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Opsonic phagocytosis in chronic obstructive pulmonary disease is enhanced by Nrf2 agonists.

Martin A. Bewley[1], Richard C Budd[1,2], Eilise Ryan[3], Joby Cole[1,2], Paul Collini[1,2], J Marshall[3], Umme Kolsum[4], Gussie Beech[4], Richard D. Emes[5], Irina Tcherniaeva[6], Guy A. M. Berbers[6], Sarah R. Walmsley[3], Gavin Donaldson[7], Jadwiga A. Wedzicha[7,] Iain Kilty[8], William Rumsey[9], Yolanda Sanchez[9], Christopher E. Brightling[10], Louise E. Donnelly[7], Peter J. Barnes[7], Dave Singh[3], Moira K.B. Whyte[3] and David H. Dockrell[11]* on behalf of COPDMAP.

[1] Department of Infection Immunity and Cardiovascular Disease and The Florey Institute for Host-Pathogen Interactions, University of Sheffield Medical School, UK, [2]Sheffield Teaching Hospitals, [3]Department of Respiratory Medicine and MRC Centre for Inflammation Research, University of Edinburgh, [4]Medicine Evaluation Unit, University of Manchester and University Hospital of South Manchester, [5] School of Veterinary Medicine and Science and Advanced Data Analysis Centre, University of Nottingham, UK, [6] Center for Infectious Disease Control (CIb),National Institute for Public Health and the Environment (RIVM), the Netherlands, [7]National Heart and Lung Institute, University of London Imperial College, UK, [8] Pfizer Inc, Cambridge, Massachusetts,[9] Stress and Repair Discovery Performance Unit, Respiratory Therapy Area, GSK, King of Prussia, USA [10]Institute for Lung Health, University of Leicester, UK , [11]Department of Infection Medicine and MRC Centre for Inflammation Research, University of Edinburgh. **Corresponding Author:** David H. Dockrell, The MRC/University of Edinburgh Centre for Inflammation Research, 47 Little France Crescent, Edinburgh, Edinburgh EH16 4TJ UK Phone: +44 (0) 131 242 658 Fax: +44 (0) 131 242 6578 email: david.dockrell@ed.ac.uk.

Author contributions: MAB, ER performed killing assays, flow cytometry, microscopy and collected data. MAB and JC produced figures. RCB, ER, UK, PC and DS co-ordinated and performed bronchoscopies to obtain patient samples. RCB, UK and GB performed clinical phenotyping. JC, ER and RE performed transcriptomic analysis. IT and GB provided multiplex immunoassays on BAL, WR and YS provided compounds and input into experimental design. GD, JAW, SRW, IK, LED, PJB, DS and CEB co-ordinated collection of the COPD patient cohort and controls, shared expertise in assays and provided reagents. MAB, MKBW and DHD designed and conceived the experiments. MAB, MKW and DHD wrote the manuscript with input from all other authors.

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At a glance summary:

Scientific Knowledge on the Subject: COPD macrophages have defective phagocytosis but the mechanism and clinical relevance remain unknown.

What This Study Adds to the Field: COPD alveolar macrophages (AM) have a specific defect in opsonic phagocytosis which correlates with clinical phenotype. COPD AM fail to engage an anti-oxidant transcriptional module following exposure to opsonized bacteria. Agonists of a key transcriptional regulator of anti-oxidant host defense, Nrf2, reverse the opsonic phagocytosis defect in COPD and offer a potential therapeutic approach to correct the defect.

Footnote:

Presented in part as "COPD alveolar macrophages have a defect in opsonic phagocytosis of serotype 14 *Streptococcus pneumoniae*". M.Bewley, R.Budd, D.Singh, P.J. Barnes, L.E. Donnelly, D.H.Dockrell and M.K.Whyte. American Thoracic Society International Conference San Diego May 18th, 2014. Am J. Respir Crit Care Med 189:2014; A1011

This article has an online data supplement, which is accessible from this issue's table of content online at **www.atsjournals.org**

Abstract

Rationale: Previous studies have identified defects in bacterial phagocytosis by alveolar macrophages (AM) 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 alveolar macrophages (AM) and monocyte-derived macrophages (MDM) from a cohort of COPD patients and controls within the MRC COPD-MAP consortium and measured phagocytosis of bacteria in relation to opsonic conditions and clinical features.

Measurements and Main Results: COPD AM and MDM have impaired phagocytosis of *S. pneumoniae*. COPD AM have a selective defect in uptake of opsonized bacteria, despite the presence of anti-pneumococcal antibodies in bronchoalveolar lavage, not observed in MDM or healthy donor's AM. 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 AM was not reduced. COPD AM have reduced transcriptional responses to opsonized bacteria, including cellular stress responses that include transcriptional modules involving antioxidant defenses and Nrf2-regualted genes. Agonists of the cytoprotective transcription factor Nrf2 (sulforaphane and Compound 7) reverse defects in phagocytosis of *S. pneumoniae* and non-type able *Haemophilus influenzae* by COPD AM.

Conclusions: Patients with COPD have clinically relevant defects in opsonic

phagocytosis by AM, associated with impaired transcriptional responses to cellular stress, which are reversed by therapeutic targeting with Nrf2 agonists.

Introduction.

Chronic Obstructive Pulmonary Disease (COPD) is a chronic inflammatory lung condition characterised 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 S*treptococcus pneumoniae* in a 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 COPD leads to an innate immune defect against *S. pneumoniae* and other bacteria.

Alveolar macrophages (AM) are the resident phagocytes enabling bacterial clearance from the lung, but COPD AM demonstrate reduced phagocytosis of *Haemophilus influenzae* and *P. aeruginosa* (8, 9), while COPD monocyte-derived macrophages (MDM) show impaired phagocytosis of *S. pneumoniae* (10). Bacterial phagocytosis by macrophages involves both non-opsonic and opsonic pathways (11, 12). Previous studies of COPD macrophages have examined non-opsonic 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 micro-organisms, 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. COPD Opsonization fails to enhance AM phagocytosis, although it enhances MDM phagocytosis. The level 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 AM. Agonists of the antioxidant transcription factor, nuclear factor (erythroid-derived2) like 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 have been previously reported in the form of an abstract (15).

Methods

Macrophage donors: COPD patients, free from exacerbation, were recruited from the UK Medical Research Council (MRC) COPD-MAP consortium with written approved consent, as outlined online.

Cells and Infection: AM were isolated from broncho-alveolar lavage (BAL) as previously described (13) Cells were >95% AM as assessed by Diff-Quick staining (Dade Behring) visualised by light microscopy (Leica DMRB 1000). Human MDM were differentiated for 14 d from peripheral blood mononuclear cells isolated from donors with written informed consent by Percoll (Sigma) gradient. Cells were cultured in RPMI (Lonza) supplemented with 10% FCS with low LPS (Lonza). Some cells were incubated with 10 μM of sulforaphane, 0.065 μM Compound 7, a selective inhibitor of the Kelch-like ECH-associated protein 1 (KEAP1) /Nrf-2 interaction (16), or vehicle control for 16 h before challenge with bacteria.

Bacteria: Serotype 14 *S. pneumoniae* (NCTC11902) represents a serotype commonly causing infection in COPD (17). Stocks were grown as previously described (18). Non-typeable *H. influenzae (*NCTC 1269) was cultured as outlined in the online supplement. Macrophages were infected at a multiplicity of infection (MOI) of 10:1. *S. pneumoniae* were opsonized for 15 min. with immune serum obtained from volunteers vaccinated with pneumococcal polysaccharide vaccine, and with detectable antibody levels against *S. pneumoniae*, prior to macrophage challenge (13). Viable intracellular bacteria were measured at 4 h post-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 h before GPA, while additional wells were placed in media containing 0.75 µg/ml vancomycin before GPA at the designated time points.

Bacterial binding: Bacterial binding and internalisation 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 online.

Transcriptomic analysis: RNA was extracted and hybridized onto the Affymetrix HG-U133 plus 2.0 Array. Data were analysed in R using affyPML and Limma. Enrichment analysis of Gene Ontology (GO) terms using a Hypergeometric model using GOstats package in R was performed for differentially expressed genes. False discovery rates (FDR) were corrected with the Benjamini-Hochberg procedure. More detailed information is included in the online supplement.

Western blot: Whole cell extracts were isolated using SDS-lysis buffer and separated by SDS gel electrophoresis, as described in the online supplement.

Statistics: Results are recorded as mean and SEM. Sample sizes were informed by standard errors obtained from similar assays in prior publications (13, 18)Decisions on use of parametric or non-parametric tests were based upon results of D'Agostino-Pearson normality tests. Comparisons were made by paired student t-test and

correlations determined by Spearman's test using Prism 6.0 software (GraphPad 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 COPD patients had a significantly greater number of pack years of cigarette exposure. Sixteen of 42 COPD patients (38.1%) had a history of frequent exacerbations (≥2/year). Vaccine history was available in 69% and of these 83% of COPD patients had received a pneumococcal vaccine.

COPD AM have selective defects in phagocytosis of opsonized S. pneumoniae.

Both COPD AM and MDM demonstrated reduced intracellular numbers of *S. pneumoniae* compared to healthy controls, irrespective of opsonic conditions (Figure 1A-D). COPD AM (but not MDM) from frequent exacerbators had reduced intracellular bacteria, irrespective of opsonic conditions (Figure 1A-D). Frequent exacerbation was set at ≥2 exacerbations/yr. and as shown in Figure E1 patients with only one exacerbation did not have a reduction in bacteria uptake, while those with ≥2 did. Paired analysis of intracellular bacteria numbers, comparing MDM with AM from the same donor, showed that intracellular *S. pneumoniae* were lower in AM than MDM in COPD (but not healthy groups,) regardless of opsonic condition or exacerbation frequency (Figure 1E-F). Opsonization significantly increased numbers of intracellular bacteria in all MDM groups, but significantly increased numbers only in healthy, not COPD, AM (Figure 1G-H).

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 AM 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 (Figure E2A-D). Opsonization appropriately increased both the percentage of healthy AM binding bacteria, and also the number of internalized bacteria binding per macrophage but did not enhance uptake in COPD (Figure E3). Binding of non-opsonized or opsonized *S. pneumoniae* was not altered by COPD or exacerbation frequency. Surface expression of Fcγ receptors, CD16, CD32 and CD64 were similar in AM/MDM of COPD patients and controls (Figure E4A-B). Studies have demonstrated a central role for the Exchange protein activated by cAMP 1 (Epac-1) in the 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 (Figure E4C-D). Similarly, there was no difference in expression of Rac1, a Rho-family GTP-binding protein that regulates lamellipodia formation and membrane ruffling in Fc receptor-mediated phagocytosis in AM (22).

Since COPD AM demonstrate a specific defect in opsonic phagocytosis we confirmed if patient bronchoalveolar lavage fluid (BAL) samples had significant levels of anti-pneumococcal antibodies. We measured antibodies against the 13 serotypes included in Prevnar-13, 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 antibody against at least one serotype, with a range of 0-9 serotypes. Antibodies were most common to serotypes 3 (38%), serotype 14 (45%) and serotype 19A (45%), see Figure E5. More specifically for the COPD sample 73% had detectable antibodies to 1 or more serotypes.

Decreased opsonic phagocytosis in COPD is associated with bacterial colonisation 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). Since AM are essential mediators of pulmonary innate immunity (24), we established whether AM phagocytic defects were associated with bacterial colonization. We found that COPD patients who were culture-positive for pathogenic micro-organisms (PPMs) in their sputum had significantly lower levels of AM phagocytosis for opsonized, but not non-opsonized *S. pneumoniae*, when compared to culture-negative patients (Figure 2A-B). In contrast, using qPCR to identify pathogenic micro-organisms in BAL, we determined that PCR-positive samples were not associated with lower levels of opsonic or non-opsonic AM phagocytosis of *S. pneumoniae* (Figure 2C-D).

Correlation analysis of non-opsonized and opsonized phagocytosis of *S. pneumoniae* against FEV_1 showed that there was a significant relationship between FEV_1 and levels of opsonic phagocytosis, but not non-opsonic phagocytosis (Figure 3A-B). However, since FEV_1 correlates poorly with symptoms in COPD (25), we also looked to see if AM phagocytosis levels were related to scores from health-related quality of life (HR-QoL) instruments, the St George's Respiratory Questionnaire (SGRQ), COPD Assessment Test (CAT) or with the 6-minute walking distance (6MW). For the SGRQ and CAT score (although not for the 6MW), there was a significant correlation between impaired opsonic phagocytosis and scores representative of increased symptom severity but, in contrast, non-opsonic uptake was not correlated with any

HR-QoL score, suggesting it was less tightly associated with COPD symptoms (Figure 4A-F).

COPD AM 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 AM we next looked at the transcriptional response of AM to opsonized *S. pneumoniae*. There are significantly fewer differentially expressed genes in the COPD AM in response to infection than in healthy AM (Figure 5A). Table E1-2 shows the top ten upregulated and downregulated gene probes in healthy and COPD AM respectively. We reviewed the enriched GO terms and noted fewer terms differentially regulated in COPD and lower levels 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 AM, it comprised significantly fewer components in the COPD AM (supplemental Table E3). Included in this response are a series of genes regulating antioxidant defense, which were prominent in the genes altered in healthy AM (Figure 5C, supplemental Figure E6 and supplemental Table E4), but these showed comparatively less differential regulation in 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 are previously described in COPD (26).

Activation of Nrf2 increases phagocytosis of non-opsonized and opsonized S. pneumoniae in AM but not MDM.

Increased oxidative stress in the COPD lung has been associated with impairment of phagocytosis of non-opsonized 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 non-type able *H. influenzae* (NTHi) and *Pseudomonas aeruginosa* in COPD AM (8). Within the differentially expressed genes in AM following pneumococcal challenge, we identified multiple Nrf2 regulated genes in healthy AM, but these were not differentially regulated in COPD AM (Figure 5C and supplemental Table E5).

Since we identified impairment of an antioxidant transcriptional module we next tested whether sulforaphane modulated phagocytosis of *S. pneumoniae*. We confirmed sulforaphane activated heme-oxygenase (HO-1), an Nrf-2 target gene, in COPD macrophages (Figure 6A-B) and did not induce either apoptosis or necrosis in macrophages (Figure E7). Sulforaphane significantly increased numbers of intracellular bacteria after challenge with non-opsonized *S. pneumoniae* in both healthy and COPD AM (Figure 6C), but after challenge with opsonized *S. pneumoniae* only in COPD (not healthy) AM (Figure 6D). In contrast, we failed to demonstrate an uplift in MDM ingestion under any of the conditions studied (Figure 6E-F). To determine if this pattern occurred with other bacteria, we confirmed sulforaphane also increased intracellular numbers of NTHi in COPD AM but not healthy AM/MDM or COPD MDM (Figure 6G-H). We also confirmed sulforaphane did not alter the rate of early intracellular killing of *S. pneumoniae* in

COPD AM (Figure 6I). Moreover, sulforaphane did not significantly induce expression of Fc-gamma expression (CD16, 32 or 64) in either AM or MDM (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 Kelch-like ECH-associated protein 1 (KEAP1) /Nrf-2 protein-protein interaction (16). Treatment with Compound 7, also induced expression of HO-1 in COPD MDM in a concentration-dependent manner (Figure 7A) and also in AM (Figure 7B). Compound 7 significantly increased phagocytosis of opsonized and non-opsonized *S. pneumoniae* by AM (Figure 7C-D). COPD MDM treated with Compound 7 also showed significant increases in uptake of opsonized bacteria and non-opsonized bacteria, although increased (Figure 7E-F). Compound 7 did not enhance uptake by healthy macrophages in any condition. As with sulforaphane Compound 7 treatment did not induce cytotoxic effects (Figure E7B). 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 AM and was the specific defect that was most predictive of clinical phenotype. COPD AM 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 COPD is associated with impaired macrophage phagocytosis of bacteria and apoptotic cells (8, 9, 10, 31). These studies suggest that there is both a local AM defect but also a systemic defect in macrophage function, which may arise from a combination of genetic, epigenetic and environmental factors. Our study extends our 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). Pneumococcal-specific IgG is detected in human BAL (32) and is required for optimal phagocytosis of *S. pneumoniae* by AM (13). Our study also confirms the presence of anti-pneumococcal antibodies in unconcentrated BAL, in a COPD population, in which available data showed >80% vaccination uptake, and an age matched population some of whom would have had vaccine on the basis of 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 since 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 remodelling 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 FEV_1 in both COPD (40) and severe asthma (41). Reduced phagocytosis of opsonized bacteria by AM was observed in patients who were culture-positive, although PCR-positivity in BAL was not associated with the level 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 $>10^4$ copies/ml to define positivity. 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 under estimate colonization. On the other hand, the detection of colonization by PCR with lower level 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 COPD patients with frequent exacerbations, a factor associated with more rapid decline in FEV_1 (44). This could explain the correlations we observed with more significant impairment of opsonic phagocytosis observed in patients with lower FEV_1 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 FEV_1 in describing disability (e.g. MRC dyspnea scale) or severity of dyspnea symptoms (e.g.

COPD assessment test) in patients living with COPD and it was noteworthy that the SGRQ and CAT correlated with the defect for phagocytosis of opsonized bacteria. FEV_1 provides a measure of COPD stage, but correlates poorly with symptoms (25). This implies the phagocytic defect may be related both to stage and symptoms.

Future studies will need to identify if the phagocytic defect for opsonized bacteria is related to a specific receptor pathway or cytoskeletal re-arrangement. 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 non-opsonized bacteria (NTHi and *P. aeruginosa*) in COPD AM and mice exposed to cigarette smoke and showed that sulforaphane corrected the defect in an Nrf2-dependent mechanism via enhanced MARCO expression. Our study in a population with very few current smokers is consistent with these findings confirming a defect in phagocytosis of non-opsonized bacteria (*S. pneumoniae* and NTHi), which is improved by Nrf2 agonists. We extend beyond this showing an additional defect for opsonized *S. pneumoniae.* In contrast to the study by Harvey and colleagues our study highlighted transcriptional changes associated with infection in healthy and COPD AM rather than the transcriptional effects of sulforaphane, but also highlights reductions in Nrf2-mediated responses in COPD AM. The range of particles, including both opsonized and non-opsonized 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 non-opsonized bacteria (8), it would not be anticipated to explain the impairment of opsonized bacteria by AM. 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 AM, in response to *S. pneumoniae* included prominent transcriptional responses involving immunometabolism. The acute responses to bacteria results in a shift to increased glucose uptake and glycolytic metabolism (47), while glucose diversion via the pentose phosphate pathway is a well-recognized mechanism of oxidative stress resistance (48). Amongst differentially expressed metabolic genes increased in healthy but not COPD AM was sirtuin (silent mating type information regulation 2 homologs) 1, a deacetylase involved in host responses to *M. tuberculosis* (49). Anti-oxidant responses were prominently upregulated in healthy AM after bacterial infection. Nrf2 regulated genes included glutamate-cysteine ligase catalytic subunit (GCLC), glutathione-S-transferase zeta 1 (GSTZ-1), glutathione peroxidase 7 (GPX7) and the SLC7A11 gene product, light chain subunit of the Xc^- (xCT) glutamine/cysteine antiporter required, all involved in glutathione maintenance and utilization, carbonyl reductase 1 (CBR1), NADPH:quinone oxidoreductase 1 (NQO1), and thioredoxin 2 (TXN2) detoxifying oxidoreductase enzymes and superoxide dismutase (SOD) 1 (48). Foxo-regulated targets including SOD 2 were upregulated, while p53 was also upregulated. Of all these anti-oxidant responses only p53 was significantly upregulated in COPD AM 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 AM after bacterial challenge (50). Collectively these anti-oxidant 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 re-arrangements altered by oxidative stress required for particular phagocytic pathways. It was noteworthy that the transcriptional response in healthy AM involved downregulation of the class B scavenger receptor CD36, a receptor for unopsonized particles (51) , which was not observed in COPD AM.

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 Nrf2 agonists correct the phagocytic defect in COPD (8), but extend these by showing they also modulate phagocytosis of opsonized bacteria. Since this also influenced uptake of non-opsonized particles it is likely 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 like 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). While 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 this could represent a 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 AM 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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Figure Legends

Figure 1. COPD AM show deficient opsonic bacterial phagocytosis which correlates with exacerbation frequency. (A-D) Alveolar macrophages (AM) (A and B) or monocyte-derived macrophages (MDM) (C and D) from healthy (H) or COPD (non-frequent (NF) and frequent (F) exacerbators) were challenged with either non-opsonized (A and C) or opsonized (B and D) serotype 14 *S. pneumoniae*. 4h post-challenge *viable* intracellular bacteria were assessed. Values for 'n=' for H/COPD-NF/COPD-F; (A) 18/27/15, (B) 10/19/13, (C) 14/18/12, (D) 14/15/12, ***= $p<0.001$, 1-way ANOVA. **(E-F)** A pairwise comparison of phagocytosis of non-opsonized (E) and opsonized (F) bacteria in MDM and AM from matched donors, ns= not significant, values for 'n=' for H/COPD-NF/COPD-F; (E) 11/12/12, (F) 10/11/12, *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, paired t-test. (**G-H)** A pairwise comparison of phagocytosis of non-opsonized and opsonized *S. pneumoniae* in matched AM (G) or MDM (H) donors, values for 'n=' for H/COPD-NF/COPD-F; (G) 10/20/11, (H) 7/8/5, *=$p<0.05$, paired t-test.

Figure 2. Defects in phagocytosis in COPD AM are associated with bacterial colonisation in the lung. (A-D) Non-opsonic (A and C) and opsonic (B and D) phagocytosis was stratified into groups dependent on if the donor had negative (-ve) or positive (+ve) culture of sputum (A and B, n=15 and n=14) or –ve or +ve (defined as $>10^4$ copies/ml) qPCR of broncho-alveolar lavage (C and D, n=27 and n=26) results indicative of bacterial colonisation, *=$p<0.05$, Student's t-test.

Figure 3. Opsonic phagocytosis correlates with FEV_1. Non-opsonic (A) and opsonic (B) phagocytosis rates were correlated against patient FEV_1 score. Pearson's correlation coefficients (r), and p values, with correlation deemed significant if $p<0.05$. n = 36 non-opsonic, 32 opsonic.

Figure 4. Opsonic phagocytosis correlates with markers of COPD disease severity. Non-opsonic (A, C and E) and opsonic (B, D and F) rates of phagocytosis were correlated against patients scores in a variety of markers for COPD disease severity, the St George's Respiratory Questionnaire (SGRQ) (A and B) (n = 29 non-opsonic, 27 opsonic), COPD Assessment Test (CAT) (C and D) (n = 34 non-opsonic, 30 opsonic) or with the 6-minute walking distance (6MW) (E and F) (n = 14 non-opsonic, 10 opsonic). Values for Pearson's (r) or Spearman's correlation coefficients (rho) and p values are shown, with correlation deemed significant if p= <0.05.

Figure 5. Transcriptional response of AM reveals less differential gene expression in COPD in response to infection. Alveolar macrophages (AM) from Healthy or COPD patients were challenged with opsonized serotype 14 *S. pneumoniae* (n=3 in each group). 4h post-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, FDR <0.05). **(B)** Plots represent the top ten enriched GO biological processes terms and the cellular response to stress term (in addition the response to oxidative stress term is plotted in the Healthy AM). The X axis represents enrichment by a hypergeometric test (–log10 (p value)). The size of the circle and colour represents the number of differentially expressed genes in that term. Figures generated using NIPA (available

at https://github.com/ADAC-UoN/NIPA). **(C)** Volcano plots represent the probe sets identified from the transcriptomic analysis. Panel a) Healthy: The red triangles are the differentially expressed probes related to the "Cellular response to stress term" with some representative terms named. In blue are the terms associated with the Nrf-2 pathway in the analysis of healthy AM. Panel b) COPD: The red triangles are the differentially expressed probes related to the "Cellular response to stress" GO term. In blue are the terms associated with NRF2 pathway seen in the Healthy analysis.

Figure 6. Treatment with the Nrf-2 agonist sulforaphane increases non-opsonic and opsonic phagocytosis in COPD AM but not MDM. (A-B) Alveolar macrophages (AM) and monocyte derived macrophages (MDM)(B) were pre-treated with the designated dose of sulforaphane (Sulf) for 16h, before cells were lysed and probed for expression of heme-oxygenase-1 (HO-1) and actin (n=3). **(C-F)** AM (C-D) or MDM (E and F) from healthy (H) or COPD non-frequent (NF) or frequent (F) exacerbators were pre-treated with vehicle (Sulf -) or Sulforaphane (Sulf +) for 16 h, before cells were challenged with non-opsonized (C and E) or opsonized (D and F) serotype 14 *S. pneumoniae*. 4h post-challenge, numbers of intracellular viable bacteria were measured, values for 'n=' for H/COPD-NF/COPD-F; (C) 11/19/14, (D) 8/ 10/4, (E) 9/9/5, (F) 8/9/5, *=$p<0.05$, paired t-test. **(G and H)** AM (G) and MDM (H) from COPD patients or healthy (H) (red lines) donors were pre-treated with sulforaphane before being challenged with non-typeable *H. influenzae* (NTHi). 4h post challenge the numbers of intracellular viable bacteria were measured, values for 'n=' for H/COPD (G)3/4, (H) 2/3, *=$p<0.05$, paired t-test. **(I)** COPD AM were pre-treated with sulforaphane (+Sulf) before being challenged with non-opsonized serotype 14 *S. pneumoniae* for 4h before extracellular bacteria were killed by the addition of

antibiotics. At the designated time post-antibiotics, viable bacteria in duplicate wells were measured to, n=3, no significant difference between vehicle and sulf.

Figure 7. The Nrf-2 agonist compound 7 also increases phagocytosis in COPD AM.

(A and B) COPD monocyte-derived macrophages (MDM) (A), or alveolar macrophages (AM) (B), were pre-treated with the Nrf-2 agonist Compound 7 for 16h at the designated dose, before cells were lysed and a probed for the expression of heme-oxygenase-1 (HO-1) by western blot. **(C and D)** Healthy donor and COPD AM were pre-treated with Compound 7 at 5x IC_{50} (0.065 μM), for 16h before being challenged with opsonized (C), n=3 healthy, n=5 COPD or non-opsonized (D), n=1 healthy, n=10 COPD, serotype 14 *S. pneumoniae* for 4h, after which numbers of intracellular viable bacteria were assessed, **=p<0.01, paired t-test. **(E and F)** Healthy donor and COPD MDM were pre-treated with compound 7 and challenged with opsonized (E), or non-opsonized (F), *S. pneumoniae* as for AM. All n=4, p **=p<0.01, paired t-test.

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3 **Table 1: Demographics of Macrophage Donors**

	Healthy Non-Smoker	Healthy Ex-Smoker	COPD
N	12	6	42
Age (years)	56 (43-65)	58 (48-69)	66(53-77)
Gender	6♀ : 6♂	2♀ : 4♂	7♀ : 35♂
FEV1 Litres	3.19 (2.25-4.77)	2.99 (2.50-3.70)	1.88 (1.00-2.72)
FEV1 %	110 (74-127)	108 (84-121)	50.8 (32-67)
FVC litres	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
Non-Frequent /Frequent**	N/A	N/A	NF 26 (0 Exacerbations = 19, 1 Exacerbation = 7 F 16 (2 Exacerbations =3, 3 Exacerbations =7, >3 Exacerbations =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 Corticosteroids use	0	0	35
Vaccine	N/A	N/A	24 Yes, 5 No, 13N/A
St George's Respiratory Questionnaire (SGRQ) Total score	N/A	N/A	39.8 (6-83)
COPD Assessment Test (CAT)	N/A	N/A	16.3 (4-33)
6 Minute Walk (m)	N/A	N/A	400 (264-496)

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