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Bronchial mucosal inflammation and illness severity in response to experimental rhinovirus infection in COPD

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Capsule Summary
Rhinovirus-induced bronchial mucosal eosinophils, neutrophils, macrophages and lymphocytes in COPD subjects are related to experimental rhinovirus-induced COPD exacerbation severity.
Anti-viral/anti-inflammatory therapies could attenuate bronchial inflammation and ameliorate virus-induced COPD exacerbation severity.

Key Messages

- Experimental rhinovirus infection increases bronchial mucosal eosinophils and neutrophils in COPD subjects only, and macrophages and lymphocytes in both COPD and non-COPD controls.
- Rhinovirus-induced bronchial mucosal inflammation is associated illness severity during virus-induced COPD exacerbations.
- Anti-viral and anti-inflammatory therapies could attenuate bronchial inflammation and ameliorate virus-induced COPD exacerbations.

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Abstract

**Background:** Respiratory viral infection causes chronic obstructive pulmonary disease (COPD) exacerbations. We previously reported increased bronchial mucosa eosinophil and neutrophil inflammation in COPD experiencing naturally occurring exacerbations. But it is unclear whether virus per se induces bronchial mucosal inflammation, nor whether this relates to exacerbation severity.

**Objectives:** To determine the extent and nature of bronchial mucosal inflammation following experimental rhinovirus (RV)-16-induced COPD exacerbations and its relationship to disease severity.

**Methods:** Bronchial mucosal inflammatory cell phenotypes were determined at pre-infection baseline and following experimental RV infection in 17 GOLD stage II COPD subjects and as controls, 20 smokers and 11 non-smokers, with normal lung function. No subject had a history of asthma/allergic rhinitis: all were negative for aero-allergen skin prick tests.

**Results:** RV-infection increased the numbers of bronchial mucosal eosinophils and neutrophils only in COPD and CD8\(^+\) T-lymphocytes in COPD and non-smokers. Monocytes/macrophages, CD4\(^+\) T- and CD20\(^+\) B-lymphocytes were increased in all subjects. At baseline, compared to non-smokers, COPD subjects and smokers had increased numbers of bronchial mucosal monocytes/macrophages and CD8\(^+\) T-lymphocytes but fewer numbers of CD4\(^+\) T- and CD20\(^+\) B-lymphocytes. The virus-induced inflammatory cells in COPD were positively associated with virus load, illness severity and reductions in lung function.

**Conclusion:** Experimental rhinovirus infection induces bronchial mucosal eosinophilia and neutrophilia only in COPD and monocytes/macrophages and lymphocytes in both COPD and control subjects. The virus-induced inflammatory cell phenotypes observed in COPD positively related to virus load, illness severity. Anti-viral/anti-inflammatory therapies could attenuate bronchial inflammation and ameliorate virus-induced COPD exacerbations.

(250 words)

**Keywords:** rhinovirus infection; eosinophils; inflammation; chronic obstructive pulmonary disease exacerbation
Abbreviations used

COPD: Chronic obstructive pulmonary disease
RV: Rhinovirus
PEF: Peak expiratory flow
FEV₁: Forced expiratory volume in 1 second
FVC: Forced vital capacity
BAL: Bronchoalveolar lavage
IL: Interleukin
Sub: Subepithelial
Epi: Epithelial
Exacerbations in chronic obstructive pulmonary disease (COPD) are a major cause of morbidity and mortality.\(^1\) Respiratory viral infections are the major cause of acute exacerbations,\(^2\) with human rhinoviruses (RV) the most common viruses detected.\(^3\) Our own previously reported studies have shown that experimental RV-infection in subjects with COPD induces lower respiratory tract symptoms, airflow obstruction, and systemic and airway inflammation that are greater and more prolonged compared with smoking control subjects without airway obstruction, indicating a causal relationship between RV-infection and COPD exacerbations.\(^4\)

COPD in its stable phase is characterised by airway inflammation that is central to the pathogenesis of the disease\(^5\) with increased numbers of airway mucosal monocytes/macrophages, CD4\(^+\) and CD8\(^+\) T and B lymphocytes and neutrophils that are associated with the severity of airflow limitation.\(^6\) \(^7\)-\(^10\) Neutrophilic inflammation has been a classical hallmark of both stable COPD\(^8,\)\(^11\) and naturally occurring COPD exacerbations.\(^12,\)\(^13\) Eosinophilic inflammation, whilst usually considered a characteristic feature of asthma, is also present in a subset of COPD patients both when stable and during exacerbations.\(^13\)-\(^15\) Increased numbers of mucosal eosinophils have been detected in bronchial biopsies from chronic bronchitis and COPD subjects experiencing naturally occurring exacerbations.\(^16\)-\(^18\) However, the role of eosinophils in COPD exacerbations, particularly in respiratory virus-induced exacerbations remains unclear. It is unknown whether virus infection per se can cause mucosal eosinophilia and neutrophilia during COPD exacerbations. Also there have been a number of confounding factors in some of the aforementioned studies, such as inclusion of mechanically ventilated patients who had received oral corticosteroids prior to sampling,\(^18\) and use of different patient groups for comparison of stable versus exacerbated states.\(^16\)-\(^18\)

We have developed an experimental model of a COPD exacerbation using human RV-16 infection in non-intubated, treatment-naïve COPD patients. As part of two completed studies using this model\(^4,\)\(^19\) bronchial biopsies were collected from COPD patients, non-COPD smokers\(^4,\)\(^19\) and non-smokers.\(^19\) at baseline prior to infection and on day 7 during the acute infection. These samples provide a unique opportunity to explore the bronchial mucosal inflammatory response and its physiological and clinical significance in virus-induced experimental COPD exacerbations, and to investigate whether these responses differ between COPD patients and non-COPD subjects.

We hypothesised that rhinovirus infection alone recruits inflammatory cells into the bronchial
mucosa and that the nature of the inflammatory response and its associated severity of clinical symptoms and airflow obstruction in subjects with COPD is distinct from that seen in non-COPD subjects.
METHODS

Participants

Table 1 shows demographic data at baseline and after infection in this study (i.e. those successfully infected and having adequate bronchial biopsy material for analysis), namely: 17 smokers with GOLD stage II COPD (FEV$_{1}$ 50%-79% predicted normal value, FEV$_{1}$/FVC <70% and β-agonist reversibility <12%), 20 smokers with normal lung function (FEV$_{1}$ ≥80% predicted; FEV$_{1}$/FVC >70%) and 11 healthy non-smokers. The inclusion/exclusion criteria are provided in Table E1 in this article’s Online Repository. All subjects were non-atopic as defined by negative reactions to a six-grass pollen mix on skin prick tests and none had any history of asthma or allergic rhinitis. No subject had symptoms of respiratory tract infection within the previous 8 weeks. COPD patients had no exacerbation and were treatment-naïve in the previous 3 months. No subject used corticosteroids (either inhaled or systemic) or antibiotics to treat the exacerbations after experimental RV infection. The only medication allowed was increased use of short-acting β2 agonists. All subjects gave informed written consent and the study protocols were approved by St Mary's NHS Trust Research Ethics Committee (study numbers 00/BA/459E and 07/H0712/138).

Experimental infection with rhinovirus-16 and confirmation of infection

Ten tissue culture infective doses 50% (TCID$_{50}$) of RV-16 was administered on day 0 by nasal spray as previously described. RV-infection was confirmed by at least one of the following: positive nasal lavage, sputum or BAL standard or qPCR for RV, positive culture of RV-16, or seroconversion defined as a titre of serum neutralizing antibodies to RV-16 >1:4 at 6 weeks as described.

Blood and sputum Inflammatory Markers

Peripheral blood eosinophils were counted at baseline and on day 7 after infection. Sputum was sampled at baseline and on days 3, 5, 9, 12, 15, 21 and 42 during/post-infection. Details of sputum processing are provided in previous publications. Sputum eosinophils in cytopsin were counted and mediators eotaxin, eotaxin-3, interleukin (IL)-4, IL-5, CXCL8/IL-8, IL-1β and TNF were measured using the Mesoscale Discovery® platform. Eosinophilic cationic protein (ECP), pentraxin3, cathelicidin (LL-37) and neutrophil elastase was measured using ELISA kits according to the manufacturer’s instructions.
**Bronchoscopy and clinic data:** Bronchial biopsies were taken ~14 days before infection (baseline), on day 7 during infection (acute infection) in all subjects, and at 42 days after infection (convalescence) in 11 COPD subjects and 12 smoking controls.

**Immunohistochemistry**
Neutrophil elastase$^+$ neutrophils, EG2$^+$ eosinophils, tryptase$^+$ mast cells, CD4$^+$ and CD8$^+$ T- and CD20$^+$ B-lymphocytes and CD68$^+$ monocytes/macrophages were immunostained as previously described.\(^6\)

**Quantification**
In slides coded to avoid observer bias, the areas of epithelium (epi) and subepithelium (sub) were assessed using an Apple Macintosh computer and Image 1.5 software. Inflammatory cells were seen and counted by light microscope. The data for cell counts were expressed as the number of positive cells per mm\(^2\) of the subepithelium and per 0.1 mm\(^2\) epithelium.

**Statistical analysis**
One way ANOVA followed by the unpaired Student's t-test was used for the analyses of age, smoking pack years and lung function data between groups. In respect of cell counts in blood, sputum and biopsies and mediators in sputum, these data were non-normally distributed and overall differences between all groups and between three time points within group were assessed firstly using the Kruskal-Wallis test, which, if significant, was followed by Wilcoxon matched pairs test for within group between baseline and infection. The between group differences were analysed by Mann-Whitney tests. Spearman’s rank correlation was used for correlations between the numbers of inflammatory cells and virus load/physiologic/clinical data/sputum inflammatory markers/blood eosinophils. A \(P\) value of <0.05 was accepted as statistically significant.

Further details of the methods are provided in this article’s Online Repository.

**RESULTS**

**Histology**
Inflammatory cells were present in both the bronchial epi- and sub-compartment. Representative
photographs are depicted in Fig 1, A-M. EG2⁺ eosinophils (Fig 1, A), Elastase⁺ neutrophils (Fig 1, B), CD68⁺ monocytes/macrophages (Fig 1, C), CD4⁺ (Fig 1, D) and CD8⁺ (Fig 1, E) T-lymphocytes and CD20⁺ (Fig 1, F) B-lymphocytes appeared to be more frequent in the bronchial mucosa of COPD on day 7 post-infection compared to their own baselines (Fig 1, G-L). Application of irrelevant antibody for the inflammatory cell markers was negative (Fig 1, M).

**Subepithelial inflammatory cells are increased from baseline to post-infection in COPD**

The most striking increase in absolute cell counts on day 7 post-infection compared to baseline in COPD was a greater than 6-fold increase in numbers of sub-eosinophils \( (P = .0005, \text{Fig 2, A and Table 2}) \). On day 7, the numbers of sub-eosinophils in the COPD subjects was significantly higher compared with non-smokers \( (P = .044) \). In COPD subjects there was a non-significant trend for an increase in sub-neutrophils \( (P = .087, \text{Table 2}) \). The numbers of sub-CD68⁺ cells were significantly increased on day 7 post-infection from baseline in all three groups \( (P = .001-.044, \text{Fig 2, B}) \). Sub-CD8⁺ cells increased significantly on day 7 from baseline in the COPD and non-smoker groups \( (P = .036 \text{ and } .010, \text{respectively, Fig 2, C}) \). Sub-CD8⁺ counts in COPD subjects and smokers were significantly higher compared with non-smokers on day 7 \( (P = .031 \text{ and } .022, \text{respectively, Fig 2, C}) \).

Sub-CD4⁺ and CD20⁺ counts significantly increased on day 7 from baseline in COPD and smoker groups \( (P = .002 - .041, \text{Fig 2, D and E}) \). The elevated numbers of sub-neutrophils and CD8⁺ cells in COPD groups persisted at week 6, remaining at similar median levels to their counts at day 7 (Table 2), whereas sub-eosinophils, CD68⁺, CD4⁺ and CD20⁺ cells had returned to their respective baseline levels (Table 2). Sub-tryptase⁺ mast cell counts were significantly decreased from baseline to day 7 post-infection in the smoker and COPD groups \( (P = .002 \text{ and } .012, \text{respectively, Fig 2, F}) \) and also decreased from baseline to week 6 in COPD \( (P = .049, \text{Table 2}) \).

**Epithelial inflammatory cells are increased from baseline to post-infection in COPD**

Compared to baseline, there was a significant increase in numbers of epi-neutrophils at day 7 post-infection in COPD only \( (P = .032, \text{Fig 3, A and Table 2}) \) and epi-neutrophils remained significantly higher \( (P = .005) \) than baseline level at week 6 post-infection (Table 2). The numbers of epi-CD68⁺ cells in smokers were significantly increased on day 7 from baseline \( (P = .031, \text{Fig 3, B}) \). Also, on day 7, epi-CD68⁺ cell counts in the smokers were significantly higher than the non-smokers \( (P = .016) \). The numbers of epi-CD4⁺ and CD20⁺ cells increased significantly from
baseline to day 7 post-infection in all three groups ($P = .002-.021$, Fig 3, C and D). The numbers of epi-CD8$^+$ cells on day 7 in the smokers and COPD subjects were significantly higher compared with the non-smoker group ($P = .004$ and .017, respectively, Fig 3, E). The elevated numbers of epi-CD8$^+$ cells in the smoker and COPD groups persisted at week 6, remaining at similar levels to their counts at day 7 (Table 2). There were no significant differences in counts of epi-eosinophils and mast cells between baseline and infection in any subject group.

Baseline CD4$^+$ T- and CD20$^+$ B- lymphocytes in smoker and COPD are decreased compared to healthy non-smoker
The baseline numbers of sub-CD68$^+$ and both epi- and sub-CD8$^+$ cells were significantly higher ($P = .002 - .039$, Fig 2, B and C, and Fig 3, E, Table 2), whereas sub-CD4$^+$ and CD20$^+$ cells were significantly lower ($P = .014 - .041$, Fig 2,D and E, Table 2) in smokers and COPD subjects compared to the baseline of non-smokers.

Greater magnitude of increase in eosinophils in COPD post-infection
To investigate differences in inflammatory responses of non-smokers, smokers and COPD subjects to RV-infection, the magnitude of the changes of inflammatory cell counts from baseline to infection were compared between groups. The change in numbers of sub- eosinophils from baseline to day 7 post-infection in COPD was significantly greater than the changes in both the non-smokers and smokers ($P = .002$ and .008, respectively, Fig 2, G) with 16 out of 17 COPD subjects experiencing an increase during exacerbation, with a median increase of 57 eosinophils / mm$^2$ of sub-epithelium in COPD vs 1 in non-smokers and 3 in smokers. In contrast, there were no significant differences between groups in changes from baseline to day 7 for any other phenotype inflammatory cells.

Blood and sputum eosinophils in COPD post-infection
There was no change in blood eosinophil numbers between baseline and after infection in any subject group, however there was a small but statistically significant increase in blood eosinophil percentages in the COPD subjects from baseline to day 7 (2.72% vs. 3.13%, $P = .001$, Fig 4, A) but not in the control subjects. We have previously reported no significant increase in sputum eosinophils when the two studies were analysed separately. $^4,^{19}$ When the two studies were combined herein, again there was no significant increase from baseline in either sputum eosinophil
numbers or percentages on any day after infection in the COPD subjects. There were no correlations between mucosal eosinophils and blood or sputum eosinophils.

**Sputum inflammatory Markers**

We measured chemokines/cytokines relevant to eosinophil biology in sputum in a subset of the subjects with sufficient sputum supernatants remaining. Following infection there were significant increases in eotaxin \( (P = .0002 \text{ and } <.0001, \text{Fig 4, B}) \) and eotaxin-3 \( (P < .00001 - P = .020, \text{Fig 4, C}) \) in the COPD subjects but not in the non-COPD controls (data not shown). There were no significant increases in IL-4, IL-5 or ECP following infection (data not shown). There were no correlations between mucosal eosinophils and any of these sputum markers.

**Associations between mucosal inflammatory cell numbers and virus load/clinical outcomes and smoking pack years**

The numbers of sub-eosinophils in COPD during infection were associated with peak sputum virus load \( (r = 0.61, P = .011, \text{Fig 5, A}) \) and also with COPD exacerbation severity as sub-eosinophils on day 7 were related to peak breathlessness scores \( (r = 0.62, P = .013, \text{Fig 5, B}) \) and to reductions in peak expiratory flow \( (r = -0.62, P = .019, \text{Fig 5, C}) \) during infection. In COPD, sub-neutrophils correlated with BAL virus load on day 7 \( (r = 0.95, P = .007, \text{Fig 5, D}) \) and higher numbers of both epi- and sub-epithelial neutrophils were significantly associated with lower pre-bronchodilator FEV\(_1\)% predicted on day 9 \( (r = -0.57 \text{ and } -0.55, P = .021 \text{ and } 0.20, \text{respectively, Fig 5, E and F}) \). Mucosal CD68\(^+\) monocytes/macrophages and lymphocytes during infection were also related with virus load, clinical symptom severity and reductions in lung function during infection, which are presented in Results and Fig. E1A-D and Fig E2A-F in this article’s Online Repository.

At baseline, the counts of epi-CD68\(^+\) and CD8\(^+\) cells in COPD subjects, and sub-CD8\(^+\) cells and both epi- and sub-tryptase\(^+\) mast cells in smokers correlated positively with smoking pack years \( (r = 0.5 - 0.68, P = 0.005 - 0.034, \text{Fig E3, A-E}) \).

**Correlations between mucosal eosinophil cell numbers and sputum inflammatory markers**

We finally examined the relationships between sub-eosinophil numbers on day 7 post-infection and sputum inflammatory markers previously measured in the COPD subjects.\(^4\,19\) Sub-eosinophils correlated with peak sputum neutrophils \( (r = 0.73, P = .001, \text{Fig 6, A}) \) but there was no significant
correlation between sub-eosinophils and peak sputum eosinophils. Sub-eosinophils also correlated strongly with peak values during infection of several sputum inflammatory mediators and antimicrobial peptides including CXCL8/IL-8 ($r = 0.86$, $P < .0001$, Fig 6, B), IL-1β ($r = 0.83$, $P = .0002$, Fig 6 C), TNF ($r = 0.77$, $P = .0007$, Fig 6, D), pentraxin-3 ($r = 0.78$, $P = .0003$, Fig 6, E), LL-37 ($r = 0.6$, $P = .012$, Fig 6,F) and neutrophil elastase ($r = 0.55$, $P = .023$, Fig 6, G). However, there were no correlations between epi- or sub-neutrophils and the sputum inflammatory markers.

Discussion

We have found that experimental RV infection induced eosinophils and neutrophils only in the COPD subjects whereas macrophages and T- and B-lymphocytes were increased in both COPD and control subjects. Statistically significant positive associations were found between inflammatory cell numbers and virus load, respiratory symptom severity and reductions in lung function in COPD. The numbers of sub-eosinophils also correlated with inflammatory markers in sputum.

Eosinophils and neutrophils: The presence and role of eosinophils in COPD exacerbations have remained unclear with conflicting results from studies using sputum. Some studies have reported increased numbers of eosinophils at exacerbation but did not include virus detection. Bafadhel et al. reported that there was only 3% of exacerbations where virus and sputum eosinophil coexisted. We reported increased sputum eosinophils restricted to virus-induced severe exacerbations. Other found no significant increase in eosinophil numbers in virus-induced exacerbations. These discrepancies may be due to heterogeneity in the aetiology of COPD exacerbations, timing of sampling, effects of treatment and variation of disease severity. Studies using bronchial biopsies have reported increased eosinophils in the bronchial mucosa of naturally occurring exacerbations but the role of viruses as a cause of such mucosal eosinophilia remains uncertain. Our present study is the first to compare the effects of experimentally administered virus on the bronchial mucosal inflammatory response using bronchial biopsies from the same subjects when stable and during exacerbations in treatment-naïve, non-intubated COPD subjects. A significant increase in mucosal, but not sputum, eosinophils was demonstrated only in the COPD subjects following RV infection. Also, the change in sub-eosinophil counts (not for other cell types) from baseline to day 7 infection in COPD subjects, was significantly greater than those in non-smoker and smoker control subjects. This demonstrated a clear difference in the mucosal
inflammatory response between COPD and non-COPD subjects. Moreover, greater numbers of sub-eosinophils were associated with greater virus load, more symptoms, bigger falls in lung function and higher sputum inflammatory markers. The findings of RV-induced eosinophilia are noteworthy given that they were observed in subjects with relatively mild COPD who had no history of asthma or allergic rhinitis and who tested negative to ten aeroallergens on skin prick tests. The data support a pathogenic role for bronchial mucosal eosinophilia in RV infection-induced COPD exacerbations. Therefore, in exacerbations of COPD where eosinophils are identified and steroid or anti-IL-5 eosinophil-targeting therapies are considered, the addition of future novel anti-viral therapies may be of particular benefit. In addition, blood eosinophils have been examined as a marker to guide corticosteroid use in COPD exacerbations, though this approach continues to be debated. Our data suggest that the relationship between blood, sputum and mucosal eosinophils is complex. The lack of a relationship between blood and mucosal eosinophils implies that using blood eosinophils alone as a marker of airway mucosal eosinophilia may result in some patients without blood eosinophilia not receiving corticosteroids when there is, indeed, mucosal eosinophilia.

Contrary to the results seen with eosinophils, sub-neutrophils were not significantly increased whereas epi-neutrophils were increased in COPD, when higher numbers were positively related to virus load and falls in lung function. We have also reported previously that neutrophils are significantly increased in the sputum of these COPD subjects with strong correlations between sputum neutrophils and sputum neutrophil elastase, IL-1β, TNF, CXCL8/IL-8, pentraxin-3 and LL-37. Surprisingly, in our present analyses, these sputum markers correlated better with sub-eosinophils than with epi/sub-neutrophils. These data suggest that virus infection induces an innate inflammatory response involving mediators such as IL-1β, TNF and CXCL8/IL-8 that contribute to recruitment of both neutrophils and eosinophils. It is considered that neutrophils transit rapidly from blood through the bronchial mucosal into the airway lumen and thus their numbers in sputum reflect mucosal tissue neutrophilic inflammation. By contrast, it is likely that eosinophils transit more slowly and are retained in the mucosal compartment. Thus, we speculate that, the contribution of eosinophils may well be underestimated in studies using sputum alone. Moreover, therapies targeting eosinophils have focussed on the Th2 pathway in both asthma and COPD. In distinction to asthma, our present data in COPD show associations between eosinophils and mediators of innate inflammation suggesting that other pathways may be involved in eosinophil...
recruitment to the airways, at least in the context of acute viral infection.

**Lymphocytes, macrophages and mast cells:** Earlier studies have suggested a pathogenic role for CD68+ monocytes/macrophages and CD8+ T cells in COPD\(^6, 8, 36, 37\) but the mechanisms of their increased recruitment in COPD are not well known. A previous study has demonstrated a positive correlation between the number of bronchial mucosal CD8+ cells in COPD and the number of pack years smoked.\(^7\) Here we have confirmed that baseline numbers of CD68+ and CD8+ cells are significantly greater in smokers and COPD than that in non-smokers and that baseline CD68+ and CD8+ counts in COPD correlate positively with smoking pack years. In addition, for the first time, we present data showing that CD8+ T cells are increased in non-smokers and those with COPD from baseline following infection but not in the smokers who had significantly higher baseline CD8+ counts compared to non-smokers at baseline. In contrast, RV-infection induced increases of CD68+ cells in all three groups. The numbers of CD8+ cells were significantly greater in smoker and COPD groups than in the non-smoker group on day 7 post-infection, respectively. At 6 weeks, CD8+ T cell numbers in both smoker and COPD were still increased. These data indicate that smoking and virus infection have an additive and prolonged effect on the pulmonary recruitment of CD8+ cytotoxic T cells.

Previously we have demonstrated that CD4+ cells are significantly fewer in COPD in its stable phase compared to non-smoker controls.\(^18\) However at that time a healthy smoker group was not available for comparison. Gosman et al have reported an increase of bronchial mucosal B lymphocytes in GOLD stage 2 and 3 COPD compared with healthy smokers.\(^38\) Hogget et al reported that the accumulated volume of B-cells in small airways was increased in stage 3 and 4 COPD and the increasing number of B cells was associated with increasing severity of COPD.\(^9\) But in the last a healthy non-smoker group was not included and the presence or absence of the virus infection was not investigated in either of the aforementioned studies. Therefore, the roles of smoking and virus infection in CD4+ and CD20+ cells recruitment into the bronchial mucosa remain unclear. Herein, we report for the first time that both smoker and COPD subjects have lower numbers of baseline sub-CD4+ and CD20+ cells compared with non-smokers at baseline whereas RV-infection recruited CD4+ and CD20+ cells into bronchial mucosa in all three groups. These findings indicate that smoking per se increases CD68+ and CD8+ cells and decreases CD4+ and CD20+ cells, whereas RV infection increases recruitment of all these cell types in the bronchial mucosa of all subjects.
Finally, we consider the reduction in the number of sub-mast cells is likely due to infection-induced degranulation, leading to fewer cells containing sufficient tryptase to stain positive for the purpose of their identification. The effects of smoking and virus infection on mast cell biology in COPD exacerbations requires further study.

**Limitations:** Our subjects had relatively mild GOLD stage II COPD and we suggest that in a more severe COPD population eosinophilic inflammation may be even more prominent. We acknowledge that our group sizes were relatively small, particularly for those where we had sputum eosinophil mediators: thus, significant correlations may have been missed. Furthermore, this is an exploratory and hypothesis-generating study and as such we did not control for type I errors arising from multiple comparisons. As a result, the observed significant differences and associations may be subject to false positives. Further hypothesis-testing studies are needed to confirm selected of our observations. However, the relative homogeneity of subjects allowed for more reliable interpretation of the data, which is difficult to obtain in naturally occurring exacerbations of COPD.

**Conclusion,** experimental RV-infection increases the numbers of bronchial mucosal eosinophils and neutrophils only in COPD whereas monocytes/macrophages, CD8\(^+\) and CD4\(^+\) T- and CD20\(^+\) B-lymphocytes increased in both COPD and non-COPD controls. The eosinophilic inflammatory response to RV infection in the bronchial mucosa of COPD differs from that seen in the airway lumen and in blood. The increased numbers of inflammatory cells in COPD correlated with virus load and illness severity and eosinophils also associated with sputum innate inflammatory mediators during the infection. In addition, chronic cigarette smoking decreased the numbers of CD4\(^+\) and CD20\(^+\) cells and increased CD8\(^+\) and CD68\(^+\) cells. Thus, our findings provide new insights into previously undescribed patterns of inflammatory response that occur during experimental RV-induced exacerbations of COPD and, also smoking per se: these data could impact on the design of future treatment modalities.

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Table 1. Demographic data at baseline and during infection

| Subjects | N = | Gender (M/F) | Age (years)* | Smoking (pack years)* | FEV₁ (% of predicted)* | FEV₁/FVC (%)* | Peak sputum virus load (Log₁₀ copies/mL) |
|----------|-----|--------------|--------------|-----------------------|------------------------|--------------|------------------------------------------|
|          |     |              |              |                       | Baseline | Day 9  | Baseline | Day 9  | Baseline | Day 9  |                                        |
| Non-smoker | 11  | 4/7          | 62±5.1‡      | 0±0                  | 101±11.8 | 89±10.8 | 78±3.7 | 87±9.0 | 7±2.4    |
| Smoker    | 20  | 10/10        | 51±7.1       | 34±10.5              | 104±14.5 | 89±26.1 | 79±5.7 | 73±9.0 | 6±3.6    |
| COPD      | 17  | 12/5         | 61±8.1‡      | 46±21.0§             | 68±5.1†  | 58±11.7† | 57±8.1† | 54±10.1† | 8±3.9    |

*Results are expressed as mean ± SD.

‡P < 0.0001 vs. non-smoker and smoker, †P = 0.0003 and §P = 0.025 vs. smokers (Student’s t test).
| Groups       | COPD  | Healthy Smokers | Healthy Non-Smokers |
|-------------|-------|-----------------|---------------------|
|             | Baseline | Day 7 | Week 6 | Baseline | Day 7 | Week 6 | Baseline | Day 7 |
| N =         | 17  | 17  | 11  | 20  | 20  | 12  | 11  | 11  |
| **Epithelium*** |       |     |     |       |     |     |     |     |
| Neutrophil  | 4   | 8†  | 14† | 3   | 8   | 10  | 4   | 4   |
| elastase*   | (1-18) | (1-46) | (2-47) | (0-30) | (0-36) | (0-47) | (16-0.4) | (0-24) |
| EG2*        | 0   | 0.4 | 0.4 | 0.2 | 0.4 | 0.3 | 0   | 0.7 |
| (0-6)       | (0-11) | (0-12) | (0-8) | (0-31) | (0-5) | (0-19) | (0-68) |
| Tryptase*   | 3   | 3   | 4   | 3   | 3   | 11  | 2   | 3   |
| (0-16)      | (0-8) | (0-14) | (0-35) | (0-21) | (0-25) | (0-9) | (0-16) |
| CD68*       | 11  | 18  | 31  | 15  | 24§ | 13  | 8   | 14  |
| (1-61)      | (1-86) | (4-56) | (1-51) | (5-88) | (0-77) | (3-27) | (4-22) |
| CD4*        | 3   | 8†  | 5   | 3   | 6†  | 2   | 4   | 8†  |
| (0-14)      | (0-96) | (2-70) | (0-13) | (1-39) | (0-18) | (0-11) | (2-44) |
| CD8*        | 55‡ | 67§ | 94  | 67‡ | 72§ | 73  | 38  | 32  |
| (31-183)    | (27-178) | (39-253) | (7-187) | (30-165) | (20-116) | (9-72) | (15-96) |
| CD20*       | 0   | 1†  | 0   | 0   | 2†  | 0   | 0   | 0.5†|
| (0-0.3)     | (0-19) | (0-2) | (0-3) | (0-5) | (0-4) | (0-2) | (0-6) |
| **Subepithelium*** |       |     |     |       |     |     |     |     |
| Neutrophil  | 109 | 135 | 155 | 135 | 148 | 117 | 125 | 149 |
| elastase*   | (40-337) | (64-445) | (72-201) | (22-492) | (27-564) | (18-289) | (44-295) | (67-469) |
| EG2*        | 11  | 72† | 23  | 11  | 25  | 34  | 12  | 38  |
| (0-87)      | (11-349) | (2-169) | (0-66) | (1-257) | (0-199) | (0-66) | (1-176) |
| Tryptase*   | 301 | 220† | 184† | 341 | 220† | 300 | 310 | 248 |
| (119-480)   | (72-347) | (140-323) | (73-661) | (77-576) | (15-605) | (109-531) | (61-459) |
| CD68*       | 201‡ | 334† | 228 | 234‡ | 302† | 217 | 127 | 294†|
| (95-388)    | (61-603) | (138-379) | (55-633) | (97-883) | (125-506) | (102-182) | (161-559) |
| CD4*        | 66‡ | 221† | 167 | 142‡ | 226† | 83  | 199 | 230 |
| (37-296)    | (12-666) | (75-406) | (14-251) | (36-1014) | (29-447) | (67-293) | (32-342) |
| CD8*        | 243‡ | 429§ | 408† | 376‡ | 491§ | 541 | 159 | 236‡|
| (142-816)   | (148-1003) | (311-593) | (90-910) | (117-1420) | (31-736) | (63-278) | (163-533) |
| CD20*       | 9†  | 23† | 13  | 9†  | 29† | 16  | 18  | 20  |
| (2-44)      | (4-270) | (6-138) | (0-39) | (0-406) | (0-51) | (5-46) | (9-215) |

*Values are medians (ranges) of positive cell counts per 0.1 mm² epithelium and per mm² subepithelium.
†P = 0.0005-0.044 vs their own baselines, respectively,
‡P = 0.0005-0.047 vs non-smoker baseline,
§P = 0.011-0.049 vs non-smoker day 7, respectively,
װP = 0.031 vs its own week 6.
FIGURE LEGENDS:

FIG 1. Immunohistochemistry stained cells are seen as red fuchsin or brown diaminobenzidine positivity: RV16-infection on day 7 increased numbers of A, eosinophils, B, neutrophils, C, CD68+ (arrows), D, CD8+, E, CD4+ and F, CD20+ (arrows) cells in bronchial mucosa of COPD subjects compared to their baseline numbers of G, eosinophils, H, neutrophils, I, CD68+ (arrows) J, CD8+, K, CD4+ and L, CD20+ (arrows) cells, M, Negative control shows an absence of signal (internal scale bar = 20 µm for all).

FIG 2. Counts for subepithelial: A, eosinophils, B, CD68+ monocytes/macrophages, C, CD8+ and D, CD4+ T-lymphocytes, E, CD20+ B-lymphocytes and F, tryptase+ mast cells in bronchial biopsies of healthy non-smokers, healthy smokers and COPD subjects at baseline and day 7 after rhinovirus-16 infection. The data are expressed as the number of positive cells per mm² of subepithelium. G, Changes in counts of subepithelial eosinophils from baseline to day 7 post-infection in bronchial biopsies of healthy non-smokers, healthy smokers and COPD subjects. The data are expressed as change in the number of eosinophils per mm² of subepithelium. Triangles show individual counts and arrows / horizontal bars show median values (Wilcoxon matched pairs test & Mann Whitney U test).

FIG 3. Counts for epithelial A, neutrophils, B, CD68+ monocytes/macrophages, and C, CD4+, D, CD20+ and E, CD8+ lymphocytes in bronchial biopsies of healthy non-smokers, healthy smokers and COPD subjects at baseline and day 7 after RV16-infection. The data are expressed as the number of positive cells per 0.1 mm² of epithelium. Triangles show individual counts and arrows show median values (Wilcoxon matched pairs test & Mann Whitney U test).

FIG 4. Blood eosinophils and sputum eosinophil-related soluble mediators in COPD subjects during experimental rhinovirus infection: A, blood eosinophil percentages at baseline and day 7 post-infection, B, eotaxin and C, eotaxin-3 in induced sputum at baseline and day 3 – 42 post-infection. Triangles show individual counts and horizontal bars show median values (Wilcoxon matched pairs test).

FIG 5. Correlations, in COPD subjects, between the numbers of sub-eosinophils on day 7 post-infection and A, peak sputum virus load, B, peak breathlessness scores and C, reduction in peak expiratory flow (% fall from baseline), recorded on day 9 post-infection, D, between BAL virus load and sub-neutrophils on day 7.
post-infection; between counts of E, epi- and F, sub-neutrophils on day 7 and pre-bronchodilator FEV1% predicted at day 9 (Spearman rank correlation, n = 17 or 9).

FIG 6. Correlations, in COPD subjects, between the numbers of subepithelial eosinophils at day 7 post-infection and peak sputum:  A, neutrophils, B, CXCL8/IL-8, C, IL-1 β , D, TNF, E, pentraxin-3, F, LL-37 and G, neutrophil elastase (Spearman rank correlation, n = 17 for all).
COPD $r = 0.61$ $P = 0.011$ $n = 17$

Peak sputum virus load (Log$_{10}$ copies/mL) vs. Eosinophils (cells/mm$^2$ subepithelium)

COPD $R = 0.62$ $P = 0.013$ $n = 17$

Peak breathlessness score vs. Eosinophils (cells/mm$^2$ subepithelium)

COPD $r = -0.62$ $P = 0.019$ $n = 17$

Reduction in peak expiratory flow (% baseline) vs. Eosinophils (cells/mm$^2$ subepithelium)

COPD $r = -0.57$ $P = 0.021$ $n = 17$

BAL virus load (Log$_{10}$ copies/mL) vs. Neutrophils (cells/mm$^2$ subepithelium)

COPD $R = -0.55$ $P = 0.020$ $n = 17$

Pre-FEV$_1$ % predicted D9 vs. Neutrophils (cells / mm$^2$ epithelium)

COPD $R = -0.55$ $P = 0.020$ $n = 17$

Pre-FEV$_1$ % predicted D9 vs. Neutrophils (cells / mm$^2$ subepithelium)
Fig. 6A: Peak sputum neutrophils (x10^6/g)

- COPD
  - r = 0.73
  - P = 0.001
  - n = 17

Fig. 6B: Peak sputum CXCL8/IL-8 (pg/mL)

- COPD
  - r = 0.86
  - P < 0.0001
  - n = 17

Fig. 6C: Peak sputum IL-1β (pg/L)

- COPD
  - r = 0.83
  - P = 0.0002
  - n = 17

Fig. 6D: Peak sputum TNF (pg/mL)

- COPD
  - r = 0.77
  - P = 0.0007
  - n = 17

Fig. 6E: Peak sputum pentraxin-3 (pg/mL)

- COPD
  - r = 0.78
  - P = 0.0003
  - n = 17

Fig. 6F: Peak sputum LL-37 (ng/mL)

- COPD
  - r = 0.6
  - P = 0.012
  - n = 17

Fig. 6G: Peak sputum neutrophil elastase (µg/mL)

- COPD
  - r = 0.55
  - P = 0.023
  - n = 17
**Fig E1A**

COPD $r = 0.54$  
$P = 0.027$  
n = 17

**Fig E1B**

COPD  
$R = 0.66$  
$P = 0.007$  
n = 17

**Fig E1C**

COPD  
$r = 0.57$  
$P = 0.028$  
n = 17

**Fig E1D**

COPD  
$r = 0.88$  
$P = 0.013$  
n = 9
COPD $r = 0.58$ $P = 0.017$ $n = 17$

Peak breathlessness score

CD8$^+$ (cells / mm$^2$ subepithelium)

Fig E2 A

Peak breathlessness score

CD20$^+$ (cells / mm$^2$ subepithelium)

Fig E2 B

Peak lower respiratory symptom score

CD68$^+$ (cells / mm$^2$ subepithelium)

Fig E2 C

COPD $r = -0.73$ $P = 0.014$ $n = 17$

Pre-FEV$_1$ %predicted

CD8$^+$ (cells / mm$^2$ subepithelium)

Fig E2 D

COPD $r = -0.72$ $P = 0.003$ $n = 17$

Pre-FEV$_1$ %predicted

CD4$^+$ (cells / mm$^2$ subepithelium)

Fig E2 E

COPD $r = -0.58$ $P = 0.015$ $n = 17$

Pre-FEV$_1$ %predicted

CD20$^+$ (cells / mm$^2$ subepithelium)

Fig E2 F
Online Repository

Bronchial mucosal inflammation and illness severity in response to experimental rhinovirus infection in COPD

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Methods

Participants: The subjects were recruited to two experimental studies that have been published previously, study one and study two. The first study ran from 2003-2007, two groups of study subjects – 13 smokers with COPD and 13 smokers with normal lung function were recruited initially. The second study ran from 2007-2010, separate cohorts of 18 smokers with COPD, 15 smokers without airway obstruction and an additional control group of 19 healthy non-smokers with normal lung function were enrolled. The inclusion criteria shown in Table E1 were same for two smoker groups in two studies. All subjects were non-atopic as defined by negative reactions to a six grass pollen mix, house dust mite, cat, dog, Aspergillus fumigatus, Cladosporium herbarium, Alternaria alternata, birch pollen, three tree pollen mix and nettle pollen (ALK Abello) on skin prick tests and none had any history of asthma or allergic rhinitis. The protocols for the two studies were identical. A total of 31 subjects with COPD, 28 smokers, and 19 non-smokers, were inoculated with low-dose rhinovirus 16 and 77 of 78 subjects completed the study; one subject with COPD withdrew because of ill health believed unconnected to the study. Rhinovirus (RV) infection was confirmed in 20 of 30 subjects with COPD (66.7%); 22 of 28 smokers
(78.6%); and 11 of 19 non-smokers (58%). Only infected subjects were included in the present biopsy study. The bronchial biopsies were not obtained from one COPD and one healthy smoker. Two day 7 biopsies obtained from two COPD subjects and one day 7 biopsies from a smoker were of inadequate quality and excluded. Thus, actual 17 COPD, 20 healthy smokers and 11 healthy non-smokers (48 pairs of baseline and day 7 bronchial biopsies) were analysed.

Subjects were recruited from number of sources including newspaper advertisements, primary care, spirometry clinics, smoking cessation clinics, and outpatient hospital clinics. Initial screening visits suitability for the study was assessed, informed consent was obtained. All subjects had no respiratory tract infection within the previous 8 weeks. Their serum neutralizing antibodies to RV-16 were measured at screening and were in a titre <1:2. COPD patients had no exacerbation and were not treated with oral, inhaled or nasal topical steroids, long-acting β-agonists or tiotropium in the previous 3 months. For those entering the study baseline clinical sampling was performed 1–4 weeks before virus inoculation, which was at study day 0. Subjects were seen daily on the 9 days immediately after inoculation. The timeline for clinical measurements and sampling is outlined in the previous papers.

**Virus inoculation:** Details regarding the preparation and safety testing of the RV-16 inoculum used in this study have been published. The virus (10 TCID<sub>50</sub>) was diluted in a total volume of 1 ml of 0.9% saline and inoculated into both nostrils, using an atomizer (no. 286; DeVilbiss Co., Heston, UK).

**Blood and sputum:** Both were collected at baseline and on days 5, 9, 12, 15, 21 and 42 post-inoculation. Blood was also collected on day 7.

**Bronchoscopy:** All bronchoscopies were carried out in the Endoscopy Unit at St Mary’s Hospital, Imperial College Healthcare NHS Trust by an experienced operator.
Subjects were administered nebulised salbutamol (2.5mg) and ipratropium bromide (0.5mg) prior to the procedure and intravenous midazolam was used to provide sedation. Bronchoalveolar lavage (BAL) and Bronchial biopsies were taken at baseline and day 7 post-infection for all subjects and 42 days (6 weeks, convalescence) in 12 smoking controls and 11 COPD subjects using an Olympus bronchovideoscope BF, type 1T 10 with sterile FB 15C forceps (Olympus Co, Tokyo, Japan) from the sub-carina of the basal segmental bronchi of the right lower lobe. Up to three biopsies were obtained from each subject and fixed immediately in 4% paraformaldehyde. Details of the collection and processing of nasal lavage, induced sputum, and bronchoalveolar lavage (BAL) are provided previously.¹ ³

**Confirmation of rhinovirus-16 infection:** RV-infection was confirmed by at least one of the following: positive nasal lavage, sputum or BAL standard or qPCR for rhinovirus, positive culture of RV16, or seroconversion defined as a titre of serum neutralizing antibodies to RV16 of at least 1:4 at 6 weeks. Serology was performed at screening and 6 weeks post-infection by microneutralization test for neutralizing antibody to RV16.⁵ Virus was cultured by adding 250μL of nasal lavage (from the day of peak virus load by qPCR) to semiconfluent HeLa cells that were cultured for up to five passages. Cultured virus was confirmed as RV16 by microneutralization assay with RV16-specific antisera (ATCC; titre 1:600).⁵ RNA was extracted from samples (QIAamp viral RNA minikit; Qiagen Ltd, Crawley, UK) and reverse-transcribed (omniscript RT kit, Qiagen) with random hexamers. Standard RV PCR (PerkinElmer 9600 GeneAmp) was performed from 2μL of cDNA in nasal lavage, an unprocessed plug of induced sputum, and unprocessed BAL.⁶ The threshold of a positive virus infection is around 25-50 copies per microliter of cDNA and is based on 2 fold mean
and plus 1 standard deviation (SD) of the minimal detectable concentration, based on how the standard curve (dsDNA) runs over many assays during the study period. To differentiate RV from other picornaviruses BglI enzyme restriction digestion was carried out on the amplicons generated by RT-PCR.\textsuperscript{7} qPCR was performed on 2μL of cDNA to detect picornavirus using AmpliTaqGold DNA polymerase. (PE Biosystems ABI Prism 7700 )\textsuperscript{8} A standard curve was produced by using serially diluted cloned product and results expressed as copies/mL. The sensitivity of this assay was 104 copies/ml. Virus load was measured with a real-time quantitative RT-PCR assay\textsuperscript{9}

**PCR for other respiratory viruses:** Infection with viruses other than RV was excluded by testing nasal lavage by PCR on random hexamer primed cDNA for *Mycoplasma* and *Chlamydophila pneumoniae*, adenoviruses, respiratory syncytial virus, influenza AH1/AH3/B, parainfluenza 1–3, human metapneumoviruses (HMPV), and coronaviruses 229E and OC43 as described\textsuperscript{10} except HMPV which was adapted from.\textsuperscript{11}

**Clinical Procedures:** Daily diary cards of upper and lower respiratory symptoms were commenced at screening and continued until 6 weeks after inoculation. Upper respiratory symptoms were measured using the Jackson scale assessing eight symptoms – sneezing, headache, malaise, chilliness, nasal discharge, nasal obstruction, sore throat, and cough – graded 0 (absent) to 3 (severe).\textsuperscript{12} The daily cold score was summated from the individual scores and a clinical cold was defined using the Jackson criteria.\textsuperscript{12} The scoring system for the lower respiratory symptoms of shortness of breath, cough, wheeze, sputum quantity and sputum quality is shown in previous paper.\textsuperscript{13} The daily lower respiratory score was summated from the individual scores and a COPD exacerbation was defined as an increase in the lower respiratory score of at least two points over baseline for at least two consecutive
For both upper and lower respiratory daily symptom scores the mean scores on days -6 to 0 were calculated and subtracted from subsequent daily scores to correct for baseline symptoms.

**Pulmonary function:** Spirometry was performed with a Micromedical MicroLab spirometer (MicroMedical, Rochester, UK) according to British Thoracic Society guidelines\(^\text{15}\) before and 15 minutes after administration of salbutamol (200mg) via metered dose inhaler and large volume spacer for pre- and post-bronchodilator values. The spirometry data were collected at baseline and on days 5, 9, 12, 15, 21, 28, 35 and 42 post-infection.

**Inflammatory Markers**

Peripheral blood eosinophils were counted at baseline and on day 7 after infection and in the Haematology Laboratories of St. Mary’s Hospital, Imperial College Healthcare NHS Trust. The ELISAs for detection of antimicrobial peptides and inflammatory mediators in sputum were carried out according to the manufacturers’ instructions and have been published previously.\(^\text{1, 2}\) Briefly, plates were read on a Spectramax Plus 384 plate reader and the results read using SoftMax Pro software. Initial experiments were carried out to determine whether sample dilution was required. Experiments were carried out to determine the recovery of antimicrobial peptides from sputum. A sputum sample and PBS were spiked with the relevant protein at the same concentration and the levels detected compared. For all the proteins measured the recovery in sputum was >80% that of the PBS sample. The sensitivities and sources of the individual ELISAs were as follows: pentraxin 3 (0.025ng/mL) (R&D Systems, Abingdon UK); HBD-2 (8pg/mL) (PeproTech, London UK); α-defensins 1-3 (156pg/mL) (Hycult Biotech, Cambridge UK); cathelicidin LL-37...
(0.1ng/mL) (Hycult Biotech, Cambridge UK) and neutrophil elastase (0.12ng/mL) (Immunodiagnostik, Bensheim, Germany).

**Meso Scale Discovery**

The mediators eotaxin, eotaxin-3, interleukin (IL)-4, IL-5, CXCL8/IL-8, IL-1β and TNF in sputum were measured using the Mesoscale Discovery® platform. The technique enables quantitative detection of between 1 and 9 mediators per well in a 96 well plate format using a Multi-spot® technique which has been published previously. Briefly, the protocol requires addition of 25μL blocking solution before incubation. Following plate washing either sample or standard was added to the plate, followed by incubation and washing and addition of detection antibody. Finally read buffer was added and the plate passed through the Sector imager for reading. The lower limits of detection of the individual analyse were as follows: Eotaxin (10pg/ml), Eotaxin 3 (80pg/ml), IL-4 (0.1pg/ml), IL-5 (0.3pg/ml), CXCL8/IL-8 (0.6pg/ml), IL-1β (1.17pg/ml) and TNF (0.376pg/ml).

**Immunohistochemistry (IHC):** EnVision-alkaline phosphatase technique (Dako Ltd, Cambridge, UK) was used to label EG2+ eosinophils, neutrophil elastase+ neutrophils, tryptase+ mast cells and CD68+ monocytes/macrophages. EnVision peroxidase staining method (Dako) was used to identify CD4+, CD8+ T- and CD20+ B-lymphocytes. The immunostaining procedures for detecting the phenotypes of inflammatory cells were conducted by Techmate ‘Horizon’ automated immunostainer (LJL Biosystems Inc, USA) as previously described but with modification. Irrelevant mouse IgG1 kappa antibody (MOPC21) was used to substitute for the primary layer as negative control for staining specificity of mouse monoclonal antibodies. The following panel of monoclonal mouse anti-human antibodies (Dako) was applied to tissue sections: anti-
neutrophil elastase (M0752), tryptase mast cell (M7052), CD4 (M0716), CD8 (M0707) CD20 (M0755) and CD68 (M0876). Mouse anti-EG2 (EG2) was from Pharmacia & Upjohn Ltd, Milton Keynes, UK.

**Quantification** In histological slides, coded to avoid observer bias, areas of epithelium and subepithelium, excluding muscle and gland, were assessed using an Apple Macintosh computer and Image Version 1.55 (US National Institute of Mental Health). Distinct phenotypes of inflammatory cells were counted using a Leitz Dialux 20 light microscope (Leitz Wetzlar, West Germany). Two to three bronchial biopsies for each subject were measured and counted in order to take account of within subject variability. The total epithelial and subepithelial areas of two or three biopsies that were more than 0.2 mm$^2$ and 1.6 mm$^2$, respectively, were accepted as adequate size. The epithelial / subepithelial areas and positive cells of two or three biopsies from each bronchoscopy were summed, respectively. Then the total counts were divided by total area to normalise the counts as the number of cells per unit area. The data for bronchial biopsy cell counts were expressed as the number of cut cell profiles with a nucleus visible (i.e. positive cells) per 0.1 mm$^2$ of the epithelial area and per 1 mm$^2$ of the subepithelial area. The coefficient of variation for repeat counts of cells immunopositive for sub-type markers of inflammatory cells by one observer this ranged between 5% and 6%.

**Statistical analysis**

Statistical analysis was performed using StatView (SAS Institute. Inc) and GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). One way ANOVA followed by the unpaired Student’s t-test was used for the analyses of age, smoking pack years and lung function data between groups. In respect of cell counts in blood, sputum and biopsies and mediators in sputum, these data were non-normally
distributed and overall differences between all groups and between three time points
within group were assessed firstly using the Kruskal-Wallis test, which, if significant, was
followed by Wilcoxon matched pairs test within group between baseline and infection.
Differences between groups were analysed by Mann-Whitney tests.
The coefficient of variation (CV = SD/mean × 100) was used to express the error of
repeat cell counts in the biopsies. Spearman’s rank correlation was used as a test for
correlations between the numbers of specific types of inflammatory cell and virus
load/physiologic/clinical data/sputum inflammatory markers. A P-value of < 0.05 was
accepted as indicating a significant difference. All reported P values are two-sided.

Results

Time for peaked virus load, respiratory symptom and lung function

In COPD, individual virus load peaked on days 4-8 in nasal lavage, on day 5 in sputum
and on day 7 in BAL, the individual lower respiratory symptom and breathlessness scores
peaked around day 9 and individual lowest PEF and FEV\textsubscript{1} were detected between day 5
and day 12, most of them on day 9.\textsuperscript{1} The virologic and blood, sputum or BAL
inflammatory data from these subjects have been reported previously.\textsuperscript{1,3}

Associations between numbers of mucosal monocytes/macrophages and
lymphocytes and virus load/clinical outcomes

Virus load: In COPD on day 7 post-infection, the numbers of sub-CD68\textsuperscript{+}, and CD4\textsuperscript{+}
cells were associated with peak nasal lavage virus load (r = 0.54 and 0.66, P = .027
and .007, respectively, Fig E1, A, B); sub-CD20\textsuperscript{+} cells correlated with peak sputum virus
load (r = 0.57, P = .028, Fig E1, C); sub-CD8\textsuperscript{+} cells correlated with BAL virus load (r =
0.88, P = 0.013, Fig E1, D).

Clinical symptoms and Lung function: In COPD only, those subjects with higher sub-
CD8\textsuperscript{+} and CD20\textsuperscript{+} counts on day 7 post-infection had significantly greater peak
breathlessness scores \((r = 0.58 & 0.50, \ P = .017 & .033, \ \text{respectively, Fig E2, A, B})\) and sub-CD68\(^+\) counts on day 7 correlated positively with peak lower respiratory symptom scores recorded between day 9 and 14 \((r = 0.58, \ P = .021, \ \text{Fig E2, C})\). In COPD, higher numbers of sub-CD8\(^+\), CD4\(^+\), and CD20\(^+\) cells on day 7 were significantly associated with lower pre-bronchodilator FEV\(_1\)% predicted at day 9 \((r = -0.58 - -0.73, \ P = 0.003 - 0.015, \ \text{Fig E2, D-F})\).

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# Supplementary Table E1. Inclusion criteria for study subjects. CO denotes chronic obstructive pulmonary disease, FEV\textsubscript{1} forced expiratory volume in one second and FVC forced vital capacity

| All subjects                                                                 | COPD group                                                   | Smokers                                                                                                                                 | Non-smokers                                                                 |
|------------------------------------------------------------------------------|--------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------|
| • Age 40-75 years.                                                           | • FEV\textsubscript{1} 50% - 79% predicted normal value and β-agonist reversibility <12%. | **Smokers**                                                                                                                         | **Non-smokers**                                                              |
| • No history of asthma or allergic rhinitis and not atopic on skin testing. | • FEV\textsubscript{1}/FVC<70%.                              | • FEV\textsubscript{1}≥80% predicted normal value.                                                                                 | • FEV\textsubscript{1}/FVC≥70%.                                             |
| • Absence of a current or previous history of bronchiectasis, carcinoma of the bronchus or other significant respiratory disease (other than COPD). | • Current or ex-smokers with at least 20 pack years' cumulative smoking | • Current or ex-smokers with at least 20 pack years cumulative smoking                                                             | • Non-smokers                                                                |
| • Absence of significant systemic disease.                                    | • FEV\textsubscript{1}/FVC>70%.                              |                                                                                                                                       |                                                                             |
| • No COPD exacerbation or respiratory tract infection within the previous eight weeks. | • Current or ex-smokers with at least 20 pack years' cumulative smoking |                                                                                                                                       |                                                                             |
| • Serum antibodies to rhinovirus 16 at screening in a titre <1:2.            | • Non-smokers                                                |                                                                                                                                       |                                                                             |
| • No treatment with antibiotics, oral, inhaled or nasal topical steroids, long-acting β-agonists or tiotropium in the previous three months. |                                                                             |                                                                                                                                       |                                                                             |
FIGURE E LEGENDS:

FIG E1. In COPD subjects, correlations between the numbers of A, sub-CD68$^+$ and B, CD4$^+$ on day 7 and peak nasal lavage virus load; C, between sub-CD20$^+$ on day 7 and peak sputum virus load; D, between sub-CD8$^+$ cells on day 7 and BAL virus load (Spearman rank correlation, n = 17 or 9).

FIG E2. In COPD subjects, correlations between the numbers of A, sub-CD8$^+$ and B, CD20$^+$ cells on day 7 post-infection and peak breathlessness scores; C, between counts of sub-CD68$^+$ cells and peak lower respiratory symptom score; and between the numbers of D, sub-CD8$^+$, E, CD4$^+$ and F, CD20$^+$ cells on day 7 and pre-bronchodilator FEV$_1$% predicted at day 9 (Spearman rank correlation, n = 17).

FIG E3. Correlations between smoking pack years and numbers of A, baseline epi-CD68$^+$ and B, CD8$^+$ cells in COPD subjects and C, baseline sub-CD8$^+$ cell counts, D, epi- and E, sub-tryptase$^+$ mast cell counts in healthy smokers (Spearman rank correlation, n = 17 for COPD and n = 21 for healthy smokers).