and, importantly, GCLC (glutamate-cysteine ligase catalytic subunit) and NQO1 (NADPH:quinone oxidoreductase 1), two of the Nrf2-regulated targets upregulated by infection, in AMs (Figure 7B). Compound 7 significantly increased phagocytosis of opsonized and nonopsonized *S. pneumoniae* by COPD (but not healthy) AMs and MDMs (Figures 7C–7F). As with sulforaphane, Compound 7 treatment did not induce cytotoxic effects (Figure E7B). To complete the data set with Compound 7 (and for healthy donors shown in Figure 6G), we added some additional patients recruited outside COPDMap whose demographics are shown in Table E6. These findings illustrate the potential to reverse opsonic phagocytic defects with Nrf2 agonists.

Discussion

We have demonstrated that COPD macrophages have reduced phagocytosis of bacteria. Although we observed defects in MDM phagocytic function, failure to induce phagocytic uplift by opsonization was unique to COPD AMs and was the specific defect that was most predictive of clinical phenotype. COPD AMs exposed to opsonized bacteria had decreased transcriptional responses involving antioxidant defenses. Importantly, AM defects in bacterial uptake were reversed with Nrf2 agonists.

Several prior publications have demonstrated that COPD is associated with impaired macrophage phagocytosis of bacteria and apoptotic cells (8–10, 31). These studies suggest that there is both a local AM defect and a systemic defect in macrophage function, which may arise from a combination of genetic, epigenetic, and environmental factors. Our study extends prior understanding by showing an additional select defect in AM function that inhibits phagocytosis of opsonized bacteria.

Both complement and immunoglobulin are present in alveolar lining fluid (14). Pneumococcus-specific IgG is detected in human BAL (32) and is required for optimal phagocytosis of *S. pneumoniae* by

Figure 5. Transcriptional response of alveolar macrophages (AMs) reveals less differential gene expression in chronic obstructive pulmonary disease (COPD) in response to infection. AMs from healthy subjects or patients with COPD were challenged with opsonized serotype 14 *Streptococcus pneumoniae* ($n = 3$ in each group). At 4 hours after challenge, cell total RNA was collected for transcriptional analysis. (A) Venn diagram showing the number of probes differentially expressed in response to infection (moderated t test, <0.05 ; false discovery rate, <0.05). (B) Plots represent the top 10 enriched Gene Ontology (GO) biological process terms and the cellular response to stress terms; in addition, the response to oxidative stress term is plotted in the healthy AMs. The x-axis represents enrichment by a hypergeometric test $[-\log_{10}(P \text{ value})]$. The size of the circle and color represent the

AMs (13). Our study also confirms the presence of antipneumococcal antibodies in unconcentrated BAL in a COPD population in which available data showed greater than 80% vaccination uptake and in an age-matched population, some of whom would have had vaccine on the basis of their age. Therefore, a defect in opsonic uptake could reduce the efficacy of vaccination despite the presence of pneumococcal antibodies in the airway. In a murine model, cigarette smoke reduced complement-mediated *S. pneumoniae* uptake but not phagocytosis of IgG-coated beads (33). Impaired phagocytosis of opsonized *S. pneumoniae* and other encapsulated bacteria is likely to contribute to COPD pathogenesis. *S. pneumoniae* remains a leading cause of exacerbations in COPD (5), and in one study, monoculture of *S. pneumoniae* proved a specific risk factor for exacerbation (17). *S. pneumoniae* also have indirect effects on exacerbations because they promote growth, biofilm formation, and synergy in inflammatory responses with other bacteria causing exacerbations (34–36). In addition, *S. pneumoniae* is the major cause of CAP in these patients (37), and COPD increases the susceptibility and risk of complications with CAP (7).

Recent observations involving polymeric immunoglobulin receptor-deficient mice illustrate how bacterial persistence drives inflammation and small airway remodeling in a model of COPD (38). Bacterial colonization of the airways is linked to decline in lung function (6, 39), and recently, bacterial phagocytosis has been shown to correlate with FEV₁ in both COPD (40) and severe asthma (41). Reduced phagocytosis of opsonized bacteria by AMs was observed in patients who were culture positive, although PCR positivity in BAL was not associated with the degree of phagocytosis. It would be of interest in the future to determine if opsonic phagocytosis correlates with quantitation of PCR, but our numbers did not allow this analysis. We used a threshold of greater than 10⁴ copies per milliliter to define positivity. On one hand, although this threshold ensures

sensitivity and a high negative predictive value in studies on the detection of lower respiratory tract infection due to organisms such as *S. pneumoniae* and *H. influenzae* (42, 43), it may underestimate colonization. On the other hand, the detection of colonization by PCR with lower PCR thresholds is problematic, and diagnostic accuracy may be influenced by increasing numbers of false-positive results. Therefore, the sputum detection may have been more predictive of colonization status. The defect in AM phagocytosis of opsonized bacteria was more severe in patients with COPD with frequent exacerbations, a factor associated with more rapid decline in FEV₁ (44). This could explain the correlations we observed with more significant impairment of opsonic phagocytosis observed in patients with lower FEV₁ or more severe symptoms with quality-of-life assessments. Assessment scales are widely used to describe COPD patient cohorts and stratify them for interventions, such as pulmonary rehabilitation (45), and to predict survival (46). Quality-of-life scales are complementary to FEV₁ in describing disability (e.g., MRC dyspnea scale) or severity of dyspnea symptoms (e.g., CAT) in patients living with COPD, and it was noteworthy that the SGRQ and CAT scores correlated with the defect for phagocytosis of opsonized bacteria. FEV₁ provides a measure of COPD stage, but it correlates poorly with symptoms (25). This implies that the phagocytic defect may be related both to stage and to symptoms.

Future studies will need to identify if the phagocytic defect for opsonized bacteria is related to a specific receptor pathway or cytoskeletal rearrangement. A prior study identified a defect in macrophage receptor with collagenous structure (MARCO)-mediated phagocytosis in COPD (8). This important study identified impaired phagocytosis of two nonopsonized bacteria (NTHi and *P. aeruginosa*) in COPD AMs and mice exposed to cigarette smoke and showed that sulforaphane corrected the defect via an Nrf2-dependent mechanism through enhanced MARCO expression.

Our study in a population with very few current smokers is consistent with these findings, confirming a defect in phagocytosis of nonopsonized bacteria (*S. pneumoniae* and NTHi), which is improved by Nrf2 agonists. We extend these findings by showing an additional defect for opsonized *S. pneumoniae*. In contrast to the study by Harvey and colleagues (8), our study highlights transcriptional changes associated with infection in healthy and COPD AMs rather than the transcriptional effects of sulforaphane, but it also highlights reductions in Nrf2-mediated responses in COPD AMs. The range of particles, including both opsonized and nonopsonized bacteria and apoptotic bodies, for which defects have been identified in COPD argues against involvement of any single receptor system underlying all these defects. Although MARCO likely contributes to defects in uptake of nonopsonized bacteria (8), it would not be anticipated to explain the impairment of opsonized bacteria by AMs. An unbiased approach is more likely to identify mechanisms underpinning the broad systemic defect in phagocytosis and the more localized pulmonary defect for opsonized bacteria.

The transcriptional responses seen in the healthy AMs in response to *S. pneumoniae* included prominent transcriptional responses involving immunometabolism. The acute responses to bacteria result in a shift to increased glucose uptake and glycolytic metabolism (47), whereas glucose diversion via the pentose phosphate pathway is a well-recognized mechanism of oxidative stress resistance (48). Among differentially expressed metabolic genes increased in healthy but not COPD AMs was sirtuin (silent mating type information regulation 2 homologs)-1, a deacetylase involved in host responses to *Mycobacterium tuberculosis* (49). Antioxidant responses were prominently upregulated in healthy AMs after bacterial infection. Nrf2-regulated genes included GCLC, GSTZ-1

Figure 5. (Continued). number of differentially expressed genes of interest in that term. Figures were generated using NIPA (available from: <https://github.com/ADAC-UoN/NIPA>). (C) Volcano plots represent the probe sets identified from the transcriptomic analysis. In the left plot, which displays findings for healthy AMs, the red triangles are the differentially expressed probes related to the “Cellular response to stress” with some representative terms named. In blue are the terms associated with the Nrf2 (nuclear factor erythroid 2–related factor 2) pathway in the analysis of healthy AMs. In the right plot, which displays COPD AMs, the red triangles are the differentially expressed probes related to the “Cellular response to stress” GO term. In blue are the terms associated with the Nrf2 pathway seen in the healthy AM analysis. GOI = gene of interest.

Figure 6. Treatment with the Nrf2 (nuclear factor erythroid 2-related factor 2) agonist sulforaphane increases nonopsonic and opsonic phagocytosis in chronic obstructive pulmonary disease (COPD) alveolar macrophages (AMs) but not in monocyte-derived macrophages (MDMs). (A) AMs and (B) MDMs were pretreated with the designated dose of sulforaphane (Sulf) for 16 hours before cells were lysed and probed for expression of heme oxygenase (HO)-1 and actin ($n=3$). (C–F) AMs (C and D) or MDMs (E and F) from healthy (H) or COPD nonfrequent (NF) or frequent (F) exacerbators were pretreated with vehicle (Sulf–) or sulforaphane (Sulf+) for 16 hours before cells were challenged with nonopsonized (C and E) or opsonized (D and F) serotype 14 *Streptococcus pneumoniae*. Four hours after challenge, numbers of intracellular viable bacteria were measured, and n values for H/COPD-NF/COPD-F, respectively, were as follows: (C) $n=11/19/14$; (D) $n=8/10/4$; (E) $n=9/9/5$; (F) $n=8/9/5$. * $P < 0.05$, paired t test. (G and H) AMs (G) and MDMs (H) from

Figure 7. The Nrf2 (nuclear factor erythroid 2-related factor 2) agonist Compound 7 also increases phagocytosis in chronic obstructive pulmonary disease (COPD) alveolar macrophages (AMs). (A and B) COPD monocyte-derived macrophages (MDMs) (A) or AMs (B) were pretreated with the Nrf2 agonist Compound 7 for 16 hours at the designated dose before cells were lysed and probed for the expression of heme oxygenase (HO)-1, GCLC (glutamate-cysteine ligase catalytic subunit), or NQO1 (NADPH:quinone oxidoreductase 1) by Western blotting. (C and D) Healthy donor and COPD AMs were pretreated with Compound 7 at $5 \times IC_{50}$ (0.065 μ M) for 16 hours before being challenged with nonopsonized ($n = 3$ healthy; $n = 5$ COPD) (C) or opsonized ($n = 3$ healthy; $n = 10$ COPD) (D) serotype 14 *Streptococcus pneumoniae* for 4 hours, after which numbers of intracellular viable bacteria were assessed. ** $P < 0.01$, paired t test. (E and F) Healthy donor and COPD MDMs were pretreated with Compound 7 and challenged with nonopsonized (E) or opsonized (F) *S. pneumoniae* as was done for AMs. All $n = 4$. ** $P < 0.01$, *** $P < 0.001$, paired t test. IC_{50} = half-maximal inhibitory concentration.

(glutathione-S-transferase zeta 1), glutathione peroxidase 7 (GPX7), and the SLC7A11 gene product xCT (light chain subunit of the Xc^- glutamine/cysteine antiporter required, all involved in glutathione maintenance and use; CBR1 (carbonyl reductase 1), NQO1, and TXN2

(thioredoxin 2) detoxifying oxidoreductase enzymes; and SOD1 (superoxide dismutase 1) (48). Foxo-regulated targets, including SOD2, were upregulated, whereas p53 was also upregulated. Of all these antioxidant responses, only p53 was significantly upregulated in COPD AMs after bacterial

challenge. We identified upregulation of a series of genes involved in regulation of ubiquitination (including ubiquitin-conjugating E2 enzymes B, D3, and N), a process controlling signaling via pattern recognition receptors, in healthy AMs after bacterial challenge (50). Collectively, these antioxidant responses have the potential to alter cytokine-induced activation of specific phagocytic pathways, expression of receptors or molecules involved in signaling cascades associated with receptors, or the susceptibility of the cytoskeleton to rearrangements altered by oxidative stress required for particular phagocytic pathways. It was noteworthy that the transcriptional response in healthy AMs involved downregulation of the class B scavenger receptor CD36, a receptor for unopsonized particles (51), which was not observed in COPD AMs.

The Nrf2 transcription factor regulates a cluster of antioxidant, cytoprotective, and detoxifying genes and influences susceptibility to COPD in murine models involving cigarette smoke exposure by modifying inflammation and tissue injury (52). We confirmed prior observations suggesting that Nrf2 agonists correct the phagocytic defect in COPD (8), but we extend these by showing that they also modulate phagocytosis of opsonized bacteria. Because this also influenced uptake of nonopsonized particles, it is likely that Nrf2 agonists have pleiotropic effects in the modulation of phagocytosis. Nrf2 agonists represent a promising class of agents with which to modulate oxidative stress in conditions such as COPD, particularly with the development of highly selective agents that bind to the Kelch domain of KEAP1 and prevent Nrf2 ubiquitination and proteasomal degradation (16). Whereas sulforaphane activates Nrf2 by targeting cysteine residues in the BTB domain of KEAP1 and can potentially interact with other targets (53), we demonstrate significant enhancement of phagocytosis in COPD macrophages with the selective Nrf2 agonist Compound 7, suggesting that this could represent a

Figure 6. (Continued). patients with COPD or healthy (H) donors were pretreated with sulforaphane before being challenged with nontypeable *Haemophilus influenzae* (NTHi). Four hours after challenge, the numbers of intracellular viable bacteria were measured, and n values for H/COPD, respectively, were as follows: (G) $n = 3/4$; (H) $n = 3/3$. * $P < 0.05$, paired t test. (I) COPD AMs were pretreated with sulforaphane (+Sulf) before being challenged with nonopsonized serotype 14 *S. pneumoniae* for 4 hours before extracellular bacteria were killed by the addition of antimicrobials. At the designated time after addition of antimicrobials, viable bacteria in duplicate wells were measured ($n = 3$), with no significant difference between vehicle and sulforaphane. ns = not significant.

potent pharmacological approach with which to correct the COPD-associated defects in phagocytosis.

In conclusion, we have identified that, although COPD induces a systemic defect in a range of forms of phagocytosis, a specific defect in phagocytosis of opsonized bacteria is observed specifically in AMs and correlates closely with clinical phenotype in COPD. Moreover, this defect is amenable

to therapeutic targeting with novel and selective inhibitors of the KEAP1–Nrf2 protein–protein interaction. ■

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