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Clinical Implications: Our findings suggest that airway neutrophilia in paediatric severe, therapy resistant asthma may be a beneficial host response, and thus should be enhanced not reduced therapeutically.

Capsule Summary: Children with STRA have increased intra-epithelial neutrophils which were associated with better lung function, higher ACT score and taking less inhaled corticosteroids. Furthermore, STRA is characterised by augmented epithelial responses to IL-17A, with increased IL-17RA expression and IL-8 production.

Running head: Neutrophils and IL-17A in paediatric severe therapy resistant asthma

Key words: Paediatric asthma, severe therapy resistant asthma, IL-17A, IL-17A receptor, neutrophils

Author contributions: Conception and design: CA, AA, SS, CL; Analysis and data acquisition: CA, AA, PN, Interpretation of data: CA, AA, PN, AB, SS, CL; Drafting the manuscript for important intellectual content: CA, AB, SS, CL
ABSTRACT

BACKGROUND: Neutrophils and IL-17A have been linked mechanistically in models of allergic airways disease and have been associated with asthma severity. However, their role in paediatric asthma is unknown.

OBJECTIVES: To investigate the role of neutrophils and the IL-17A pathway in mediating paediatric severe therapy resistant asthma (STRA).

METHODS: Children with STRA (n=51, age 12.6 (6 -16.3) years) and non-asthmatic controls (n=15, age 4.75 (1.6-16) years) underwent clinically indicated fiberoptic bronchoscopy, bronchoalveolar lavage (BAL), endobronchial brushings and biopsy. Neutrophils, IL-17A and IL-17RA expressing cells and levels of IL-17A and IL-22 were quantified in BAL and biopsies and related to clinical features. Primary bronchial epithelial cells (PBECs) were stimulated with IL-17A and/or IL-22, with and without Budesonide.

RESULTS: Children with STRA had increased intra-epithelial neutrophils, which positively correlated with FEV₁ %predicted (r=0.43, p=0.008). Neutrophil-high patients also had better symptom control, despite lower dose maintenance inhaled steroids. Submucosal neutrophils were not increased in STRA. Submucosal and epithelial IL-17A positive cells and BAL IL-17A and IL-22 levels were similar in STRA and controls. However, there were significantly more IL-17RA positive cells in the submucosa and epithelium in children with STRA compared to controls (p=0.001). Stimulation of PBECs with IL-17A enhanced mRNA expression of IL-17RA and increased release of IL-8, even in the presence of Budesonide.

CONCLUSIONS: A proportion of children with STRA exhibit increased intra-epithelial airway neutrophilia that correlated with better lung function. STRA was additionally characterised by increased airway IL-17RA expression. These data suggest a potential beneficial rather than adverse role for neutrophils in paediatric severe asthma pathophysiology.
Word count: 247

ABBREVIATIONS

ACT  asthma control test
ASM  airway smooth muscle
BAL  bronchoalveolar lavage
BEGM bronchial epithelial growth medium
BMI  body mass index
ECM  extracellular matrix
ELISA enzyme-linked immunosorbent assay
FEV1 Forced expiratory volume in 1 second
GR  glucocorticoid receptor
HBE  human bronchial epithelium
PBEC primary bronchial epithelial cell
FMO  fluorescence minus one
PBMC peripheral blood mononuclear cells
PCR  polymerase chain reaction
RBM reticular basement membrane
STRA severe therapy resistant asthma
INTRODUCTION

Severe asthma is heterogeneous and can be divided into sub-phenotypes (1-3). Severe therapy resistant asthma (STRA) affects a small proportion of children with asthma and is characterised by persistent symptoms, acute severe exacerbations and/or fixed airflow obstruction despite treatment with high-dose steroid therapy, and after modifiable factors such as poor adherence and persistent allergen exposure have been addressed (4). Since Th2 mediated eosinophilic asthma generally responds well to steroid treatment, the lack of responsiveness to steroids in STRA suggests an alternative inflammatory pathway in these children. We have previously shown that children with STRA exhibit airway eosinophilia and remodelling, but a paucity of classical Th2 cytokines (IL-4, -5 and -13) (5).

In adults, airway neutrophils have been associated with increased asthma severity (1, 6, 7) but their functional role in mediating disease pathophysiology is unclear. Elevated levels of IL-17A have been reported in sputum, bronchoalveolar lavage (BAL) fluid and peripheral blood (8-11) in severe asthmatics and have been implicated in pathogenesis (10, 12). In contrast, little is known about the role of neutrophils and the IL-17 pathway in children with STRA. IL-17A can induce bronchial epithelial secretion of pro-inflammatory cytokines including the neutrophil chemoattractants GRO and IL-8. It is proposed that IL-17A can therefore induce neutrophilic airway inflammation and promote steroid resistance in severe adult asthma (13). Moreover, IL-17A may also be involved in airway remodelling. IL-17A has been reported to increase airway smooth muscle (ASM) contraction upon methacholine stimulation and drive migration of ASM cells in murine models of allergic inflammation and in asthmatic patients (9, 14, 15). IL-17A binds to the receptor subunits IL-17RA and C which are expressed on epithelial and mesenchymal cells as well as on some immune cell populations such as lymphocytes, dendritic cells and monocytes/macrophages in the lung (16). Mice that lack IL-17RA produce less CXCL1 and CXCL2 upon pulmonary challenge with Klebsiella pneumonias (17) and IL-17RA signalling is also necessary for host defence against Candida albicans.
In two mouse models, blocking IL-17R and IL-17RB reduced airway inflammation and airway hyperreactivity (14). Th17 cells also secrete IL-22, and IL-22 mRNA is elevated in PBMCs in paediatric severe asthma, and rhinitis (19). Although IL-22 has been described as a pro-inflammatory cytokine causing airway hyperreactivity in mice and remodelling in both epithelial and airway smooth muscle cells in humans, it has also been shown to reduce inflammation by suppressing cytokine production from epithelial cells. Taken together, data suggests IL-22 plays different roles in various phases of airway inflammation (20, 21).

We have previously reported that there was no increase in mucosal or BAL neutrophils in STRA. However, when re-examining biopsy slides, we noted that some but not all of these children had intra-epithelial neutrophils. We therefore hypothesised that intra-epithelial neutrophils, together with elevated IL-17A and IL-22, would be associated with worse asthma severity. We determined the extent of neutrophilic inflammation in a new cohort of children with STRA and investigated IL-17A and IL-22 levels and their cellular sources in BAL. We assessed the response of bronchial epithelial cells to IL-17A, IL-22 and steroids in STRA compared to controls. The findings were then related to key clinical features and airway remodelling and were confirmed in archived biopsies from an older cohort of children with STRA to look specifically at intra-epithelial neutrophils which have not previously been reported.
PATIENTS, MATERIAL AND METHODS

Subjects

Fifty-one school-aged children with STRA (22) (age 12.6 (6-16.3) years) were recruited from the Royal Brompton Hospital. They underwent detailed clinical assessments including spirometry, exhaled nitric oxide measurements and symptom scores followed by a clinically indicated bronchoscopy, endobronchial brushings, BAL and endobronchial biopsies to characterize airway pathology and develop customised treatment plans (4). All children had previously undergone a detailed assessment to ensure any modifiable factors such as poor adherence or persistent allergen exposure had been addressed prior to the bronchoscopy (23). Atopy was defined as at least 1 positive serum IgE (sIgE) RAST result (≥0.35 kUI/L) to aeroallergens (HDM, cat, dog, and grass pollen) and quantified as the sum of sIgE levels of these aeroallergens. 15 non-asthmatic control subjects (age 4.75 (1.6-16) years) were either (A) having a bronchoscopy to investigate upper airway symptoms and agreed to extra research samples being taken or (B) were undergoing general anaesthesia for cardiac catheterization and agreed to have a research bronchoscopy at the same time (see Table 1 and online repository (OR) Table E2 for details). The study was approved by the NRES Committee London - Chelsea, and informed parental consent and child assent were obtained.

In addition to the above mentioned cohort, findings relating to intra-epithelial inflammation were confirmed in biopsies obtained from a previously published cohort (reference (5)) of children with STRA (n=21) and controls (n=5). In all figures the latter will appear as grey symbols and their clinical details are described in detail in the OR and Table E1. Inclusion of both cohorts of children is of vital importance to the strength of the findings since we were able to replicate the finding even in this second cohort. There is no duplication of the data as we merely used the archived biopsy samples to examine neutrophils, which had not previously been examined in these samples. Further details of investigations are given in the Methods section in this article’s Online Repository at www.jacionline.org.
Epithelial culture and stimulation

Primary bronchial epithelial cells (PBECs) were seeded into tissue culture flasks containing BEGM and used at passage 3 for all experiments. PBECs were stimulated with recombinant human IL-17A (eBioscience, San Diego, CA) and IL-22 (eBioscience) alone, or to cells pre-treated with Budesonide (Breath Limited, UK) as stated in (24). Culture supernatants were collected after 24h and samples for mRNA extraction were harvested after 8h stimulation (see OR).

Quantification of cytokines

Cell culture supernatant was collected and cytokines analysed using ELISA and Milliplex human cytokine panel I and II (see OR).

Cytomix

BAL cytokine quantification was performed using Flowcytomix™ Human TH1/TH2/TH9/TH17/Th22 13 plex multiplex (eBioscience, San Diego, CA) (OR).

Flow cytometry

Lymphocytes used for compensations and fluorescence minus one (FMOs) were extracted from peripheral blood and cells from the airway lumen were obtained after centrifuge of broncho-alveolar lavage fluid. Cells were stained for extracellular markers CD3, CD4, CD8, CD161, γδTCR and intracellular IL-17A (eBiosciences, mouse anti-human 5µl per well) (OR).

RNA extraction and real-time PCR
Total RNA was extracted from epithelial cells using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized from 500ng of total RNA and analysed by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster city, CA) (OR).

**Histopathology**

Endobronchial biopsies were processed to paraffin. Five-micrometre sections were stained with haematoxylin and eosin and used to assess morphology and consecutive sections were used for Masson’s trichrome (Sigma Aldrich) for collagen staining, Congo red for staining of eosinophils and immunohistochemical staining for IL-17A, IL-17RA and neutrophil elastase (OR).

**Statistical analysis**

Sample size was opportunistic since there are no data to inform a power calculation. Nonparametric tests including Mann-Whitney U test and Kruskal Wallis test with Bonferroni post hoc test were used to detect differences between 2 groups or more than 2 groups, respectively using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). Correlations were assessed using the Spearman rank correlation test. $P<0.05$ was considered significant.
RESULTS

Patient demographics

Clinical characteristics are shown in Table 1. BAL bacterial culture was positive in 9/51 (15.6%) and BAL viral PCR was positive in 5/51 (9.8%) of STRA patients. There was a parental report of exposure to tobacco smoke in 15/41 (37%) STRA patients which was confirmed using urinary cotinine levels.

A subgroup of STRA is characterised by increased intra-epithelial neutrophils

Previous findings from our group (5) which showed increased BAL and submucosal eosinophils in children with STRA compared to controls were confirmed in the present study (Figure 1A-B and D-E). However, when investigating intra-epithelial eosinophils in both the present and the previous cohort (5) we found no eosinophils in the epithelium of controls or children with STRA (Figure 1C and F).

Since severe asthma in adult patients (13) and in murine models (10, 25-27) has been associated with neutrophilia, we quantified the number of neutrophils in the submucosa and epithelium in endobronchial biopsies from children with STRA and controls. In keeping with our previous findings (5), neutrophil counts were not increased in the submucosa of children with STRA compared to controls (p=0.9, Figure 1G-H and J-K). However, intra-epithelial neutrophils, expressed both as proportion of all neutrophils in the biopsy as well as neutrophils per length basement membrane, were significantly higher in children with STRA compared to controls (p=0.01 and 0.007, respectively) (Figure 1I and L). We also confirmed the increase in intra-epithelial neutrophils in STRA in biopsies obtained from a previous cohort (5) of children with STRA (Figure 1I, shown as grey symbols; See Table E1 OR for clinical details of these patients). The patients with intra-epithelial neutrophils (referred to as Neutrophil\textsuperscript{high}) had (median (range)) 0.02 (0.004-0.04) neutrophils per length of RBM compared to the Neutrophil\textsuperscript{low} (0 (0-0)) (p=0.007). There was no difference in number of intra-epithelial neutrophils between STRA patients treated with maintenance oral and inhaled steroids compared to those treated with only inhaled steroids (p=0.8).
Children with STRA did not have increased BAL or tissue IL-17A

To investigate any relationship between neutrophils and airway IL-17A in STRA, endobronchial biopsies were stained for IL-17A. IL-17A expression was observed in small mononuclear cells as well as in a larger mononuclear cell population in the adventitia of the bronchial wall, but there was no difference in number of IL-17A positive cells between controls and patients with STRA (Figure 2A-B). No IL-17A expression was seen within the epithelium (Figure 2A and C). Although undetectable in most samples, IL-17A levels in BAL were similar in children with STRA and controls (Figure 2D). IL-22 was detected in BAL fluid in 15 of 25 controls (60%) and 25 of 37 STRA patients (68%). However, IL-22 levels were similar in both groups (Figure 2E). There was also no difference in the proportion of Th17 (CD4^+IL-17^+) (Figure 2F) or IL-17^-γδT cells (Figure 2G) in BAL from children with STRA compared to controls.

Children with STRA had increased submucosal and epithelial expression of IL-17RA

We further wanted to investigate the tissue expression of the receptor for IL-17A. IL-17RA was expressed in small mononuclear and polymorphonuclear cells in the submucosa in controls (Figure 3A) and patients with STRA (Figure 3B). Strong expression was also present in the epithelium of patients with STRA (Figure 3B). There was significantly increased expression of IL-17RA in both submucosa (Figure 3C) and epithelium (Figure 3D) expressed as positive pixels per area (positivity) in STRA compared to controls. Epithelial IL-17R expression remained elevated in STRA even after excluding the patients with a positive bacterial culture or viral detection (p=0.006). There was no difference in IL-17RA expression between the patients taking maintenance oral steroids and inhaled steroids compared to those only on inhaled steroids (p=0.3).

Since intra-epithelial IL-17R expression was significantly increased in STRA, we determined how the bronchial epithelium responds to stimulation with IL-17A alone or in combination with Budesonide. mRNA expression of IL-17RA and C was comparable between STRA and controls at baseline, but expression was significantly increased in STRA compared to controls following IL-17A stimulation.
alone, and with the addition of Budesonide to cultures (Figure 3E and F). Furthermore, better symptom control, measured using the asthma control test (ACT), correlated with increased IL-17RA expression in the epithelial compartment in children with STRA (Figure 3G).

**IL-17A stimulation of bronchial epithelial cells from STRA children induced IL-8 secretion**

Since IL-8 is a neutrophil chemo-attractant and we had seen increased intra-epithelial neutrophils in STRA, levels of IL-8 were measured in BAL fluid. IL-6 was measured as a comparative marker of a general inflammatory response. BAL IL-8 and IL-6 levels were similar in STRA and controls (Figure 4A-B). This was unchanged after patients with a positive BAL bacterial culture or viral detection were excluded. IL-17A has previously been shown to induce IL-8 from bronchial epithelial cells (28). In order to investigate the relationship between IL-17A, epithelial cells and neutrophils we measured IL-8 secretion from PBEC culture supernatants following IL-17A and IL-22 stimulation, with or without Budesonide. Primary bronchial epithelial cells from patients with STRA secreted significantly higher amounts of IL-8 compared to cells from control patients. This effect was observed following stimulation with IL-17A alone, in combination with IL-22, or with IL-22 alone (Figure 4C). Importantly, IL-8 secretion was unaffected by the presence of Budesonide (Figure 4C). In contrast, levels of epithelial IL-6 secretion were comparable between STRA patients and controls (Figure 4D). Levels of interleukin (IL) -13 (Th2 cytokine), and interferon (IFN)-γ (Th1 cytokine) in epithelial cell culture supernatants were similar between STRA and controls. However, levels of the neutrophil chemoattractant GRO was increased in STRA compared to controls upon IL-17A stimulation (Figure E1 (OR)).

To further investigate the link between increased IL-17A receptor expression, intra-epithelial neutrophils and a clinical phenotype, correlations with clinical parameters were performed. We found no correlations with any confounding factor such as age or BMI in STRA children. Neither was there any association between intra-epithelial neutrophils and parental smoking status or BAL.
bacteriology/virology (Table 2). Tissue expression of IL-17A and IL-17RA was also compared in patients with and without intra-epithelial neutrophils. Although no significant differences were found, there was a trend for increased epithelial IL-17RA expression in the group with intra-epithelial neutrophils (Figure E3 OR). Two subpopulations of children with STRA were apparent based on the presence or absence of intra-epithelial neutrophils. Patients were therefore divided into an intra-epithelial Neutrophil^{high} and Neutrophil^{low} group. There were no differences in the number of submucosal, epithelial or BAL eosinophils (Figure 4E and F), total IgE (Figure 4G) or exhaled nitric oxide (FeNO) (Figure 4H) between the Neutrophil^{high} and Neutrophil^{low} group. There was no relationship between reticular basement membrane thickness, a marker of airway remodelling, and intra-epithelial neutrophils (Fig E5E). However, the Neutrophil^{high} STRA patients had a significantly higher ACT score (Figure 4I) and were prescribed lower dose maintenance inhaled corticosteroids compared to the Neutrophil^{low} STRA patients. FEV1 %predicted (Figure 4K) was significantly higher in the Neutrophil^{high} STRA patients, and the proportion of intra-epithelial neutrophils positively correlated with FEV1 %predicted (Figure 4L).
DISCUSSION

We have shown that a sub-group of children with STRA have increased intra-epithelial neutrophils compared to younger non-asthmatic controls, which surprisingly and contrary to our hypothesis, was associated with better FEV\textsubscript{1} %predicted, symptom score and lower maintenance inhaled steroids. In contrast, there were no intra-epithelial eosinophils apparent in STRA. Although there was no difference in tissue or luminal IL-17A, paediatric STRA patients exhibited increased submucosal and epithelial expression of IL-17R. Collectively our data indicates a potential beneficial role for intra-epithelial neutrophils in this sub-phenotype of asthma.

The association between intra-epithelial neutrophils and higher FEV\textsubscript{1}, higher ACT score and less inhaled corticosteroid treatment suggests neutrophils specifically within the epithelium may have a protective role in this asthma phenotype. Most previous studies were in adult asthma patients and very few (including ourselves) have looked at the specific localisation of neutrophils within the airway (6, 7, 29). Having found a relationship between intra-epithelial neutrophils and lung function in the children reported here, we retrospectively quantified epithelial neutrophils in archived biopsies from our previously published cohort (5). The biopsies from the older cohort had not previously been assessed for intra-epithelial neutrophils and confirmed an association between intra-epithelial neutrophils and better spirometry, a significant strength of the study. Unlike intra-epithelial neutrophils, eosinophils were not present within the epithelium of children with STRA. However, the increased eosinophils in the submucosa and lumen that we have reported previously (5), were confirmed here (Figure 1A&B). Although we cannot confirm a direct functional role of neutrophils in paediatric STRA, the localisation of these cells within the epithelium may point to a specific role in host defence in this phenotype. Adult data suggest there may be at least two phenotypes associated with airway neutrophilia; beneficial as a response to infections, or harmful potentially driven by environmental irritants such as tobacco smoke (30). Exposure to viruses, bacterial endotoxins and air pollution are common triggers of a neutrophil-rich inflammation and
consequently could lead to asthma symptoms. Other studies have shown increased numbers of intra-epithelial neutrophils in various bacterial, viral and fungal infections as well as in cystic fibrosis and COPD (31-35). However, we found no association between intra-epithelial neutrophils and BAL bacteriology/virology or parental smoking status. Only 1% of bacteria can be cultured (36), therefore we cannot rule out that the children had other infections or an altered microbiota that we could not detect (37). It is also important to highlight that the children in this study were clinically stable and had at least 2 weeks since any exacerbation at the time of bronchoscopy. Since neutrophils have a high turnover and short lifespan it is likely that the neutrophils observed within the epithelium in these patients are inherently present as opposed to recruited in response to an insult during an exacerbation.

There is an increasing need to find biomarkers that will indicate sub-groups of patients (Th2-low, neutrophil-high) to enable effective personalised therapies. Other studies have identified that BAL and sputum neutrophils are more abundant in severe asthmatics (38) (7). In a study investigating the molecular phenotype of severe asthma in children, both neutrophils and eosinophils were found to be elevated in BAL fluid from children with severe asthma compared to adult controls (39). These children also had high levels of GRO and IL-8 in BAL fluid (39). However, the specific location of neutrophils in the sub compartments of the bronchial wall was not investigated. The neutrophils in our study are intra-epithelial and hence unlikely to result in a predictive biomarker in the periphery or airway lumen. However, molecules that represent intra-epithelial neutrophils could be investigated in induced sputum in future studies. Unfortunately we were unable to collect sputum from enough patients with intra-epithelial neutrophils to undertake such an analysis.

It is possible that the intra-epithelial neutrophils might be migrating through the epithelium into the lumen (33-35). But the increased epithelial expression of IL-17R, as well as the increased local epithelial production of neutrophil chemoattractants including IL-8 and GRO that we have demonstrated suggest that neutrophils are trapped in the epithelial layer in children with STRA.
Although speculative and further investigation is warranted, this might indicate an epithelial microenvironment that is dependent on altered expression of epithelial adhesion molecules that results in trapping of neutrophils in the epithelial layer (36, 37).

There is scant information regarding the role of IL-17A in paediatric STRA. Higher numbers of IL-17A positive cells have been found in the bronchial submucosa from adults with severe asthma compared to controls (8). IL-17 mRNA and numbers of IL-17 positive lymphocytes are also increased in BAL from asthmatic patients (10, 11, 40). A recent study that has investigated the Th17 high phenotype in adult asthma has shown that a Th17 high signature is associated with steroid dependent moderate to severe asthma and eosinophilia (41). But despite this signature there were no significant differences in lung function between patients. In addition, when IL-17 was blocked in a murine model of HDM induced allergic airways disease, there was no impact on lung function, eosinophils or neutrophils (41). Other studies have shown that Th17 mediated airway inflammation is steroid resistant(42). Furthermore, Nanzer et al., reported PBMCs from adult severe asthmatics exhibited increased levels of Th17 cytokines, which were not inhibited by steroids (43).

We were careful to ensure all reagents were IL-17A specific, eliminating effects of contamination with IL-17F. Although significantly higher serum levels of IL-17A have been reported in children with asthma exposed to diesel exhaust particles (44) we found no increase in IL-17A in BAL fluid. Importantly, IL-17A levels were detectable, but similar between STRA and controls, suggesting little influence from dilution effects of BAL. However, this might be because samples were collected during stable disease rather than following challenge or during disease exacerbation. After performing a double stain with IL-17A and the major leukocyte populations we found that the major cell types in the submucosa expressing IL-17A were CD3+ lymphocytes and neutrophils. However, neither the epithelial cells nor infiltrating leukocytes within the epithelium were IL-17A positive. In contrast, our patients with STRA did show enhanced IL-17RA immunoreactivity in submucosa and epithelium as well as increased mRNA expression of IL-17RA and C upon stimulation of PBECs with IL-17A when compared to non-asthmatic controls. IL-17A and IL-17F both signal through IL-17RA
(25), and have fundamental roles as neutrophil chemoattractants (26, 33). However, we found no association between neutrophils or IL-17A levels and infection in our patients, albeit molecular microbiological techniques were not used.

Deficiency in IL-17RA results in impaired neutrophil responses to allergens in mouse models (45, 46). Elevation of IL-17RA and C suggests a role in neutrophil migration, either via a direct response to IL-17A or via effector molecules such as IL-8. Although no significant difference was found between the STRA patients with or without intra-epithelial neutrophils with regard to IL-17A and IL-17RA expression in submucosa or epithelium, there was an indication of higher epithelial IL-17RA expression in the Neutrophil\textsuperscript{high} patients. We found no increased production of IL-6 in our study, which indicates a specific IL-17A driven epithelial response, characterised by the neutrophil chemoattractants IL-8 and GRO which were significantly increased with IL-17A stimulation of PBECs from STRA compared to controls. These findings question the rationale for treating children with STRA with anti-IL-17A antibody. A monoclonal antibody against IL-17RA, Brodalumab, has shown no benefit in adult patients with moderate-to-severe asthma (47) and our data suggests its use in children with STRA is unlikely to be beneficial.

All of the STRA patients in our manuscript were symptomatic and had poor control despite a high dose of inhaled steroids. Corticosteroids have been shown to increase airway neutrophils in asthma (48, 49) and molecules that are associated with suppression of neutrophil apoptosis are upregulated by glucocorticosteroids. This may be an unwanted effect of asthma therapy. In contrast, our data indicate patients with lower epithelial neutrophils were prescribed higher doses of maintenance inhaled steroids, suggesting an altered relationship between intra-epithelial neutrophils and glucocorticosteroids. This suggests the neutrophil low group may be less sensitive to steroids, since they were on a higher median dose as a group.

We have shown an association between increased IL-17RA expression and STRA. IL-17A induced epithelial secretion of IL-8 was also unaffected by the presence of Budesonide. Previous studies
have also shown a lack of effect of dexamethasone on IL-17A production from PBMCs in both mice and humans (27, 43). These data suggest a lack of association between IL-17A and steroids in patients with severe asthma. Interestingly, it has been shown that the release of GRO and IL-8 induced by IL-17 in the bronchial epithelial cell line 16-HBE is sensitive to hydrocortisone treatment (50). We have found similar results with this immortalised cell line (Figure E4, OR), confirming critical functional differences between PBECs and 16-HBEs and emphasising the importance of using primary cells from asthmatic patients.

The strengths of this study include the large number of carefully characterised children with STRA, a phenotype of asthma that has been little studied, and the comparison of clinical parameters with morphological studies and functional in vitro assays using primary airway epithelial cells from the same patients. However, we acknowledge some limitations. We did not include a control group of mild to moderate asthma, nor did we include true healthy controls. Although an invasive procedure involving a general anaesthetic cannot be ethically justified for research studies in children, the controls included did not have lower respiratory symptoms, and when we have used similar patients previously we have found meaningful group differences (5, 51, 52). In addition, the controls were younger because isolated upper airway problems are less common in school-aged children.

In conclusion, our study shows that children with STRA compared to younger non-asthmatic controls have an exaggerated epithelial response to IL-17A, with increased expression of IL-17RA in the airway submucosa and epithelium. Primary bronchial epithelial cells from children with STRA responded to IL-17A stimulation with elevated production of the neutrophil attracting mediators IL-8 and GRO compared to controls. Furthermore, increased numbers of neutrophils were found only in the epithelial compartment in a sub-group of children with STRA and this finding was associated with better lung function, better symptom control and lower dose maintenance inhaled steroids. The critical role of the bronchial epithelium and its interactions with airway leukocytes in determining downstream functional effects has been highlighted. Our study shows that there are two sub-groups
within the paediatric STRA phenotype: intra-epithelial Neutrophil$^{\text{high}}$ and Neutrophil$^{\text{low}}$, further demonstrating that STRA is heterogeneous and requires careful sub-phenotyping to identify optimal personalised molecular therapies.
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**FIGURE LEGENDS**

**Figure 1.** Eosinophils and neutrophils in different lung compartments. Eosinophil percentage in BAL (A) (controls n=11, STRA n=31), numbers per area of submucosa (B) (non-asthmatic controls n=7, STRA n=20) and intraepithelial eosinophils per length of RBM (C) (controls n=7, STRA n=20). Representative micrographs of Congo Red stain for eosinophils in non-asthmatic controls (D) and STRA (E). (F) Show high magnification picture of epithelium with no eosinophils in STRA. Neutrophil percentage in BAL (G) (non-asthmatic controls n=12, STRA n=52), numbers per area of submucosa (H) (non-asthmatic controls n=10, STRA n=28) and proportion of intraepithelial neutrophils (I) (non-asthmatic controls n=13, STRA n=37). Representative micrographs of immunohistochemical stain for elastase in non-asthmatic controls (J) and STRA (K). (L) Show high magnification picture of intraepithelial neutrophils in STRA. Scale bar: 200 μm (D, J), 100 μm (E, K), 20 μm (F) and 10 μm (L). Statistical significance between non-asthmatic controls and STRA was tested using Mann–Whitney test.

**Figure 2.** Sources and expression of IL-17A in children with STRA compared to non-asthmatic controls. Representative micrograph of immunohistochemical stain for IL-17A in STRA (A). Scare bar: 100 μm. Quantification of the number of IL-17A expressing cells per area in submucosa (B) (non-asthmatic controls n=6, STRA n=39) and bronchial epithelium (C) (non-asthmatic controls n=6, STRA n=32). BAL levels of IL-17A (D) (non-asthmatic controls n=11, STRA n=23) and IL-22 (E) (non-asthmatic controls n=11, STRA n=23) non-asthmatic controls and children with STRA. Percentages of IL-17A expressing TH17 (F) (non-asthmatic controls n=10, STRA n=13) and gamma delta (G) (non-asthmatic controls n=8, STRA n=11) lymphocytes in children with STRA compared to non-asthmatic controls. Statistical significance between non-asthmatic controls and STRA was tested using Mann–Whitney test.

**Figure 3.** Representative micrographs of immunohistochemical stain for IL-17RA in non-asthmatic controls and STRA patient (A and B, respectively). Scare bar 100 μm (A) and 50 μm (B).
Quantification of the expression (positive pixels per area) of IL-17RA in submucosa (non-asthmatic controls n=6, STRA n=31) and epithelium (non-asthmatic controls n=6, STRA n=24) (C, D, respectively). mRNA expression of IL-17RA and C in PBECs after stimulation with IL-17A and Budesonide (non-asthmatic controls n=3, STRA n=3) (E and F, respectively). Correlations between ACT score and IL-17RA expression in epithelium (G) (n=21). Statistical significance between non-asthmatic controls and STRA was tested using Mann–Whitney test and correlation analysis was performed using Spearman rank test, where p<0.05 was considered significant.

Figure 4. Levels of IL-8 (A) (non-asthmatic controls n=9, STRA) and IL-6 (B) (non-asthmatic controls n=12, STRA n=23) in BAL. Levels of IL-8 (C) and IL-6 (D) in culture supernatant from PBECs after stimulation with IL-17A, IL-22 and Budesonide D n=24 (controls n=4, STRA n=7 and non-asthmatic controls n=4, STRA n=7, respectively). Differences in submucosal eosinophils (E) (Neu^{hi} n=10, Neu^{lo} n=15), BAL eosinophils (F) (Neu^{hi} n=12, Neu^{lo} n=20), total IgE (G) (Neu^{hi} n=10, Neu^{lo} n=21), FeNO (H) (Neu^{hi} n=11, Neu^{lo} n=18), ACT score (I) (Neu^{hi} n=12, Neu^{lo} n=20), dose of inhaled corticosteroids (J) (Neu^{hi} n=12, Neu^{lo} n=21) and FEV_{1} % predicted (K) within the group of children with STRA based on the presence of intraepithelial neutrophils (Neu^{hi} n=13, Neu^{lo} n=21). Correlations between FEV_{1} % predicted and proportion of intraepithelial neutrophils (L) (n=36). Patients from the cohort previously published in (S) are shown in grey. Statistical significance between controls and STRA was tested using Mann–Whitney test and correlation analysis was performed using Spearman rank test, where p<0.05 was considered significant.
Table 1. Demographics of STRA and control patients undergoing bronchoscopy.

<table>
<thead>
<tr>
<th></th>
<th>STRA (n=51)</th>
<th>Non-asthmatic controls(^\dagger) (n=15)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atopy</td>
<td>45/51 (88.2%)</td>
<td>1/15 (6.6%)</td>
<td>0.005</td>
</tr>
<tr>
<td>Male: Female</td>
<td>30:21</td>
<td>8:7</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>12.6 (6.16.3)</td>
<td>4.75 (1.6-16)</td>
<td>0.005</td>
</tr>
<tr>
<td>Duration of symptoms, years</td>
<td>7 (3.5 - 14.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (Kg)</td>
<td>41.7 (22.3-99.9)</td>
<td>19.2 (8.9-68)</td>
<td></td>
</tr>
<tr>
<td>Weight z score</td>
<td>0.5 (-3.4 - 3.7)</td>
<td>0.4 (-3.1-3.4)</td>
<td></td>
</tr>
<tr>
<td>Height (cm)</td>
<td>150 (106 -188)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height z score</td>
<td>0.02 (-3.9 - 2.88)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intubation for asthma</td>
<td>4/51 (7.8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total IgE(IU/ml)</td>
<td>419 (20-4867)</td>
<td>46.5 (1-210)</td>
<td>0.003</td>
</tr>
<tr>
<td>Sum of inhalant slgE (IU/mL)</td>
<td>7.4 (0-321)</td>
<td>0.54 (0-20)</td>
<td>0.1</td>
</tr>
<tr>
<td>Sum of all slgE (IU/mL)</td>
<td>11.3 (0-321)</td>
<td>1.1 (0-20)</td>
<td>0.2</td>
</tr>
<tr>
<td>BAL Neutrophils(%)(^\dagger)</td>
<td>3.7 (1-21.7)</td>
<td>5.4 (4-9)</td>
<td>0.35</td>
</tr>
<tr>
<td>BAL Eosinophils (%)**</td>
<td>3 (0.3-23)</td>
<td>2 (0-5)</td>
<td>0.17</td>
</tr>
<tr>
<td>Blood Eosinophils (%)</td>
<td>7.5 (0-21.4)</td>
<td>3.5 (0.9-9.8)</td>
<td>0.06</td>
</tr>
<tr>
<td>Blood Neutrophils (%)</td>
<td>51 (6-87)</td>
<td>49 (31-71)</td>
<td>0.4</td>
</tr>
<tr>
<td>ACT score</td>
<td>13 (5-23)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACT normal (&gt;19/25)</td>
<td>10/51 (19.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline FEV(_1) (litres)</td>
<td>1.75 (0.45 - 3.95)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline % predicted FEV(_1)* (&gt;80% predicted)</td>
<td>31/51 (60.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline FVC (litres)</td>
<td>2.36 (1.37-5.39)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline % predicted FVC</td>
<td>99.5 (63 - 133)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline bronchodilator reversibility (%)</td>
<td>11.4 (-3 - 66.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline FeNO(_50) (ppb)(^\dagger)</td>
<td>46.2 (5.4 - 164.8)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Medications**

<table>
<thead>
<tr>
<th></th>
<th>Daily dose Inhaled (^\dagger) Budesonide equivalent</th>
<th>Leukotriene receptor antagonist</th>
<th>Systemic corticosteroids</th>
<th>Daily dose (mg/day)</th>
<th>Theophylline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1400 (500-2000)</td>
<td>46/51 (90.1%)</td>
<td>6/51 (11.7%)</td>
<td>6.25 (2.5 - 20)</td>
<td>4/51 (7.8%)</td>
</tr>
</tbody>
</table>

\(^\dagger\) BAL Neutrophils (<3% is normal). **BAL eosinophils (<3% is normal). \(*FEV \_1*: presented as percentage predicted. \(^\dagger\)FeNO: fractional exhaled nitric oxide measured at 50L/min. \(^\dagger\)ICS: inhaled corticosteroids/day. \(\_80\%\) of the non-asthmatic controls symptoms such as stridor (tracheal stenosis, laryngotracheomalacia), reflux, dry cough, or haemoptysis. The remaining 20% were undergoing general anaesthesia for elective cardiac catheterisation and had agreed to a research bronchoscopy. For details of diagnosis in the non-asthmatic controls see OR, Table E2. Differences between groups were assessed by Mann-Whitney test where P<0.05 is significant.
Table 2. Neutrophil, IL-17A and IL-17RA quantification in relation to BAL bacteriology, virology and parental smoking status.

<table>
<thead>
<tr>
<th></th>
<th>STRA (n=51)</th>
<th>Non-asthmatic control (n=15)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAL Bacteriology (positive)</td>
<td>8/51 (15.4%)</td>
<td>1/15 (6.6%)</td>
<td></td>
</tr>
<tr>
<td>BAL Virology (positive)</td>
<td>5/51 (9.8%)</td>
<td>1/15 (6.6%)</td>
<td></td>
</tr>
<tr>
<td>Parental smoking (positive)</td>
<td>15/41 (37%)</td>
<td>1/15 (6.6%)</td>
<td></td>
</tr>
<tr>
<td>Cotinine levels (ng/ml)</td>
<td>1.3 (1- 4.8)</td>
<td>1/15 (6.6%)</td>
<td></td>
</tr>
<tr>
<td>IL-17A (submucosa)*</td>
<td>0.09 (0.03-0.3)</td>
<td>0.03 (0.02-0.07)</td>
<td>0.001</td>
</tr>
<tr>
<td>IL-17RA (submucosa)#</td>
<td>20 (0-291)</td>
<td>9 (0-47)</td>
<td>0.4</td>
</tr>
<tr>
<td>IL-17RA (epithelium)#</td>
<td>0.2 (0.01-0.5)</td>
<td>0.02 (0.007-0.08)</td>
<td>0.0006</td>
</tr>
<tr>
<td>Neutrophils (submucosa)*</td>
<td>8 (0-202)</td>
<td>8 (0-36)</td>
<td>0.9</td>
</tr>
<tr>
<td>Neutrophils (epithelium), %</td>
<td>0 (0-60)</td>
<td>0 (0-0)</td>
<td>0.04</td>
</tr>
<tr>
<td>Eosinophils (submucosa)</td>
<td>54 (4-263)</td>
<td>8 (0-56)</td>
<td>0.01</td>
</tr>
<tr>
<td>Eosinophils (epithelium), %</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
<td>&gt;.999</td>
</tr>
</tbody>
</table>

Comparison of intraepithelial neutrophils in STRA groups based on:

<table>
<thead>
<tr>
<th></th>
<th>Positive</th>
<th>Negative</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAL bacteriology</td>
<td>0 (0-17)</td>
<td>0 (0-60)</td>
<td>0.7</td>
</tr>
<tr>
<td>BAL virology</td>
<td>8 (0-17)</td>
<td>0 (0-60)</td>
<td>0.3</td>
</tr>
<tr>
<td>Parental smoking</td>
<td>0 (0-20)</td>
<td>0 (0-60)</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Data presented as median (range). * >4.8 ng/ml indicates exposure to tobacco smoke and >50 ng/ml is indicative of active tobacco smoking. **Cells per mm². #Positivity (positive pixels per all pixels). Differences between groups were assessed by Mann-Whitney test where P<0.05 is significant.
Figure 1.

A. BAL Eosinophils

B. Eosinophils within submucosa

C. Eosinophils within epithelium

D, E, F. Representative images of eosinophils in the submucosa.

G. BAL Neutrophils

H. Neutrophils within submucosa

I. Neutrophils within epithelium

J, K, L. Representative images of neutrophils in the submucosa.
Figure 3.

A

B

C

D

E

F

G

ACT vs. IL-17RA expression in epithelium

$\text{ACT score}$

$0.0$ $0.2$ $0.4$ $0.6$

$\text{IL-17RA ep (positivity)}$

$\rho = 0.48$

$p = 0.03$
Figure 4.
Figure E2

A  IL-17A submucosa

B  IL-17RA submucosa

C  IL-17RA epithelium

- Neu^lo vs Neu^hi

- Positive pixels/mm²

- p = 0.6

- p = 0.6

- p = 0.2
Figure E3

A

IL-6

pg/µL

B

IL-1β

pg/µL

Controls

STRA

C

IL-13

pg/µL

D

IP-10

pg/µL

E

MCP-1

pg/µL

F

MDC

pg/µL

G

FGF-2

pg/µL

H

IFN-γ

pg/µL

 Controls

STRA
Figure E4

A. IL-6 (16-HBEs culture supernatant)

B. IL-8 (16-HBEs culture supernatant)
Intra-epithelial Neutrophils in Paediatric Severe Asthma are Associated with Better Lung Function

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*These authors contributed equally to this work

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Corresponding author: Professor Clare M Lloyd or Dr Sejal Saglani, Inflammation, Repair & Development, National Heart and Lung Institute, Imperial College London, Sir Alexander Fleming Building, Exhibition Road, London SW7 2AZ, UK; c.lloyd@imperial.ac.uk or s.saglani@imperial.ac.uk

Running head: Neutrophils and IL-17A in paediatric severe therapy resistant asthma
MATERIAL AND METHODS

Subjects

Fifty-one school-aged children with STRA who were undergoing a clinically indicated bronchoscopy were recruited from the Royal Brompton Hospital between 2009 and 2015. Patients remained poorly controlled despite being prescribed high doses of inhaled steroids (800mcg/day budesonide or equivalent) and receiving additional long-acting β-agonists, and current or a previous failed trial of montelukast (Table 1). 12% of the patients with severe asthma were also taking regular oral steroids. Only children with true STRA were included in the study (1-3). Those with difficult asthma, in whom underlying modifiable factors such as persistent allergen exposure, poor adherence and/or inhaler technique that might contribute to poor asthma control were excluded (4). The remaining children with STRA underwent invasive analysis with bronchoscopy, bronchial brushings, bronchoalveolar lavage (BAL) and endobronchial biopsies to characterize airway pathology and develop customised treatment plans. Atopy was defined as 1 or more positive specific IgE RAST (≥0.34 kU/L) or 1 or more positive skin prick test results to aeroallergens. Asthma control was assessed by using the Asthma Control Test (ACT) (5, 6); poor control was defined as a score of less than 20 of 25. Spirometry and bronchodilator reversibility (BDR) defined as a greater than 12% change from baseline FEV1 were measured according to American Thoracic Society/European Thoracic Society guidelines (7, 8). Exhaled nitric oxide measurements at a flow rate of 50 mL/s were made with a chemiluminescence analyzer (NIOX; Aerocrine AB, Solna, Sweden) in accordance with American Thoracic Society/European Thoracic Society guidelines(9). Clinical details of the children with STRA are shown in Table 1. 15 non-asthmatic control subjects were either having a bronchoscopy to investigate upper airway symptoms and agreed to extra research samples being taken or were undergoing general anaesthesia for cardiac symptoms and agreed to have a research bronchoscopy at the same time (see Table 1 and Table E2 for details). The study was approved by the Brompton and Harefield
National Heart and Lung Institute Ethics Committee, and informed parental consent and child assent were obtained.

Findings relating to epithelial inflammation in this group were confirmed in biopsies already obtained from a previously published cohort of children with STRA (n=21) and non-asthmatic controls (n=5) (Table E1 OLS). The inclusion criteria in this cohort were the same as for the patients described above (see also reference (1). Children aged 5 to 16 years with severe therapy resistant asthma were included. Definitions of uncontrolled disease and entry criteria were:

1. Persistent (most days for ≥3 months) chronic symptoms (use of short-acting β2-agonists ≥3 times per week) of airway obstruction despite high doses of inhaled corticosteroids (ICSs; ≥800 μg/d budesonide equivalent) and/or regular oral corticosteroids, long-acting β2-agonists, and current (or previous failed trial of) montelukast.

2. Recurrent severe exacerbations requiring 1 or more admission to the intensive care unit or 2 or more hospital admissions requiring intravenous medications or 2 or more courses of oral corticosteroids in the past year despite therapy for persistent symptoms as described in above.

3. At least 1 very sudden (≤6 hours) severe attack (requiring hospitalization) without warning despite therapy for persistent symptoms, as described in above.

Evaluations were performed to assess medication, dose, device and technique, atopic status, asthma understanding and assessment of adherence, environment, and any psychosocial issues were addressed. After assessment, those classified as true STRA were further investigated. The study was approved by the local research ethics committee, and all procedures were performed after obtaining written informed parental consent and, where appropriate, child assent.

Epithelial cell culture and stimulation
Epithelial brushings were obtained at bronchoscopy using a fiberoptic bronchoscope (Olympus, Keymed, UK). A sterile single-sheathed nylon cytology brush was used to sample epithelial cells from the bronchial mucosa of the second- and third-generation bronchi. Cells were transferred to 5mL bronchial epithelial growth medium (BEGM, Lonza, Walkerville, MD, USA) and the samples were centrifuged at 1500rpm× g for 5 min to pellet the cells. Primary cultures were established by reconstituting the pellet in BEGM and cells were seeded into collagen coated T25 tissue culture flasks containing 5mL BEGM. Cultures were routinely tested for mycoplasma infection.

Primary paediatric bronchial epithelial cells were grown to 80–90% confluence in 12 well plates. On the day of stimulation, the growth medium was replaced with bronchial epithelial basal medium (Lonza) 4h before start of experiment. The primary epithelial cells were used at passage 3 for all experiments. Primary bronchial epithelial cells were stimulated with recombinant human IL-17A (eBioscience, San Diego, CA) and IL-22 (eBioscience) at 10ng/mL alone, or with added budesonide at a final concentration of 10^{-8} mol/L. All experiments were done in triplicates. After 24h cell culture supernatants were taken, and cell lysates were prepared in RIPA buffer containing protease inhibitor tablets (Roche Diagnostics, Mannheim, Germany). Samples for mRNA extraction were undertaken in separate 12-well plates and cells were harvested in Trizol (Sigma, St Louis, MO) after 8h stimulation.

16HBEs, a differentiated SV-40 transformed bronchial epithelial cell line, was used in the same way as the primary HBECS and stimulated as described above with IL-17, IL-22, IL-17 and IL-22 in combination with or without Budesonide.

Quantification of cytokines

Cell culture supernatant was collected and cytokines were analysed using ELISA and Milliplex. Milliplex human cytokine panel I and II (HCYP2MAG-62K and MPXHCYTO-60K, Millipore Corporation, Billerica, MA) were used to detect levels of IL-4, -5, -6, -8, -9, -10, -13, -17, IP-10, MCP-1, MDC,
RANTES, TNF-α, VEGF, Eotaxin, Eotaxin-2, Eotaxin-3, FGF-2, GRO, IFN-γ, TARC, SCF, TSLP and IL-33 according to manufacturer's instructions. Levels of IL-6 and IL-8 were confirmed with standardized sandwich ELISAs, according to the manufacturer's protocol (88-8086 and 88-7066, eBiosciences).

Cytomix

BALF cytokine quantification was performed using Flowcytomix™ Human TH1/TH2/TH9/TH17/Th2 13 plex multiplex (eBioscience, San Diego, CA) to detect levels of IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p70), IL-13, IL-17A, IL-22, and TNF-α according to manufacturer's instructions. Samples were assayed using the BD Fortessa flow cytometer. Data analysis was performed using Flowcytomix™ software (eBioscience, San Diego, CA) and GraphPad Prism (V.6 for Mac OS X, GraphPad Software Inc).

FACS

Lymphocytes used for compensations and fluorescence minus one (FMOs) were extracted from peripheral blood using a density gradient medium (Lymphoprep™) cell concentration was optimized to 2-4 x 10^5 cells per 100µl. Cells from the airway lumen were obtained after centrifuge of broncho-alveolar lavage fluid to obtain a cell pellet. Cells were re-suspended in RPMI complete and plated at 2-4 x 10^5 cells per 100µl. Cells were stimulated for 4 hours with phorbol 12-myristate 13-acetate (PMA), Ionomycin and Brefeldin A. Cells were stained for extracellular markers CD3 (1:50), CD4 (1:50), CD8, CD161, γδTCR and intracellular staining for IL-17A (eBiosciences, mouse anti-human 5µl per well).

Samples were assayed using the BDFortessa flow cytometer (BD, UK). Data were analysed using FlowJo (V.9.2, TreeStar Inc) and GraphPad Prism (V.6 for Mac OS X, GraphPad Software Inc).
RNA extraction and real-time PCR

Total RNA was extracted from epithelial cells by using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized from 500ng of total RNA and analysed by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster city, CA). Reactions were run with TaqMan primers against IL-17RA, IL-17RC, Fibronectin, Collagen I (Applied Biosystems) and TaqMan Fast Advanced Master Mix (Applied Biosystems) according to manufacturer’s directions on a Viaa-7 (Life Technologies) instrument and gene expression was analysed using the change-in-threshold ΔΔCt- method.

Histopathology

Endobronchial biopsies were processed to paraffin. Five-micrometre sections were stained with haematoxylin and eosin and used to assess morphology and consecutive sections were used for Masson’s trichrome staining (Sigma Aldrich) and immunohistochemical staining for IL-17A, IL-17RA and neutrophil elastase. Human polyclonal goat anti–IL-17 antibody (R&D, Rocky Hill, NJ; dilution 1:50) and human monoclonal mouse anti–IL-17RA antibody (R&D, Rocky Hill, NJ; dilution 1:50) was detected by using Dako EnVision (K5007) or anti-goat HRP (Dako). Staining was visualised with 3’,3’-diaminobenzidine (DAB kit, Vector Laboratories, Peterborough, UK) and counterstained with haematoxylin. Human monoclonal mouse anti-elastase (Dako, Glostrup, Denmark, dilution 1:100) was used for detection of neutrophils. Primary was detected with Vector Laboratories ABC kit (AK5000) according to manufacturer’s instructions and was visualised with Fast Red and counterstained with haematoxylin. IL-17A positive cells were quantified in the submucosa and expressed as cells per square millimetre of tissue using ImageScope (Aperio, Vista, CA). IL-17RA was quantified using ImageScope positive pixel count algorithm in the submucosa and epithelium and expressed as...
positive pixels divided by total number of pixels (positivity). Neutrophil density was quantified in the epithelium and the subepithelial compartment using ImageScope. Neutrophils in the submucosa were expressed as cells per square millimetre tissue and intraepithelial neutrophils as the proportion of intraepithelial neutrophils of all neutrophils present in the biopsy as well as number of cells per micrometre reticular basal membrane length. Since only a proportion of patients with STRA had intraepithelial neutrophils we included biopsies from another cohort with similar patients (patient details have been previously published (1)) to be able to do a statistically assured comparison and correlation to FEV$_1$ % predicted.

Double staining with immunofluorescence was used to simultaneously visualise IL-17A together with the following molecules: CD68 (1:200, Dako, Glostrup, Denmark), elastase (1:200, Dako), tryptase (Dako) and CD3 (1:50, Dako). The markers were selected to represent multiple leukocyte populations that might express IL-17A. After antigen retrieval (citrate buffer, pH 6), sections were stained for IL-17A using specific and validated primary antibody (IL-17A, R&D systems) and visualised by an Alexa-Flour 555-conjugated secondary antibody (Molecular Probes, Oregon, USA). Next, antibodies against CD68, elastase, tryptase or CD3 were added and visualised using a AlexaF-488 conjugated secondary antibody. Sections were mounted with Prolong gold mounting medium with DAPI (Molecular Probes) for visualisation of cell nuclei.

Statistical analysis

Nonparametric tests Mann-Whitney U tests and Kruskal Wallis test with Bonferroni post hoc test were used to detect differences between 2 groups or more than 2 groups, respectively, by using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA). Correlations were assessed by using the Spearman rank correlation test. Results were considered significant at a p-value < 0.05.
RESULTS

Stimulation of bronchial epithelial cells with IL-17A induced secretion of GRO in children with STRA

Since IL-17A is associated with increased numbers of neutrophils in adult asthma, levels of GRO were measured in BAL fluid as well as in PBEC culture supernatants following IL-17A and IL-22 stimulation, with or without Budesonide. Epithelial GRO secretion from bronchial epithelial cells was significantly increased in patients with STRA compared to non-asthmatic controls with IL-17A stimulation in combination with budesonide (Figure E1A). No effect was seen with IL-22 stimulation alone.

Detectable levels of IL-6, -8, -13, IP-10, MCP-1, MDC, FGF-2, IFN-γ were also found in cell culture supernatants using Milliplex but no difference was found between STRA and non-asthmatic controls expect for IL-8 which confirmed the ELISA data (Figure E2B). The other mediators in the Milliplex kit were below detection level.

Correlations between specific serum IgE levels and clinical parameters, neutrophil numbers and IL-17A/RA expression

We did not find any correlation of submucosal or intraepithelial neutrophils, IL-17A and IL-17RA expression to slgE levels to any specific allergen nor to the sum of inhaled allergen or to the total sum of slgE to all allergens. However, positive correlations were found between sum of slgE to inhaled allergens and RBM thickness (rs=0.43, p=0.05) and sum of slgE to inhaled allergens and submucosal eosinophils (rs=0.63, p=0.03).

16-HBEs cell line appears to be sensitive to treatment with Budesonide
In contrast to the primary HBECs, the 16HBE cell line did not respond with increased IL-6 and -8 production upon stimulation with IL-17A, IL-22 or in combination. However, they did display sensitivity to Budesonide and showed decreased production of IL-6 and IL-8 upon treatment with Budesonide (Figure E3A and B).

**IL-17A stimulation induced epithelial expression of remodelling mediators**

Increased reticular basement membrane (RBM) thickness and airway smooth muscle mass is a distinct feature of children with STRA (1). Since IL-17A and IL-22 has been associated with airway remodelling (10-12) we measured epithelial secretion of remodelling mediators in response to stimulation with these cytokines. VEGF was significantly increased in STRA compared to non-asthmatic controls when IL-17A and IL-22 were used in combination (Figure E5A, p=0.04).

Fibronectin mRNA was increased in STRA compared to non-asthmatic controls when cells were stimulated with IL-17A alone (p=0.02, Figure E5B), while collagen I was increased in STRA compared to non-asthmatic controls when cells were stimulated with either IL-22 (p=0.02), IL-17 (p=0.005) or IL-17 with Budesonide (p=0.05) (Figure E5C). We confirmed our previous findings (1) that children with STRA have increased RBM thickness (p=0.03, Figure E5E). However, no significant difference in submucosal collagen deposition was found in bronchial biopsies from non-asthmatic controls and patients with STRA (p=0.09, Figure E3F and G).
FIGURE LEGENDS

Figure E1. Measurement of GRO (A) in culture supernatants from PBECs after stimulation with IL-17A, IL-22 and budesonide (A) (non-asthmatic controls n=4, STRA n=4). Statistical significance between non-asthmatic controls and STRA was tested using Mann–Whitney test, where p<0.05 was considered significant.

Figure E2. Tissue density of IL-17A in submucosa (A), IL-17RA in submucosa (B) and IL-17RA in epithelium (C) in bronchial biopsies from children with STRA divided into Neutrophil\textsuperscript{high} vs. \textsuperscript{low} groups. Statistical significance was tested using Mann–Whitney test, where p<0.05 was considered significant.

Figure E3. Measurement of IL-6 (A), IL-8 (B), IL-13 (C), IP-10 (D), MCP-1 (E), MDC (F), FGF-2 (G), and IFN-\gamma (H) in culture supernatants from PBECs after stimulation with IL-17A, IL-22 and budesonide (non-asthmatic controls n=4, STRA n=4) using Milliplex assay. Statistical significance between non-asthmatic controls and STRA was tested using Mann–Whitney test, where p<0.05 was considered significant.

Figure E4. Measurement of IL-6 (A) and IL-8 (B) in culture supernatants from 16HBEs after stimulation with IL-17A, IL-22 and budesonide using ELISA.

Figure E5. The effect of IL-17A on airway remodelling parameters. Measurement of VEGF in culture supernatants from PBECs after stimulation with IL-17A, IL-22 and Budesonide (A) (non-asthmatic controls n=4, STRA n=4). mRNA expression in PBECs after stimulation with IL-17A, IL-22 and Budesonide of fibronectin (B) and collagen I (C) (non-asthmatic controls n=3, STRA n=3). RBM thickness in non-asthmatic controls compared to STRA (D) (non-asthmatic controls n=7, STRA n=21) and RBM thickness within the STRA group when divided into Neutrophil\textsuperscript{high} vs. \textsuperscript{low} subgroups (E). Representative micrographs of bronchial biopsies stained with Masson’s trichrome staining for collagen (blue) (F control and G STRA). Subepithelial collagen deposition (H) (non-asthmatic controls n=7, STRA n=9) in bronchial biopsies from non-asthmatic controls and children with STRA expressed as positive pixels per area. Statistical significance between non-asthmatic controls and STRA was tested using Mann–Whitney test, where p<0.05 was considered significant.

Figure E6. Immunohistochemical double staining of bronchial biopsies of IL-17A (red – AlexaF 555) and elastase (A) or CD3 (B) (green – AlexaF 488). Corresponding isotype controls are shown in C-D). Nuclei are stained blue with DAPI. Scale bar: 100 um.
REFERENCES


Table E1. Demographics of STRA and control patients undergoing bronchoscopy in validation cohort collected between 2007-2009.

<table>
<thead>
<tr>
<th></th>
<th>STRA (n=21)</th>
<th>Healthy control (n=5)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Atopy</strong></td>
<td>21/21 (100%)</td>
<td>0/5 (0%)</td>
<td></td>
</tr>
<tr>
<td><strong>Male: Female</strong></td>
<td>14:7</td>
<td>3:2</td>
<td></td>
</tr>
<tr>
<td><strong>Age, years</strong></td>
<td>11.2 (8 -16)</td>
<td>11.2 (9-16)</td>
<td>0.80</td>
</tr>
<tr>
<td><strong>Weight (Kg)</strong></td>
<td>42.4 (24.1-74.0)</td>
<td>44.7 (37.8-60.2)</td>
<td>0.96</td>
</tr>
<tr>
<td><strong>Height (cm)</strong></td>
<td>141 (114 -159)</td>
<td>160 (144-168)</td>
<td>0.054</td>
</tr>
<tr>
<td><strong>Total IgE(IU/ml)</strong></td>
<td>415 (16-18645)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><strong>BAL Neutrophils(%) †</strong></td>
<td>3 (0-19)</td>
<td>1.3 (0-14)</td>
<td>0.22</td>
</tr>
<tr>
<td><strong>BAL Eosinophils (%)</strong></td>
<td>3 (0-51)</td>
<td>0 (0-10)</td>
<td>0.33</td>
</tr>
<tr>
<td><strong>ACT score§</strong></td>
<td>11 (6-22)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><strong>Baseline % predicted FEV₁</strong></td>
<td>69 (29 - 98)</td>
<td>98 (78- 106)</td>
<td>0.0072</td>
</tr>
<tr>
<td><strong>Number FEV₁ ’normal’ (&gt;80% predicted)</strong></td>
<td>6/21 (28.6%)</td>
<td>4/5 (80%)</td>
<td></td>
</tr>
<tr>
<td><strong>Baseline % predicted FVC</strong></td>
<td>99.5 (63 - 133)</td>
<td>93 (89-116)</td>
<td>0.56</td>
</tr>
<tr>
<td><strong>Baseline bronchodilator reversibility (%)</strong></td>
<td>13.3 (-2 - 135)</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

**Medications**

| Daily dose Inhaled ‡ | 1600 (800-3200) | 0 |
| Budesonide equivalent |             |   |
| **Systemic corticosteroids** | 12/21 (57%) | 0 |
| **Daily dose (mg/day)** | 6.88 (5 - 40) | 0 |

Data presented as median (range). † BAL Neutrophils (<3% is normal). **BAL eosinophils (<3% is normal). §Asthma control test (>19 is normal). *FEV₁: presented as percentage predicted. ‡ICS: inhaled corticosteroids/day. Differences between groups were assessed by Mann-Whitney test where P<0.05 is significant.

Table E2. Clinical diagnosis and reason for bronchoscopy of non-asthmatic controls.

<table>
<thead>
<tr>
<th>Non-asthmatic controls (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elective cardiac catheter</td>
</tr>
<tr>
<td>Unexplained hypoxia</td>
</tr>
<tr>
<td>Reflux</td>
</tr>
<tr>
<td>Tracheal stenosis</td>
</tr>
<tr>
<td>Chronic dry cough</td>
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<tr>
<td>Haemoptysis</td>
</tr>
<tr>
<td>Laryngotracheomalacia</td>
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</tbody>
</table>

Data presented as number of patients.