INTRODUCTION
Severe asthma represents approximately 10% of asthma but disproportionately impacts the mortality, morbidity and costs of asthma.1 Eosinophil inflammation has long been implicated in severe asthma, with several reports suggesting a more prominent role in a late onset phenotype.2–5

Eosinophil trafficking into the airways is a complex process likely regulated by a host of cells and chemotactic factors, including interleukin 5 (IL-5), eotaxins 1–3 (CCL11, 24 and 26) and regulated on activation normal T-cell expressed and secreted (RANTES) among others.6 IL-5 stimulates proliferation, differentiation and survival of eosinophils in the bone marrow and contributes to airway inflammation.7 Three recent studies of monoclonal antibodies to IL-5 in eosinophil-predominant asthma demonstrated reductions in blood/sputum eosinophils and improved asthma control.5,7–9 Yet, despite a general reduction in eosinophils, tissue eosinophils remained and eosinophil exacerbations still occurred.3,10 Thus, it is likely additional factors are involved.

Eotaxin 1–3 are CC chemokines of divergent structure which engage the CCR3 receptor and stimulate eosinophil chemotaxis. IL-4 and IL-13 induce eotaxins in vitro through activation of the IL-4Rα receptor/STAT-6 pathway, a process generally reported to be attenuated by corticosteroids (CS) in vitro.11–13 All three eotaxins have variably been reported to be increased in epithelial cells from endobronchial biopsies of patients with mild asthma, and in response to allergen challenge.14–17 However, their relative contribution to asthma severity, control or eosinophilic phenotypes remains poorly understood.

We hypothesised that epithelial eotaxin expression would contribute to eosinophil trafficking towards the airway lumen in association with asthma severity, control and eosinophilic phenotypes.
severity/control and in relation to airway eosinophilia. mRNA and protein expression for the three eotaxin isoforms was evaluated in freshly harvested bronchial brushing cells (primarily airway epithelial cells (AECs)) and bronchoalveolar lavage (BAL) cells/fluid from patients with asthma ranging in severity and normal controls (NCs). The relationship of this expression to physiological and clinical markers of severity/control, luminal eosinophilic inflammation and age at asthma onset was addressed.

**METHODS**

**Study participants**
Subjects were recruited as part of the National Heart Lung and Blood Institute’s Severe Asthma Research Program (SARP). Asthma was defined using American Thoracic Society criteria and subjects divided into mild, mild/moderate and severe asthma as previously described.\(^{18}\) See online supplement for details.

**Bronchoscopy with airway brushing and lavage**
Bronchoscopy was performed as previously described.\(^{19} 20\) See online supplement for details.

**Sputum induction and processing**
Sputum was processed according to the method of Fahy et al.\(^{21}\) Please see online supplement for details.

**Real-time quantitative PCR analysis**
Eotaxin 1–3 mRNA expression from epithelial and BAL cells was determined by reverse transcription and quantitative real-time PCR, as previously described. Please see online supplement for details.

**Enzyme-linked immunosorbent assays**
Eotaxin protein levels in AEC and BAL cell lysates and BAL fluid were measured by ELISA with antibodies from R&D Systems (Minneapolis, Minnesota, USA) by ELISA Tech (Aurora, Colorado, USA), as described in the online supplement.

**Eotaxin 1–3 stability to the mast cell protease tryptase**
Eotaxin 1–3 (R&D Systems) final concentration 500–700 pg/ml in phosphate-buffered saline were incubated with or without tryptase (recombinant lung, 13 mUnits/ml; Promega, Madison, Colorado, USA) and heparin (0.33 units/ml) for 20 h at room temperature. Samples were quantified for eotaxin 1–3 by ELISA as above.

**Corticosteroid effects on eotaxin expression in vitro**
Primary human bronchial epithelial cells were cultured under air–liquid interface (ALI) as previously described.\(^{22}\) From day 0 of ALL, cells were stimulated with IL-15 (10 ng/ml) every 48 h. On day 6, in addition to IL-15, dexamethasone (100 nM) was added to the cells. Cells were harvested on day 8 and analysed for eotaxin-2 and eotaxin-3 protein using the above methods.

**RESULTS**

**Subject characteristics**
A total of 100 subjects underwent bronchoscopy, including 27 NCs, 9 patients with mild asthma, 20 with mild to moderate asthma on inhaled corticosteroids (mild/mod+ICS) and 44 with severe asthma (table 1). The groups did not differ by sex, race or age at onset but there was a significant difference in age (p=0.004), with patients with severe asthma older than NCs and those with mild/mod+ICS. As expected, patients with severe asthma had the lowest pre-bronchodilator forced expiratory volume in 1 s (FEV\(_1\)%)\(^{6.4}\), but the greatest reversal post bronchodilator. Twenty-one (48%) of the patients with severe asthma used regular systemic corticosteroids (SCS). Atopy was less common in NCs (overall p=0.002), but there were no differences among asthmatic groups.

**Epithelial eotaxin-1 expression is low and does not differ by subject type**
Eotaxin-1 mRNA levels were extremely low and did not differ according to asthma or severity (n=74, p=0.76, data not shown). Eotaxin-1 protein was undetectable in epithelial cell lysates from six NCs, seven patients with severe asthma, four with mild/mod+ICS asthma and one with mild asthma. Further studies of eotaxin-1 were not performed.

**Statistical analyses**
Epithelial and BAL cell mRNA and protein data were generally not normally distributed and subsequently analysed non-parametrically. The Kruskal–Wallis variation of the Wilcoxon rank sum test compared eotaxin 1–3 expression between groups. Intergroup comparisons were made by Wilcoxon rank sum when the overall p value was <0.05. Bonferroni correction was applied with a p value of 0.008 considered significant. Spearman’s rho was used for correlations. In addition, a multivariable non-parametric regression of medians was modelled to test the association between eotaxin-2 protein with age of asthma onset, adjusting for severity, gender, age and age at enrolment.\(^{25}\) A p value <0.05 was regarded as significant. Racial data were analysed by Fisher’s exact t test, taking into consideration cells with ‘zero’ values. Data are presented as medians with 25th–75th percentile ranges. Graphs include maximums and minimums. Data are occasionally presented on a log\(_{10}\) scale for illustrative purposes. Statistics were performed using JMP8 software (SAS).

| Table 1 | Subject characteristics |
|---|---|---|---|---|---|
| **Age (±SD)** | Normal | Mild | Mild/mod+ICS | Severe | p Value |
| Age (±SD) | 34±12 | 33±15 | 33±11 | 43±12 | 0.004 |
| Race*, n (%) | 22/2/3 (81/7/11) | 8/0/1 (89/0/11) | 12/4/4 (60/20/20) | 34/7/3 (77/16/7) | 0.42 |
| Sex (M/F), n (%) | 14/13 (52/48) | 2/7 (22/78) | 6/14 (30/70) | 11/33 (25/75) | 0.10 |
| FEV\(_1\)% pred (±SD) | 100±2 | 91±3 | 90±6 | 56±3 | <0.0001 |
| FEV\(_1\) reversal % (±SEM) | 6±0.8 | 9±3 | 11±2 | 33±5 | <0.0001 |
| Eosinophils in sputum, % (25th–75th percentiles) | 0.9 (0.3–8.3) | 5 (0.3–13.8) | 1 (0.4–2.8) | 6.9 (1.8–31.3) | 0.0012 |
| Age at onset, median (25th–75th percentiles) | NA | 12 (5–40) | 5 (2–14) | 8 (2–32) | 0.26 |
| Atopy (Y/N) | 14/13 | 9/0 | 16/2 | 29/8 | 0.002 |

* *Caucasian/African American/other.

FEV\(_1\)% pred, forced expiratory volume in 1 s % predicted; ICS, inhaled corticosteroid; NA, not applicable.
Epithelial eotaxin-2 expression is increased in severe asthma

AEC eotaxin-2 mRNA levels differed among groups (n=72, overall p value=0.0155) and were higher in severe than in mild asthma (figure 1A). AEC lysate eotaxin-2 protein was detectable in 19/25 patients with severe asthma, 5/13 with mild/mod+ICS asthma, 1/5 with mild asthma and 4/12 NCs (p=0.0003, figure 1B). Patients with severe asthma expressed higher eotaxin-2 protein levels than NCs (p=0.006) and those with mild/mod+ICS asthma (p=0.008). Patients with asthma on SCS expressed higher eotaxin-2 protein and mRNA compared with those not on SCS (protein: no SCS=5 (5–28) vs SCS=58 (5–151) pg/ml, p=0.006; mRNA/per glyceraldehyde 3-phosphate dehydrogenase (GAPDH): no SCS=0.15 (0.07–0.27) vs SCS=0.34 (0.18–0.82) pg/ml, p=0.004, respectively). Given the high levels of eotaxin-2 protein in those with severe asthma on SCS, the effect of the addition of CS on eotaxin-2 protein was evaluated in ALI-cultured epithelial cells. The addition of dexamethasone decreased eotaxin-2 protein levels by 25±6%, p<0.0001. There were no differences by subject group. AEC eotaxin-2 mRNA and protein modestly correlated (r=0.40, p=0.01).

Epithelial eotaxin-3 mRNA is increased in severe asthma

AEC eotaxin-3 mRNA also differed across groups (overall p value=0.0003, figure 2), being higher in severe asthma compared with NCs (p=0.0001) and mild/mod+ICS (p=0.002). Eotaxin-3 protein from 26 AEC lysates was detectable in only five subjects (3/11 with severe asthma). Given the small lysate sample volumes and the low eotaxin-3 protein levels, further eotaxin-3 protein measurements were not performed. Similar to eotaxin-2, eotaxin-3 mRNA levels indexed to GAPDH were higher in those on SCS (0.3 (0.11–0.73)) compared with those not on SCS (0.12 (0.02–0.55)) (p=0.04). Eotaxin-2 and eotaxin-3 mRNA levels only modestly correlated (r=0.24, p=0.04). Similar to eotaxin-2, the impact of CS on eotaxin-3 protein expression was also evaluated in asthmatic epithelial cells in ALI. Dexamethasone decreased eotaxin-3 protein levels by 24±12%, p<0.0001 (n=11), without difference by severity.

Impact of mast cell tryptase on eotaxin 1–3 protein levels

Eotaxin-2 has previously been reported to be resistant to mast cell proteases, known to be present in the airway epithelium.24 To determine whether, like eotaxin-1, eotaxin-3 might also be susceptible to tryptase and explain the lower protein levels, known levels of eotaxin 1–3 were incubated with tryptase and heparin. Tryptase selectively reduced eotaxin-1 and eotaxin-3 protein levels, while having little effect on eotaxin-2 protein. Eotaxin-1 was reduced by 85%, SEM±0.9% and eotaxin-3 by 70%, SEM±11.5%. Under the same conditions eotaxin-2 remained stable (5% reduction, SEM±1.98%).

BAL cells express high levels of eotaxin-2 mRNA/protein but with variable relationship to severity

BAL cells expressed high levels of eotaxin-2 mRNA, being 100-fold higher than AEC levels (table 2). Unlike epithelial
Brushings, the levels did not differ by subject group (overall \( p = 0.83 \)). Eotaxin-2 protein levels in BAL cell lysates and uncentranted BAL fluid samples were relatively high and strongly correlated (\( r = 0.74, p = 0.0002, n = 28 \)). Given the limited BAL cell lysate volume and the strong correlations between BAL fluid and BAL lysates, further comparisons among groups were made on BAL fluid samples (\( n = 75 \)). BAL fluid eotaxin-2 levels differed among groups (\( p = 0.046 \)). In contrast to epithelial cells, BAL fluid eotaxin-2 protein levels were lower in severe asthma than in NCs (\( p = 0.006 \)), but not different from the two milder asthma groups (\( p = 0.28, n = 23 \) severe vs milder asthma). In contrast, AEC eotaxin-2 protein also inversely correlated with FEV1% predicted (\( r = -0.33, p = 0.013 \)). There were no correlations of eotaxin levels in any compartment with reversibility. However, higher eotaxin-2 mRNA and protein associated with three or more oral CS bursts in the previous year (\( p = 0.006 \) and \( p = 0.004 \), respectively) (table 5). Higher eotaxin-3 mRNA was marginally associated with a history of three or more CS bursts (\( p = 0.051 \)). Higher eotaxin-2 (mRNA and protein) and eotaxin-3 mRNA levels all associated with an emergency room visit in the previous year (table 5). BAL fluid eotaxin-2 mRNA or protein levels did not correlate with any control characteristics.

**Relation to eosinophilia and age at onset**

Evaluating patients with asthma only, AEC eotaxin-2 and eotaxin-3 mRNA levels inversely correlated with FEV1% predicted (\( r = -0.39, p = 0.0005 \) and \( r = -0.31, p = 0.005 \) respectively). AEC eotaxin-2 protein also inversely correlated with FEV1% predicted (\( r = -0.35, p = 0.013 \)). There were no correlations of eotaxin levels in any compartment with reversibility. However, higher eotaxin-2 mRNA and protein associated with three or more oral CS bursts in the previous year (\( p = 0.006 \) and \( p = 0.004 \), respectively) (table 5). Higher eotaxin-3 mRNA was marginally associated with a history of three or more CS bursts (\( p = 0.051 \)). Higher eotaxin-2 (mRNA and protein) and eotaxin-3 mRNA levels all associated with an emergency room visit in the previous year (table 5). BAL fluid eotaxin-2 mRNA or protein levels did not correlate with any control characteristics.

**DISCUSSION**

This study identified differential expression of the three eotaxin isoforms from freshly brushed epithelial cells of patients with severe asthma compared to mild asthma and NCs. Eotaxin-2 protein levels were lower in severe asthma compared to mild asthma and NCs, whereas eotaxin-3 mRNA levels were higher in severe asthma compared to mild asthma and NCs. These findings suggest that eotaxin-2 and eotaxin-3 may play different roles in the pathogenesis of asthma, with eotaxin-2 possibly playing a protective role against severe asthma and eotaxin-3 possibly contributing to the development of severe asthma.

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**Table 2 Bronchoalveolar lavage eotaxin levels by severity**

<table>
<thead>
<tr>
<th>Eotaxin-2 mRNA/GAPDH (n=42)</th>
<th>Normal</th>
<th>Mild</th>
<th>Mild/mod+ICS</th>
<th>Severe</th>
<th>Overall p</th>
</tr>
</thead>
<tbody>
<tr>
<td>19 (1.9–58.7)</td>
<td>24.7 (4.6–310.9)</td>
<td>73.5 (3.9–502.2)</td>
<td>10.3 (3.4–47.5)</td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td>Eotaxin-2 cell lysate protein (pg/million cells) (n=19)</td>
<td>1178 (135–3134)</td>
<td>816 (370–3263)</td>
<td>795 (456–1195)</td>
<td>511 (315–2670)</td>
<td>0.81</td>
</tr>
<tr>
<td>Eotaxin-2 BAL fluid (pg/ml) (n=75)</td>
<td>309 (87–654)</td>
<td>221 (41–425)</td>
<td>196 (37–435)</td>
<td>77 (30–222)</td>
<td>0.046</td>
</tr>
<tr>
<td>Eotaxin-1 mRNA/GAPDH (n=40)</td>
<td>0.0001 (0–0.04)</td>
<td>0.0001 (0–0.1)</td>
<td>0 (0–0.04)</td>
<td>0 (0–0.0001)</td>
<td>0.62</td>
</tr>
<tr>
<td>Eotaxin-3 mRNA/GAPDH (n=40)</td>
<td>0.0001 (0–0.08)</td>
<td>0.0001 (0–0.15)</td>
<td>0.42 (0–0.11)</td>
<td>0.05 (0.15–0.25)</td>
<td>0.14</td>
</tr>
</tbody>
</table>

All values are median (25th–75th percentiles).

BAL, bronchoalveolar lavage; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
asthma and normal controls. While eotaxin-1 expression was negligible, eotaxin-2 and eotaxin-3 mRNA were higher in patients with asthma and higher in those with severe asthma. At the protein level, eotaxin-2 was measurable and significantly higher in patients with severe asthma compared with those with milder asthma. Eotaxin-2 and eotaxin-3 expression related to lower lung function, asthma exacerbations and importantly eosinophilia. Eotaxin-2 protein was strongly predicted by increasing age at asthma onset, suggesting a distinct role for eotaxin-2 in the eosinophilia associated with later onset asthma.\(^4\) Unlike epithelial cells, BAL cells constitutively expressed eotaxin-2 mRNA and protein, with generally lower BAL fluid eotaxin-2 protein levels in severe asthma.

In contrast to eotaxin-2 and 3, eotaxin-1 mRNA expression in epithelial and BAL cells was nearly undetectable. This result differs from previous studies.\(^12\)\(^15\)\(^26\)\(^27\) The reasons for this difference are not clear, although highly quantifiable quantitative PCR and ELISA were used here. Interestingly, a previous study did not find eotaxin-1 expression in primary human epithelial cells in either submersed or ALI cultures.\(^25\)

The current study suggests eotaxin-2 and 3 are relevant to luminal migration of eosinophils. Epithelial eotaxin-2, at the biologically relevant protein level, was quantifiable, differentiated severe from milder asthma and was associated with eosinophilic inflammation. This is consistent with a previous report of increased eotaxin-2 and 3 (as measured by immunohistochemistry) in the epithelium of patients with asthma following allergen challenge.\(^14\) However, this study expands on those results by evaluating a broad range of carefully phenotyped patients with chronic asthma recruited through SARP.\(^\)\(^23\)\(^24\)\(^25\)\(^26\)\(^27\) The expression in BAL cell lysates strongly correlated with BAL fluid eotaxin-2 protein levels in severe asthma. Epithelial eotaxins in relation to exacerbations

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Epithelial eotaxins in relation to exacerbations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No ER visit</td>
</tr>
<tr>
<td>Eotaxin-2 protein (pg/million cells)</td>
<td>5 (5–26)</td>
</tr>
<tr>
<td>Eotaxin-2 mRNA/GAPDH</td>
<td>0.16 (0.07–0.27)</td>
</tr>
<tr>
<td>Eotaxin-3 mRNA/GAPDH</td>
<td>0.10 (0.03–0.38)</td>
</tr>
</tbody>
</table>

All values median (25th–75th percentiles).

CS, corticosteroid; ER, emergency room; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
reflect the BAL cell lysates. The extremely low levels of eotaxin-1 mRNA/protein and eotaxin-3 mRNA in the BAL cells suggest that analysing greater numbers of these small volume samples would not add to the findings. Finally, sputum samples were not available on every subject due to FEV1 restrictions and inability to produce sputum. Greater numbers of sputum samples may have improved our ability to address the specificity of the eotaxin-2 increases to late onset eosinophilic asthma.

In summary, this study shows significant increases in eotaxin-2 and 3 in the epithelium in human severe asthma in association with poor asthma control and sputum eosinophilia. Higher eotaxin-2 protein levels (in the epithelial and BAL cell compartment) were strongly associated with older age at asthma onset, and marginally with the eosinophilia associated with late onset asthma. Whether eotaxin-2 plays a specific contributory role in this persistent and often CS-refractory eosinophilic asthma awaits further study.

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Competing interests None.

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