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Airway inflammation and illness severity in response to experimental rhinovirus infection in asthma

Short title: Airway inflammation in rhinovirus infected asthma

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ABSTRACT

Background: The nature of bronchial mucosal inflammation and its physiological/clinical significance in rhinovirus-induced asthma exacerbations is unclear.

We investigated bronchial mucosal inflammatory response and its association with physiological and clinical outcomes in an experimental model of rhinovirus-induced asthma exacerbations.

Methods: We applied immunohistochemistry to phenotype-inflammatory cells infiltrating the bronchial mucosa before and after experimental rhinovirus infection in 10 asthmatic and 15 normal subjects.

Results: Compared to baseline, virus infection significantly increased epithelial ($P=0.005$) and subepithelial ($P=0.017$) neutrophils in asthmatics only and subepithelial $CD68^+$ macrophages in both asthmatic ($P=0.009$) and normal ($P=0.018$) subjects, but more so in asthmatics ($P=0.021$). Numbers of $CD45^+$, $CD68^+$ and $CD20^+$ cells, neutrophils and eosinophils at day 4 post-infection were positively associated with virus load ($r=0.50-0.72$, $P=0.016-0.03$). In asthmatics, at acute infection, $CD4^+$ cells correlated with chest symptom scores ($r=0.69$, $P=0.029$), the fall in PC_{10} correlated with neutrophils ($r=-0.89$, $P=0.029$), the PC_{10} correlated inversely with $CD4^+$ ($r=-0.67$, $P=0.023$) and $CD8^+$ cells ($r=-0.65$, $P=0.03$), the PC_{20} was inversely associated with $CD20^+$ cells ($r=-0.65$, $P=0.03$), higher epithelial $CD8^+$ cell counts were significantly associated with a greater maximum fall in FEV_1 ($r=-0.72$, $P=0.03$), whereas higher subepithelial mast cell counts were significantly associated with a lower maximum % fall in PEF ($r=0.8$, $P=0.024$).

Conclusion: In asthmatics, rhinovirus infection induces bronchial mucosal neutrophilia and more severe monocyte/macrophage infiltration than in normal subjects. Airway neutrophils, eosinophils, T and B lymphocytes during infection are related to virus load, physiological and clinical severity whereas mast cells are related to greater lung function.

Abbreviations: RV = Rhinovirus.

INTRODUCTION

Rhinoviruses (RVs) are the major cause of acute exacerbations of asthma.¹⁻³ Human experimental RV infection in mild asthmatic volunteers is associated with augmented physiological and inflammatory responses to allergen challenge,^{4,5} reductions in PEF,⁶ FEV₁⁷ and increases in bronchial reactivity.⁸ In agreement, our own previously reported study showed that experimental RV16 infection in asthma induced significant increases in bronchial reactivity, lower respiratory tract symptoms and lung function impairment and that significant changes in these outcomes did not occur in normal subjects.⁹

Regarding RV induced airway inflammation, two previous studies of experimental RV infection reported increases in submucosal CD3⁺ lymphocytes and eosinophils in asthmatic and normal groups when combined¹⁰ or in asthmatics alone.¹¹ The increased mucosal CD3⁺ cells were accompanied by an increase in airway responsiveness and correlated positively with cold symptoms.^{10,11} However, it is unclear if RV-induced bronchial mucosal cellular inflammatory responses differ between asthmatic and non-asthmatic subjects. Here we extend our previous work⁹ in a comparison of the nature of the bronchial mucosa inflammatory response to experimental RV infection in asthmatic and normal healthy subjects and explore its physiological and clinical significance in asthma.

We hypothesised that the bronchial mucosal cellular inflammatory response to RV infection will be exaggerated and of a distinctive predominant inflammatory cell phenotype in asthmatic as compared with normal healthy subjects and that the inflammatory cells recruited to the bronchial mucosa will be associated with virus load and the increased clinical symptoms, airways responsiveness and airflow obstruction associated with an exacerbation.

METHODS

Subjects: we here extended our investigation of the same 10 atopic asthmatics and 15 normal non atopic adults (Table 1) reported previously.⁹ These were human RV16 neutralizing antibody sero-negative. This study was conducted in accordance with amended Declaration of Helsinki. All subjects gave informed written consent and the study protocol was approved by St Mary's NHS Trust Research Ethics Committee (99/BA/345). See online supplement (OLS) for other details.

Experimental infection with human RV16: Subjects were administered 10,000 tissue culture infective dose 50% of RV16 on day 0 by nasal spray.⁹ See OLS for Virologic confirmation of RV16 infection.

Bronchoscopy and clinical data: Bronchial biopsies were taken 14 days before infection (baseline), day 4 (acute infection) and 6 weeks after infection (convalescence). For details of physiological and clinical data obtained see OLS.

Immunohistochemistry: CD45⁺ pan leukocyte/inflammatory cells, eosinophils, neutrophils and mast cells, CD3⁺, CD4⁺, CD8⁺ and CD20⁺ cells were immunostained using the methods as previously described.¹² For details of the immunostaining see OLS.

Quantification: The areas of epithelium and subepithelium in bronchial biopsies were assessed using an Image 1.5 software. The inflammatory cells were counted using a light microscope. The data for cell counts were expressed as the number of positive cells per mm² of the subepithelium and per 0.1 mm² epithelium. For details of counting information see OLS.

Statistical analysis: Differences in cell counts within group between baseline and infection were assessed using Wilcoxon matched pairs test. Mann-Whitney U-test was used to compare differences between groups. Spearman's rank correlation was used as a test for correlations between the numbers of phenotypes of inflammatory cells and physiologic/clinical data. A P-value of < 0.05 was accepted as indicating a significant difference. For further details of the statistical analyses see OLS.

RESULTS

Inflammatory cells were present in both the bronchial epithelial and the subepithelial compartments—representative photographs are depicted in Figs 1A-E. Elastase⁺ neutrophils (Fig 1A) and CD68⁺ monocytes/macrophages (Fig 1B) appeared to be more frequent in the bronchial mucosa of asthmatics at acute infection compared to their baselines (Figs 1C, 1D). Application of irrelevant antibodies for the inflammatory cell markers were negative (Fig 1E).

Absolute counts of inflammatory cells

Total leukocytes: The numbers of subepithelial CD45⁺ cells in asthmatics were significantly greater at both baseline ($P=0.014$) and day 4 infection ($P=0.025$) (Fig 2A) as compared to the same time points in the normal controls. Compared to their respective baselines and day 4, the numbers of subepithelial CD45⁺ cells were significantly less numerous at week 6 after infection in both normal ($P=0.038$ and $P=0.006$) and asthmatic subjects ($P=0.003$ and $P=0.0006$) (Fig 2A). There were no significant differences in counts of epithelial CD45⁺ cells within and between groups.

Granulocytes: Compared to baseline, the numbers of neutrophils in both epithelium ($P=0.005$) (Fig 2B and Table 2) and subepithelium ($P=0.017$) (Fig 2C) of the asthmatic, but not normal subjects, were significantly increased at day 4. At this time point there were significantly greater numbers of epithelial neutrophils in the asthmatics than in normal subjects ($P=0.01$) (Fig 2B). EG2⁺ eosinophils were not significantly increased from baseline in either normal or asthmatic subjects after infection (Table 2). Compared to normal subjects there were significantly higher numbers of epithelial eosinophils in asthma at baseline ($P=0.011$), day 4 ($P=0.046$) and week 6 ($P=0.01$) (Table 2), and trends towards greater numbers of subepithelial eosinophils in asthma at baseline ($P=0.07$) and at day 4 ($P=0.09$), which became statistically significant at week 6 ($P=0.03$) (Table 2).

Macrophages: Compared to baseline, the numbers of subepithelial CD68⁺ cells in both normal (P=0.018) and asthmatic subjects (P=0.009) were significantly increased at day 4 post-infection (Fig 2D). There was a significantly greater number of subepithelial CD68⁺ cells present in asthmatics than in normal subjects at day 4 (P=0.021). There were no significant differences in counts of epithelial CD68⁺ cells within and between groups (Table 2).

Mast cells: There were no significant differences within the asthmatics in epithelial and subepithelial mast cell counts from baseline to day 4 or week 6 (Table 2). The numbers of subepithelial mast cells of normal subjects at baseline started unexpectedly higher than at day 4 (P=0.036) and week 6 (P=0.006) (Table 2). The numbers of epithelial tryptase⁺ mast cells were significantly greater in asthmatic than in normal subjects at day 4 (P=0.049) and week 6 (P=0.01) (Table 2),

Lymphocytes: In asthmatics only, the numbers of epithelial CD3⁺ cells increased at week 6 from baseline (P=0.023) (Table 2). Unexpectedly, there were significant decreases in subepithelial CD4⁺ counts at week 6 from baseline in both normal (P=0.016) and asthmatic (P=0.013) subjects (Fig 2E). Compared to normal subjects, the number of subepithelial CD4⁺ cells was significantly greater at baseline in asthmatic subjects (P=0.043). There were no significant differences in epithelial CD4⁺, CD8⁺ or CD20⁺ and subepithelial CD3⁺, CD8⁺ or CD20⁺ cell counts in either normal or asthmatic subjects after infection (Table 2).

Changes in numbers of inflammatory cells

To investigate differences in inflammatory responses of normal and asthmatic subjects to RV infection, the magnitude of the changes of inflammatory cell counts from baseline to infection were compared. The changes in numbers of both epithelial (P=0.0036) and subepithelial (P=0.018) neutrophils from baseline to day 4 infection in asthma were significantly greater than those of normal subjects (Figs 3A, 3B). The changes in counts of subepithelial CD68⁺ cells from baseline to day 4 in asthma were significantly greater than the

changes in normal subjects ($P=0.025$) (Fig 3B). There were no significant differences between normal and asthmatic subjects at baseline to day 4 or week 6 in respect of changes of any other inflammatory cell phenotypes.

Associations

Virus load: In all subjects taken together at day 4, the numbers of subepithelial $CD45^+$ cells were associated with peak sputum virus load (Fig 4A, $r=0.5$, $P=0.025$) and subepithelial neutrophils (Fig 4B, $r=0.62$, $P=0.027$), $EG2^+$ eosinophils ($r=0.64$, $P=0.016$) and $CD68^+$ cells (Fig 4C, $r=0.65$, $P=0.016$) correlated with BAL virus load.

Clinical symptoms and airway hyperresponsiveness: In the asthmatics only at day 4, epithelial $CD4^+$ counts in asthmatic subjects alone (Fig 5A, $r=0.69$, $P=0.029$) correlated with total chest symptom scores, recorded during the 2 week post infection period, those with high subepithelial neutrophil counts had significant larger falls in PC_{10} from baseline to day 6 post-infection (Fig 5B, $r=0.89$, $P=0.029$), the PC_{10} at day 6 correlated inversely with subepithelial $CD3^+$ (Fig 5C, $r=-0.81$, $P=0.016$), $CD4^+$ (Fig 5D, $r=-0.67$, $P=0.023$) and $CD8^+$ (Fig 5E $r=-0.65$, $P=0.03$) cells and the PC_{20} at day 6 was inversely associated with subepithelial $CD20^+$ cells (Fig 5F, $r=-0.65$, $p=0.03$).

Lung function: In the asthmatics alone at day 4, higher epithelial $CD8^+$ cell counts were significantly associated with a greater maximum fall in FEV_1 (% fall from baseline) (Fig 6A, $r=-0.72$, $P=0.03$). Interestingly, higher subepithelial mast cell counts were significantly associated with a lower maximum % fall in PEF (Fig 6B, $r=0.8$, $P=0.024$). In all subjects taken together at day 4, higher epithelial ($r=0.64$, $P=0.03$) and subepithelial ($r=-0.53$, $P=0.01$) $CD4^+$ cell counts were significantly associated with a bigger maximum fall in FEV_1 .

Serum IgE: The levels of serum IgE were significantly correlated with subepithelial $EG2^+$ eosinophils in normal subjects at day 4 ($r=0.64$, $P=0.017$) and in asthmatics at both baseline ($r=0.94$, $P=0.005$) and day 4 ($r=0.83$, $P=0.013$), respectively (Figs 7A, 7B). There were no significant associations between serum IgE and other phenotypes of inflammatory cell.

DISCUSSION

We demonstrated that, compared to normal, RV-infected asthmatic subjects had an exaggerated bronchial mucosal inflammatory response particularly characterised by increased neutrophils but also of CD68⁺ monocytes/macrophages. Significant correlations were found between CD45⁺, neutrophils and CD68⁺ cells and virus load and between neutrophils, T- and B-lymphocytes and the airway hyperresponsiveness, chest symptoms and reduced lung function whereas mast cells were related to better lung function.

Previous experimental studies of RV16 infection have shown that RV16 is associated with a significant increase in peripheral blood and BAL neutrophils in allergic asthma.¹³ In sputum, virus infection in asthma exacerbations has been associated with increased numbers of neutrophils, elevation of both neutrophil and eosinophil degranulation products and cell lysis were related to clinical severity.¹⁴ Increased neutrophils were detected in the bronchial mucosa of severe asthma experiencing naturally occurring exacerbations.¹⁵ However to our knowledge, there has been no attempt to determine whether RV infection alone can initiate bronchial mucosal neutrophilia and its physiological/clinical outcomes is unknown. Our previous publication on the clinical study, from which these biopsies were collected, reported increased numbers of BAL eosinophils during acute infection in the asthmatic group relative to normal subjects, with similar, but non-significant trends for neutrophils.⁹ However, significant increases in BAL or induced sputum inflammatory cells from baseline to acute RV infection within the asthmatic group were not detected. Consistent with our BAL results, we found increased epithelial eosinophil and neutrophil counts in asthmatic subjects vs. normal volunteers during the acute RV infection. In addition we demonstrated significant increases in numbers of both epithelial and subepithelial neutrophils from baseline to acute infection in the asthmatic subjects and the changes in increased numbers of both epithelial and subepithelial neutrophils from baseline were significantly higher in asthma than that in

normal subjects. We did not find increased neutrophils at week 6, which suggests that virus induced airway neutrophilic inflammation in these mild asthmatic subjects occurs only during the virus induced acute asthma exacerbation and not at convalescence. Thus in contrast to normal subjects, acute experimental RV16 infection selectively increases bronchial mucosal neutrophils in atopic asthma and the higher numbers of neutrophils is related to the severity of infection and airway hyperresponsiveness. These data suggests that virus infection is one of the major aetiologies inducing neutrophilic asthma. As neutrophils play an important role in innate immune responses to clear virus, but they can also cause destruction of lung tissues via releasing their proteases and other mediators.¹⁶ Thereby, a fine balance between the protective and pathologic effects of neutrophils in the course of virus induced asthma exacerbations would need to be clarified when a drug treatment to control neutrophilic inflammation¹⁷ is studied in the future.

Whereas we consider that asthma severity and treatment affects the magnitude of the inflammatory response to RV infection, the nature of the inflammation is more influenced by the time at which the biopsies are taken in relation to the onset of RV infection. In respect of the latter we believe that our results should not have been affected by the mild asthmatics studied who had not taken inhaled or oral corticosteroid.

The effect of RV infection on sputum and BAL CD68⁺ cells in both normal and asthmatic subjects was not investigated in our previously reported study of these subjects.⁹ Seymour et al reported an increase in the macrophages in bronchial biopsies of normal subjects in response to experimental RV16 infection, but asthmatic subjects were not investigated.¹⁸ Thus, the present study is the first to show that acute RV infection increases the numbers of subepithelial CD68⁺ cells of both normal and asthmatics. Notably the changes in levels observed in asthmatic from baseline to acute infection were significantly greater than those in normal subjects. Other work suggests impaired macrophage production of Th1 related

cytokines is strongly implicated in asthma exacerbations pathogenesis.^{19,20} Therefore further investigation is needed to discern their role in asthma exacerbations induced by RV infection.

Airway mast cells are key cells involved in the immediate allergic response and are cells long-associated with the pathogenesis of stable asthma,^{21,22} Using chloroacetate esterase to identify lysosomal granules in mast cells, Lozewicz and coworkers found increased mast cell numbers in endobronchial biopsies from atopic asthmatics when compared with normal subjects.²³ However, the numbers of tryptase+ mast cells, tryptase+,chymase- mast cells and tryptase+,chymase+ mast cells in bronchial mucosa of symptomatic atopic asthmatics is not greater compared to normal subjects^{24,25}. We did not find a difference in number of tryptase+ mast cells in the bronchial mucosa between normal and asthmatic subjects at baseline, which is consistent with previous reports.^{24,25} Although there were no changes in numbers of epithelial mast cells within either normal or asthma groups during RV infection, we detected a greater number of epithelial mast cells in asthma day 4 and week 6 compared with normal day 4 and week 6, respectively. This implies that asthma susceptibility to immediate allergic response could be greater after RV infection. The numbers of subepithelial mast cells declined between the baseline and acute infection and further declined between acute infection and convalescent periods in normal subjects, which appears to be inconsistent with an earlier study in which the number of mast cell tryptase+ cells in bronchial submucosal declined between the cold and convalescent periods, but there was no initial increase between baseline and the cold.²⁶ Interestingly, we detected that in asthmatics, those with the highest subepithelial mast cell counts had the lowest maximum % falls in PEF at acute infection. In this regards, our findings support the results described previously in which the numbers of mast cells in severe asthma correlated with better rather than worse lung function, suggesting that recruitment of mast cells, particularly to the distal lung, may be protective for lung function in severe asthma.²⁷ The biologic mechanism for a 'protective' role remains unclear. Interestingly, Rauter et al. demonstrated that β -tryptase, a major protease released during

mast cell activation, cleaves IgE.²⁸ They suggested that IgE cleavage by effector cell proteases is a natural mechanism for controlling allergic inflammation. The role of mast cells in the pathogenesis of virus induced asthma exacerbations clearly merits further study.

We have demonstrated for the first time in asthmatics that epithelial CD4⁺ cell counts at day 4 infection correlated with total chest symptom scores and the numbers of bronchial mucosal CD3⁺, CD4⁺ and CD8⁺ T and CD20⁺ B lymphocytes are associated with increased airway hyperresponsiveness. Also, the asthmatic subjects with high epithelial CD8⁺ counts had significantly greater maximum falls in FEV₁. Our findings suggest the involvement of both T- and B-lymphocytes in severity of asthma exacerbations induced by virus infection. The absence of increases in counts of CD3⁺ and CD8⁺ T lymphocytes at day 4 during acute RV infection in asthmatic subjects alone is not in keeping with increase subepithelial CD3⁺ and CD8⁺ cells at day 6 during RV16 infection reported in the previously mentioned study.¹¹ The inconsistency between the two studies may be due to differences in timing of the biopsies as ours were taken day 4 versus day 6. The reductions in subepithelial CD4⁺ counts from baseline to convalescence may be due to decrease in blood CD4⁺ cells during acute infection,⁹ which could result in less recruitment of CD4⁺ cells into the lung.

IgE plays a critical role in the development of eosinophil recruitment into the airways after antigen provocation.²⁹ Zambrano et al demonstrated among patients with mild asthma, that those with high levels of IgE had significantly greater lower respiratory tract symptom scores during the initial 4 days of the infection than those with low IgE. They also had higher total blood eosinophil counts at baseline.³⁰ As an addition to these findings we demonstrate that asthmatics with high levels of serum IgE at both baseline and day 4 had significantly greater numbers of submucosal EG2⁺ eosinophils. Our findings imply that atopic asthmatics with higher levels of serum IgE could have more EG2⁺ eosinophils in their airway and experience more severe illness during acute RV infection. Anti-IgE therapy^{31,32} may therefore be useful for preventing exacerbations of asthma triggered by RV infection.

We acknowledge that an atopic non asthmatic group was not included in the present study. Thus, the differences that were observed between the normal and asthmatic populations may have been due, at least in part, to changes attributable to atopy versus those due to asthma. Previous work reported that compared to non atopic normal individuals, nasal biopsies of atopic subjects during experimental RV16 infection did not show increases in infiltration of inflammatory cells (including neutrophils).³³ Another study showed that there were no difference in changes of bronchial mucosa inflammatory cells between normal subjects and atopic rhinitic subjects experiencing naturally occurring common cold virus infections.³⁴ However, in order to answer this question directly, bronchial mucosa inflammatory responses to experimental RV16 infection in atopic, non asthmatic subjects requires further investigation.

We acknowledge also that we have investigated relatively small numbers of subjects, albeit in some detail, and that a large number of statistical tests were carried out. The study may therefore have failed to detect some genuine observations due to limitations of statistical power. However we consider its strengths include the comprehensive and meticulous immunochemical approach and rigorous design of the human challenge model, permitting accurate sampling before and during infection of a uniform nature and known time of onset. Despite these caveats, novel data and interesting observations have emerged.

In conclusion RV16 infection induced a more severe neutrophil and monocyte/macrophage-rich inflammatory response in the bronchial mucosa of asthmatics as compared to similarly infected normal subjects. Numbers of mucosa neutrophils, monocytes/macrophages, EG2⁺ eosinophils, CD4⁺, CD8⁺ and CD20⁺ cells in asthmatics during acute infection were related to virus load, increasing airway reactivity and worsening of asthma symptoms and lung function, whereas tryptase⁺ mast cells were related to better lung function. These data add to our understanding of the pattern of inflammatory response that occurs during RV-induced experimental exacerbations of asthma and, as such could impact on the design of future treatment modalities.

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Author contributions: Dr Zhu carried out the counting, data analyses and drafted up the paper.

Drs Message, Mallia & Contoli recruited subjects, performed bronchial biopsies, collected relevant clinical data. Dr Zhu and Dr Message contributed equally.

Dr Qiu conducted immunohistochemical staining.

Dr Keadze performed the viral detection.

Drs Ward, Barnathan, Mascelli, Kon, Papi and Stanciu were essential to the study design.

Prof. Jeffery was co-principal investigator and directed all histological aspects of the study.

Prof. Johnston was the principal investigator and directed all aspects of the project.

All authors contributed to manuscript writing and review.

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Table 1 Baseline Demographic Data

Subjects	n=	Sex (M/F)	Age (yrs)*	Serum (units/mL) *	IgE	FEV ₁ predicted*	%	Skin pick test+ subjects	Histamine PC ₂₀ (mg/mL)*
Normal	15	8/7	27±2.3	27±8.0		104±3.3	0		20±2.5
Asthmatics	10	2/8	23±1.4	241±49.8 †		106±4.4	9		3±0.7 †

*Values are means ±SEM. †P<0.0001 vs. normal (Student's t test).

Table 2 Counts of phenotype of inflammatory cells infiltrating in epithelial and subepithelial areas in bronchial mucosa of asthmatic and normal subjects

Groups	Asthma			Normal		
	Baseline	Day 4	Week 6	Baseline	Day 4	Week 6
Cells						
Epithelium*						
CD45 ⁺	126 (41-434)	164 (93-1600)	111 (62-597)	109 (42-254)	152 (84-394)	113 (27-205)
Neutrophil elastase ⁺	4.9 (0-12)	16.1 [†] (4-50)	2.6 (0-28)	5.2 (0-23)	6 (0-24)	5 (6-67)
EG2 ⁺	8.4 [‡] (0-160)	2.4 [§] (0-53)	1.2 (0-4)	0 (0-8)	0 (0-9)	0 (0-2)
Tryptase ⁺	6 (0-15)	7 [§] (0-16)	11 (3-24)	2 (0-41)	2 (0-7)	5 (0-13)
CD68 ⁺	14 (3-67)	28 (1-640)	24 (7-70)	22 (0-78)	30 (5-93)	36 (4-133)
CD3 ⁺	88 (20-145)	104 (44-452)	100 [†] (81-393)	97 (40-187)	99 (38-239)	125 (34-267)
CD4 ⁺	13 (2-38)	15 (3-65)	10 (2-55)	11 (0-26)	7 (1-35)	5 (0-27)
CD8 ⁺	64 (11-194)	80 (23-321)	77 (48-186)	79 (3-152)	70 (20-148)	76 (17-134)

CD20 ⁺	2 (0-7)	1 (0-4)	1 (0-3)	0 (0-12)	1 (0-15)	0 (0-2)
Subepithelium*						
EG2 ⁺	84 (0-572)	55 (4-717)	26 (1-133)	18 (0-214)	9 (0-202)	5 (0-50)
Tryptase ⁺	142 (78-252)	121 (8-285)	95 (16-243)	183 [§] (81-342)	92 (20-335)	58 (16- 290)
CD3 ⁺	803 (292-1280)	587 (159-2127)	458 (241-1406)	527 (225-1091)	434 (91-1261)	440 (99-1373)
CD8 ⁺	712 (129-1451)	536 (120-2354)	457 (136-1103)	407 (155-792)	418 (114-1003)	306 (41-623)
CD20 ⁺	53 (17-322)	88 (15-351)	71 (10-275)	51 (0-386)	58 (4-379)	14 (0-98)

*Values are medians (ranges) of positive cell counts per 0.1 mm² epithelium and per mm² subepithelium.

[†]P = 0.005 or 0.023 vs asthma baseline, [‡]P = 0.011 vs normal baseline, [§]P = 0.036, 0.046 and 0.049 vs normal day 4, respectively, ^{||}P = 0.006, 0.01 and 0.03 vs normal week 6, respectively.

FIGURE LEGENDS:

FIGURE 1. Immunohistochemistry stained cells are seen as red fuchsin positivity: an asthmatic patient at day 4 RV16 infection demonstrating an increased number of A, elastase⁺ neutrophils and B, CD68⁺ monocytes/macrophages in bronchial epithelium and subepithelium, an asthmatic patient at baseline showing fewer of C, neutrophils and D, CD68⁺ cells, E, Negative control shows an absence of signal (internal scale bar = 20 μm for all).

FIGURE 2. Counts for A, CD45⁺ cells, B, epithelial and C, subepithelial neutrophils, D, CD68⁺ cells, E, CD4⁺ cells in bronchial biopsies of normal and asthmatic subjects at baseline, day 4 and week 6 after RV16 infection. The data are expressed as the number of positive cells per mm² of subepithelium or per 0.1 mm² of epithelium. Dots show individual counts and horizontal bars show median values (Wilcoxon matched pairs test & Mann Whitney U test).

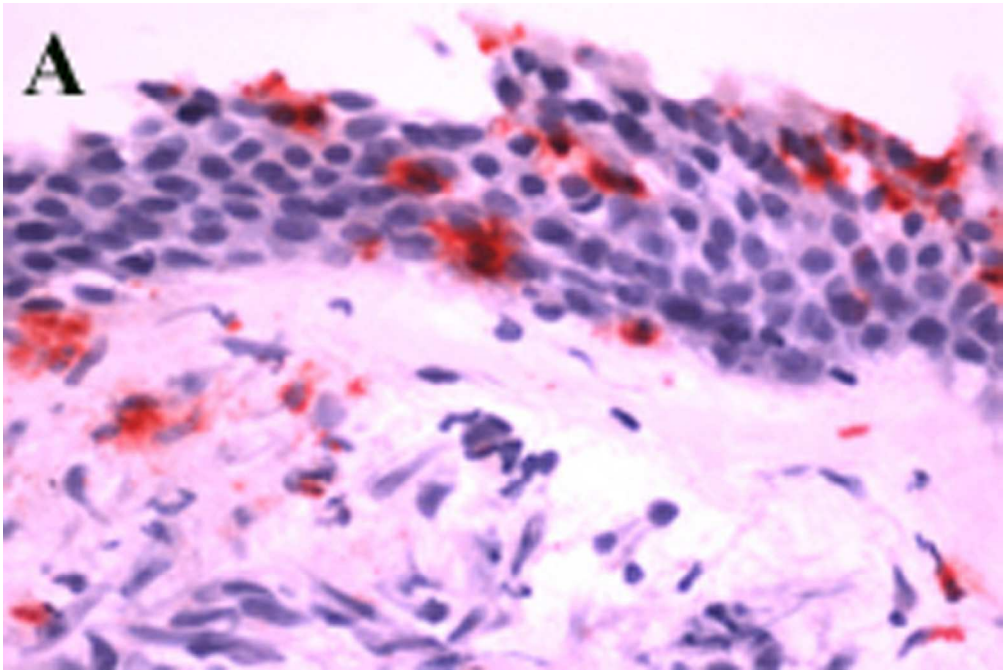
FIGURE 3. Changes in counts of A, epithelial and B, subepithelial neutrophils and CD68⁺ cells from baseline to day 4 post-infection in bronchial biopsies of normal and asthmatic subjects. The data are expressed as change in the number of positive cells per 0.1 mm² of epithelium or per mm² of subepithelium. Dots show individual change in counts and horizontal bars show median values (Mann Whitney U test).

FIGURE 4. Correlations between the numbers of subepithelial A, CD45⁺ cells at day 4 and peak sputum virus load at day 3 after infection, B, neutrophils or C, CD68⁺ cells at day 4 and BAL virus load at day 4 in all subjects taken together (asthmatics, ●; normals, ○) (Spearman rank correlation).

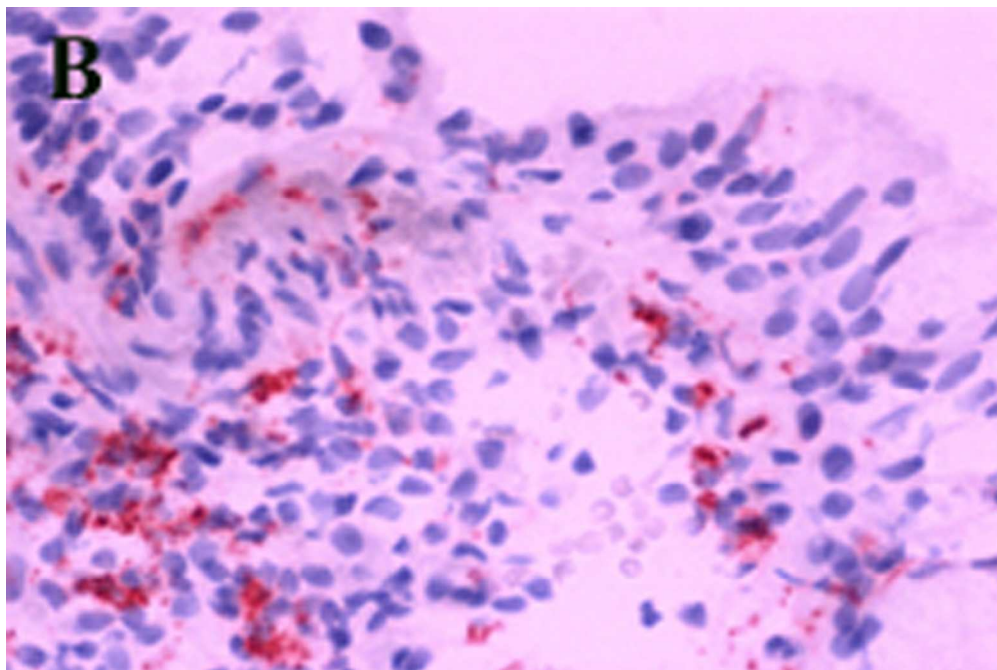
FIGURE 5. In asthmatic subjects alone, correlations A, between total chest symptom score days 0-14 after RV16 infection period and counts of epithelial CD4⁺ cells, B, between fall in PC₁₀ (Log₂) and counts of subepithelial neutrophils at day 4, between PC₁₀ at day 6 and counts of subepithelial C, CD3⁺, D, CD4⁺ and E, CD8⁺ cells at day 4 and F, between PC₂₀ at day 6 and counts of subepithelial CD20⁺ cells at day 4 (Spearman rank correlation, n = 10 for all).

FIGURE 6. In asthmatic subjects at day 4 after RV infection, correlations A, between maximum fall in FEV₁ % and counts of epithelial CD8⁺ cells and B, between maximum fall in PEF (%) and counts of subepithelial tryptase⁺ mast cells (Spearman rank correlation).

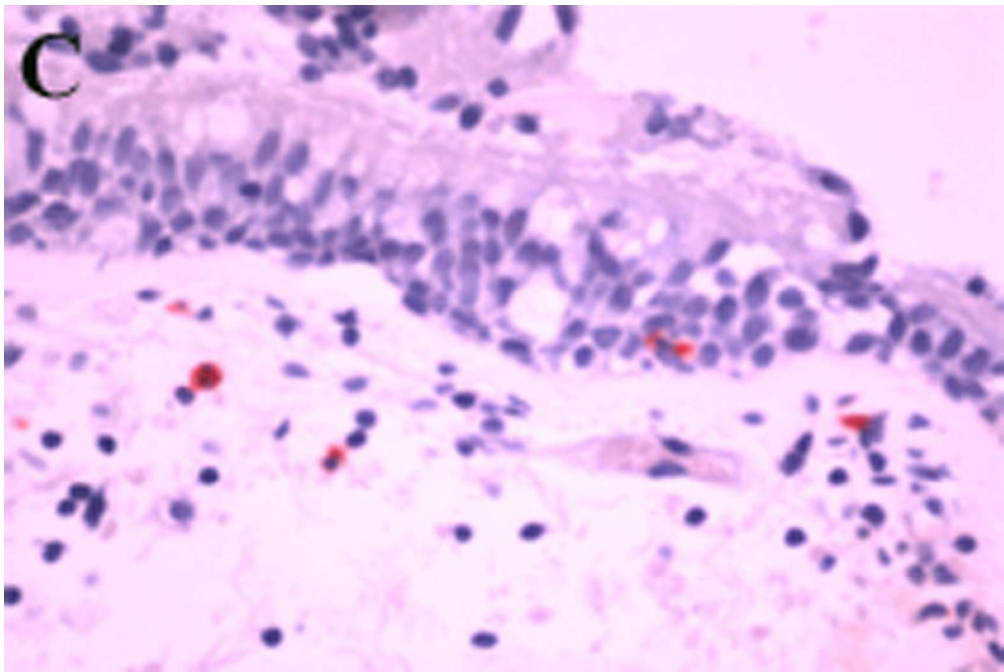
FIGURE 7. Correlations between serum IgE and counts of subepithelial EG2⁺ eosinophils in asthma at A, baseline and B, day 4 after RV infection (Spearman rank correlation).



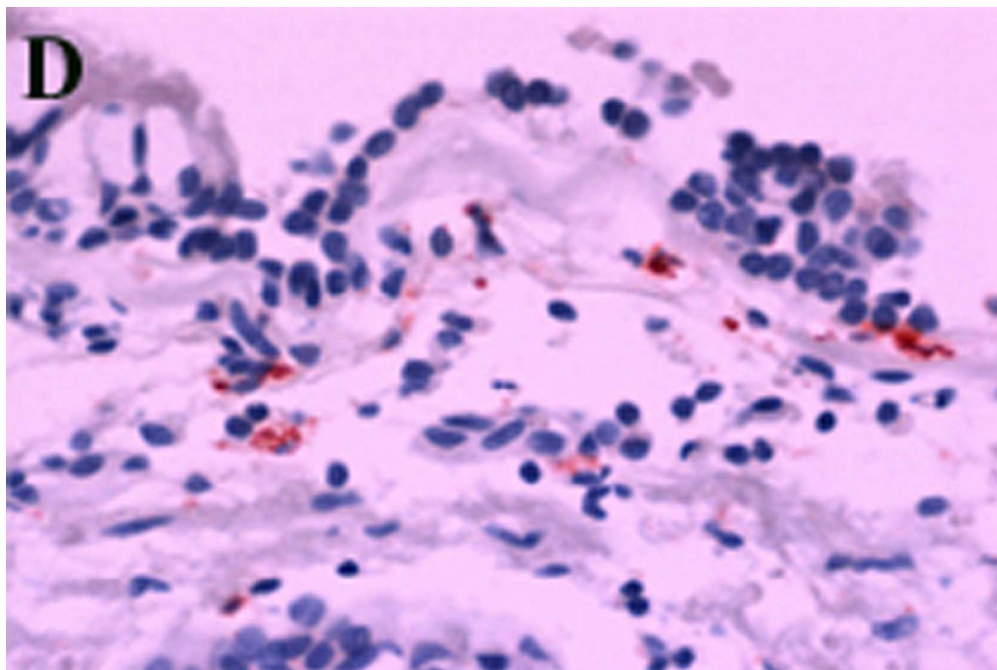
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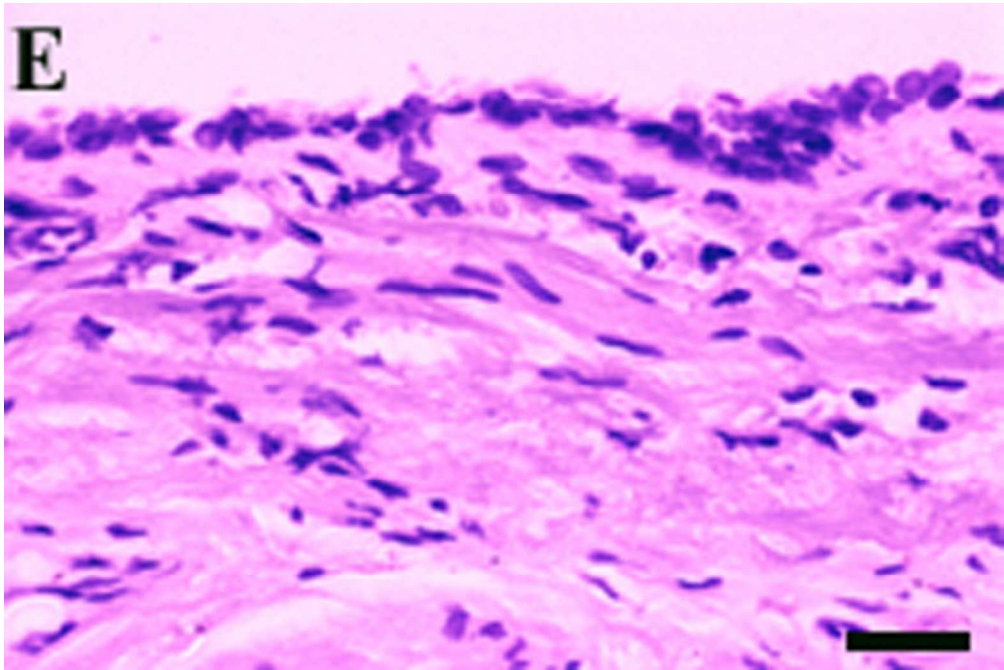
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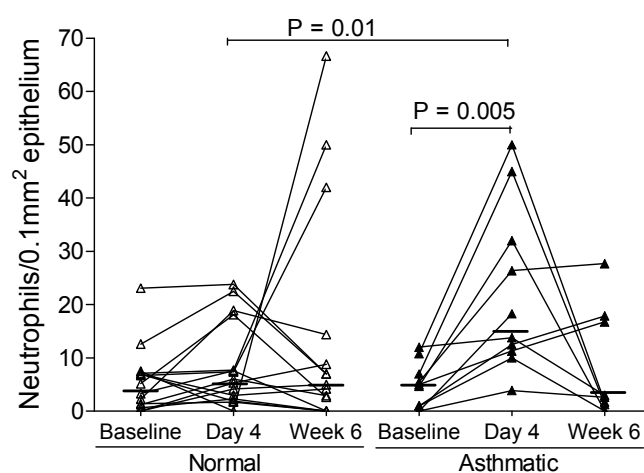
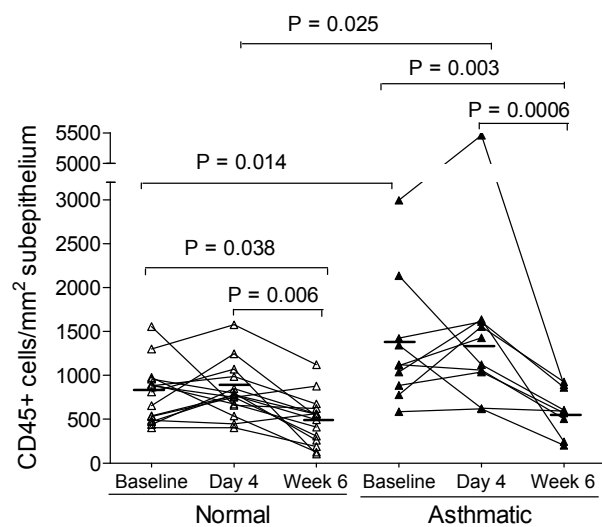
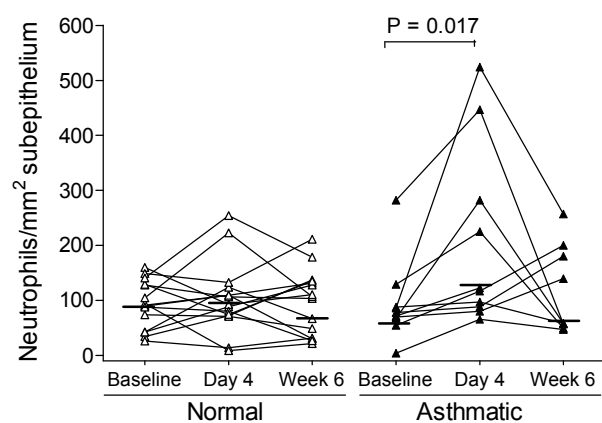
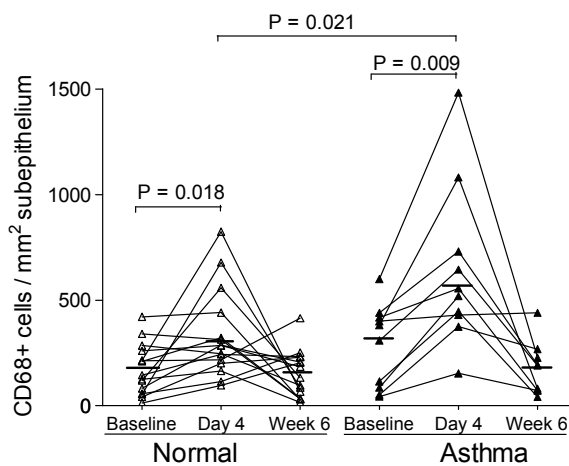
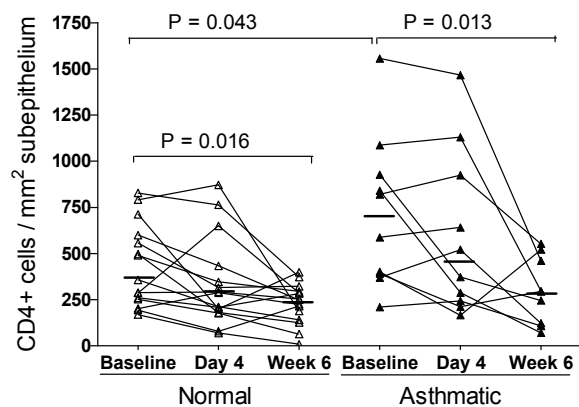
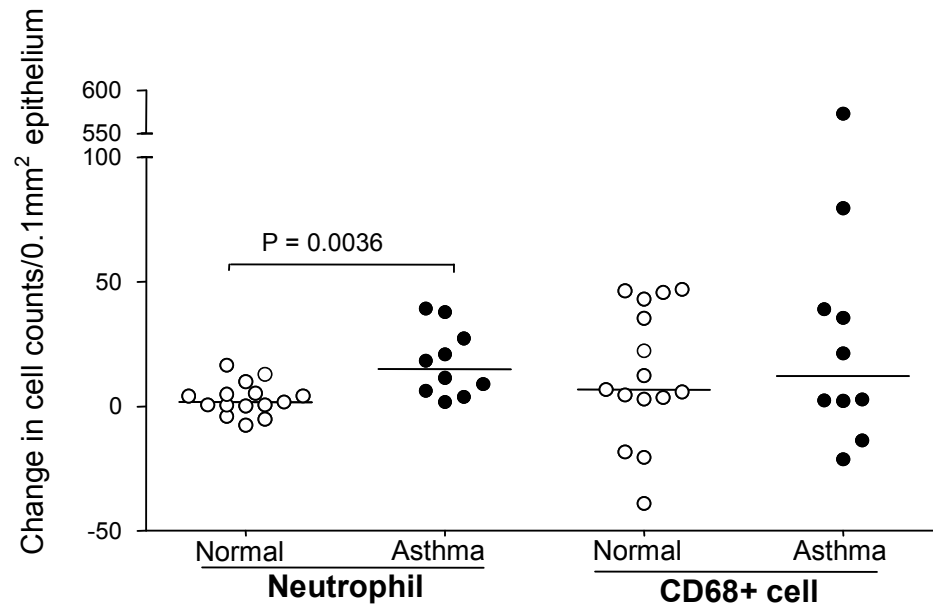
Figure 2A-E**A****B****C****D****E**

Figure 3A,B

A



B

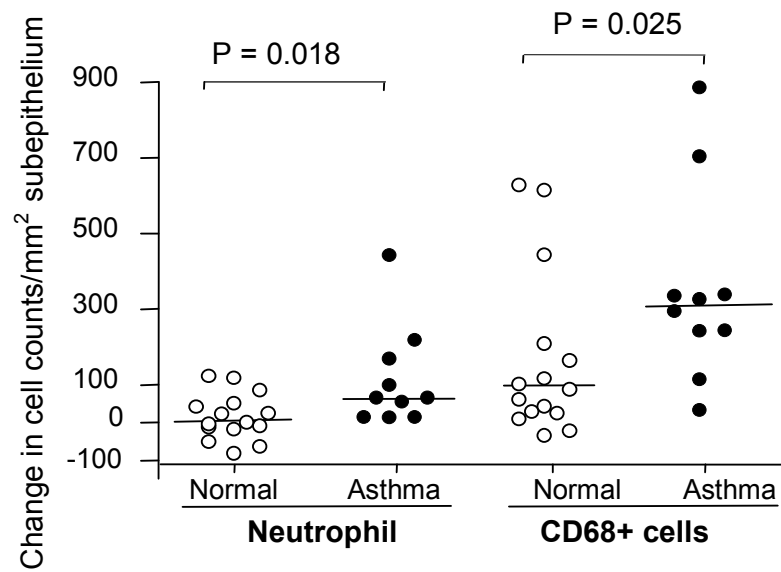


Figure 4A-C

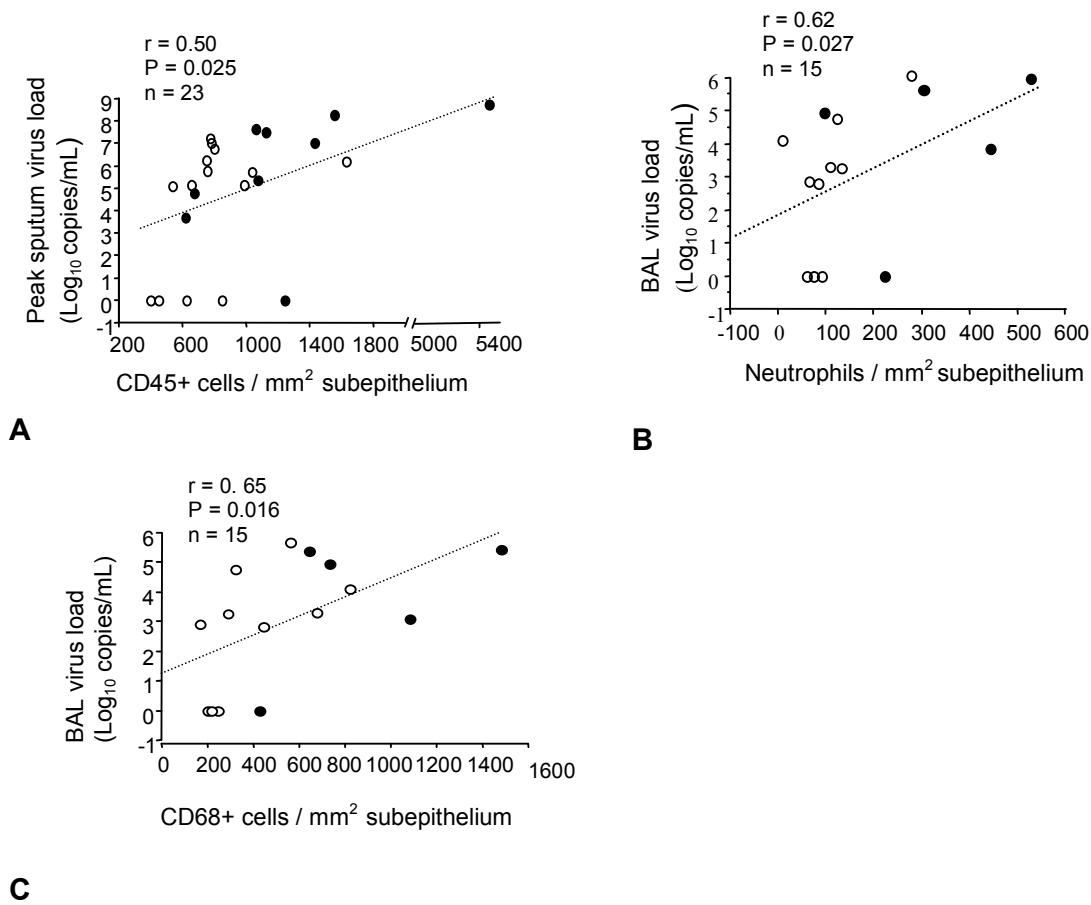


Figure 5A-F

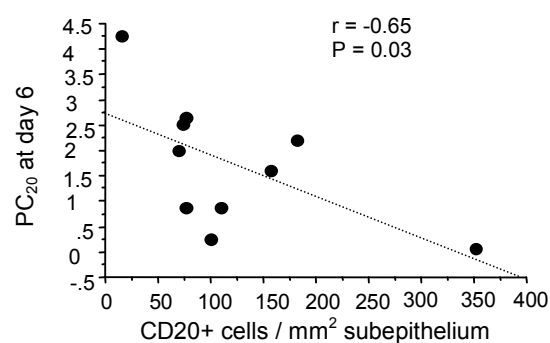
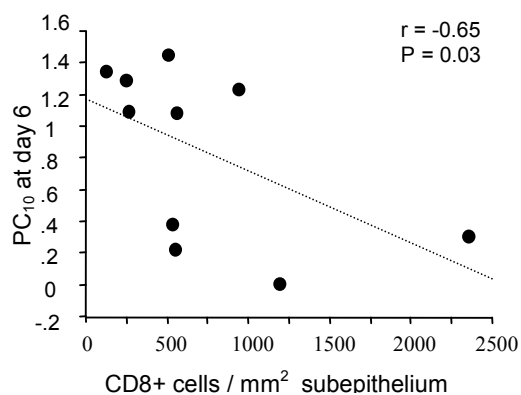
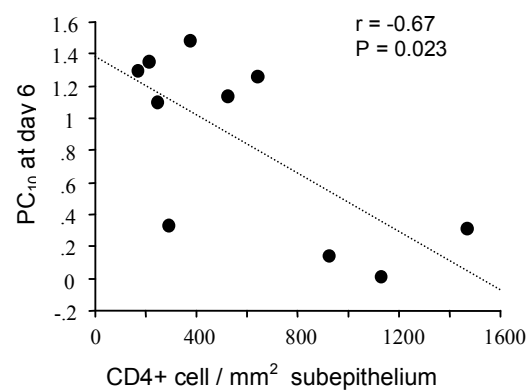
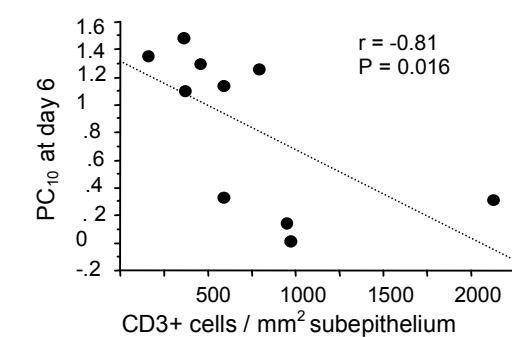
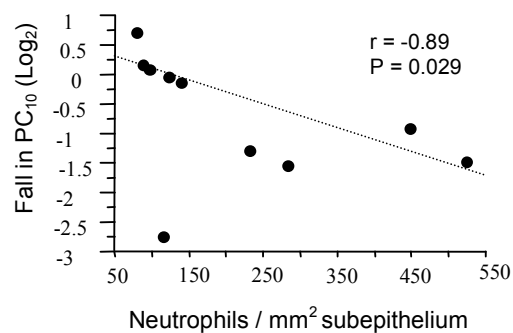
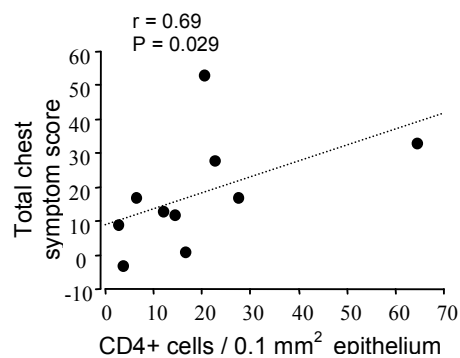
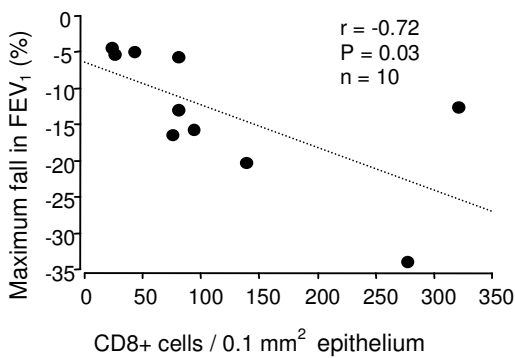
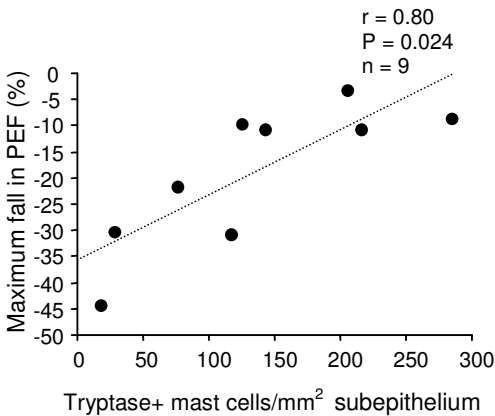


Figure 6A, B

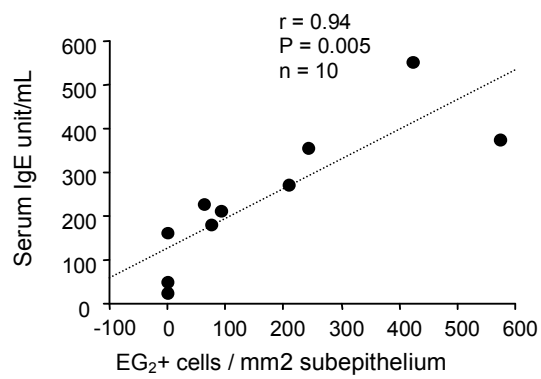
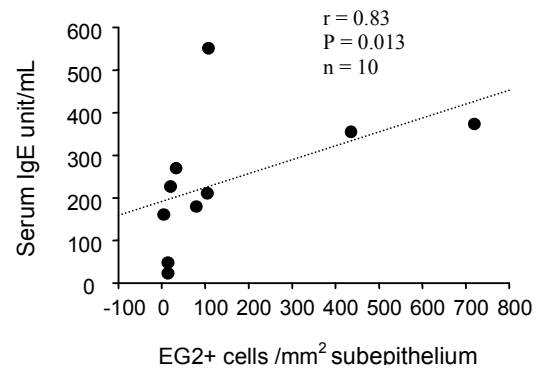


A



B

Figure 7A, B

**A****B**

Airway inflammation and illness severity in response to experimental rhinovirus infection in asthma

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Online data supplement

Subjects: we here extended our investigation of the same 25 non-smokers reported previously.² Those were human RV16 neutralizing antibody sero-negative and comprised of: 10 atopic asthmatics and 15 normal non atopic adults (Table 1).

Clinical and atopic status were defined by questionnaire, skin prick testing, serum IgE and lung function testing including PEF, FEV₁, forced vital capacity (FVC).² The normal healthy subjects were taking no medication. Asthmatics were prescribed inhaled short-acting β_2 -agonists only. Subjects were free of common cold symptoms for at least 6 weeks prior to the study start.

Histamine challenge. Bronchial reactivity was measured by histamine challenge at baseline, on day 6 after infection and convalescence. For bronchodilator reversibility, sputum induction and histamine challenge subjects used a Vitalograph Dry Wedge Bellows Spirometer. Histamine challenge (maximum concentration 32 mg/ml) was performed according to guidelines by using the 2-min tidal breathing method.¹ The asthmatic group was required to have a PC20 < 8 mg/ml, and the normal group had > 8 mg/ml. The provocative concentration of histamine causing a 20% reduction in FEV₁

(PC20) was calculated,¹ and as eight normal subjects did not achieve a 20% reduction, a PC10 was also calculated for both groups.

Skin Prick Testing and Serum IgE. Atopy was determined by skin prick testing to common aeroallergens: six grass pollen mix; house dust mite; cat; dog; *Aspergillus fumigatus*; *Cladosporium herbarum*; *Alternaria alternata*; and birch, three tree and nettle pollen (ALK Abello). One wheal 3 mm greater than negative control or total IgE > 110 units/ml was considered diagnostic of atopy.²

Virologic confirmation of RV16 infection

RV16 infection was confirmed by at least one of the following: positive standard or qPCR for RV or positive culture of RV16 from induced sputum obtained on days 3 and 7 and bronchoalveolar lavage (BAL) on day 4; sero-conversion was defined as a titre of serum neutralising antibodies to RV16 of at least 1:4 at 6 weeks.²

Diary Cards for Symptom Scores and Spirometry Recording. Symptom assessment was by daily diary cards, for 2 weeks before, during, and 6 weeks after infection. The daily cold score was summated from individual scores (sneezing, headache, malaise, chilliness, nasal discharge, nasal obstruction, sore throat, cough, fever) graded 0 (absent) to 3 (severe). The daily chest score was calculated from symptom scores (cough on waking; wheeze on waking; daytime cough; daytime wheeze; daytime shortness of breath; nocturnal cough, wheeze or shortness of breath), graded 0–3. The same diary cards recorded medication usage and home spirometry (microDL; MicroMedical) recording the best of three recordings of PEF and FEV1.²

Immunohistochemistry (IHC): EnVision-alkaline phosphatase technique (Dako Ltd, Cambridge, UK) was used to label CD45+ pan leukocyte/inflammatory cells, EG2+ eosinophils, neutrophil elastase+ neutrophils, tryptase+ mast cells and CD68+ monocytes/macrophages. EnVision peroxidase staining method (Dako) was used to identify CD3+, CD4+, CD8+ T- and CD20+ B-lymphocytes as previously described but with minor

modification.³ 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-CD45 (M0701), neutrophil elastase (M0752), tryptase mast cell (M0752), CD3+ (M0835), CD4+ (M0716), CD8 (M0707) CD20 (M0755) and CD68 (M0876). Mouse anti-EG2 (EG2) was from Pharmacia & Upjohn Ltd, Milton Keynes, UK, The immunostaining procedures for detecting the phenotypes of inflammatory cells were conducted by Techmate 'Horizon' automated immunostainer (LJL Biosystems Inc, USA).

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 1.5 software. 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 from each bronchoscopy were measured and counted, respectively. Then the total counts were divided by total area to normalise the counts as the number of cells per unit. 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 mm² of the subepithelium and per 0.1 mm² epithelium. 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 4 (GraphPad Software, Inc) softwares.

One way ANOVA followed by the unpaired Student's t-test was used for the analyses of age, serum IgE, lung function and histamine PC₂₀ data between groups. In respect of cell counts, these data were non-normally distributed and differences between groups were assessed first using the Kruskal-Wallis test, which, if significant, was followed by Wilcoxon matched pairs test within group between baseline and infection and Mann-Whitney U-test between groups at baseline and infection. The coefficient of variation ($CV = SD/mean \times 100$) was used to express the error of repeat counts. A P-value of < 0.05 was accepted as indicating a significant difference. Spearman's rank correlation was used as a test for correlations between the numbers of specific types of inflammatory cell and physiologic/clinical data. We wished to examine the relationships between virus load and the inflammatory response and believe that this is relevant in the non-asthmatic subjects also. Including the controls allowed us to examine these relationships in greater numbers of subjects and therefore avoid the possibility of missing significant correlations due to lack of power.

References

1. Sterk PJ, Fabbri LM, Quanjer PhH et al. Airway responsiveness. Standardized challenge testing with pharmacological, physical and sensitizing stimuli in adults. Report working party "Standardization of Lung Function Tests". European Community for Steel and Coal. Official statement of the European Respiratory Society. *Eur Respir J.* 1993;6 (Suppl 16):53-83.
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3. O'Shaughnessy T, Ansari TW, Barnes NC et al. Inflammation in bronchial biopsies of subjects with chronic bronchitis: inverse relationship of CD8⁺ T lymphocytes with FEV₁. *Am J Resp Crit Care Med.* 1997;155:852-857.