Pulmonary type2 innate lymphoid cells in paediatric severe asthma: phenotype and response to steroids


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Pulmonary type2 innate lymphoid cells in paediatric severe asthma: phenotype and response to steroids

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Take home message

Children with severe asthma have a distinct type2 airway molecular phenotype with higher ILC2s, Th2 cells and eosinophils than difficult asthma, while IL-17+ cells are similar. ILC2s are sensitive to systemic steroids whereas IL-17+ cells are unchanged.

Key words: severe asthma, Th2, Th17, steroid resistance, innate lymphoid cells, eosinophils
Abstract:

Children with severe therapy resistant asthma (STRA) have poor control despite maximal treatment, while those with difficult asthma (DA) have poor control from failure to implement basic management including adherence to therapy. Although recognized as clinically distinct, the airway molecular phenotype, including the role of ILCs and their response to steroids in DA and STRA is unknown.

Immunophenotyping of sputum and blood ILCs and T cells from STRA, DA and non-asthmatic controls was undertaken. Leukocytes were analysed longitudinally pre and post intramuscular triamcinolone in children with STRA. Cultured ILCs were also evaluated to assess steroid responsiveness in vitro.

Airway eosinophils, Th2 cells and ILC2s were significantly higher in STRA patients compared to DA and disease controls, while IL-17$^+$ lymphoid cells were similar. ILC2s and Th2 cells were significantly reduced in vivo following intramuscular triamcinolone and in vitro with steroids. Asthma attacks and symptoms also reduced after systemic steroids despite persistence of steroid resistant IL-17$^+$ cells and eosinophils.

Paediatric STRA and DA have distinct airway molecular phenotypes with STRA characterized by elevated type2 cells. Systemic corticosteroids but not maintenance inhaled steroids resulted in improved symptom control and exacerbations concomitant with a reduction in functional ILC2s despite persistently elevated IL-17$^+$ lymphoid cells.
Introduction:
Paediatric severe therapy resistant asthma (STRA) is characterized by persistent poor control despite maximal doses of treatment and optimal assessment of modifiable factors such as adherence, allergen and smoke exposure. STRA affects approximately 2% of children with asthma, but results in significant morbidity [1] utilizing up to 50% of all healthcare resources for asthma [2, 3]. Difficult Asthma (DA) in children is characterized by poor control despite maximal prescribed therapy, but detailed clinical assessments reveal modifiable factors, most commonly lack of adherence to maintenance therapy, as a reason for the apparent poor control [4, 5]. We have shown that paediatric DA and STRA have distinct clinical phenotypes [6]. In contrast to STRA, when the basics of asthma management are addressed, children with DA have lower exhaled nitric oxide levels, improved lung function, and are able to reduce their daily dose of inhaled corticosteroids while maintaining control. Moreover DA continue to have significantly fewer exacerbations than STRA up to six years later [6]. This suggests children with DA have steroid sensitive disease, while STRA have disease that is resistant to maximal maintenance corticosteroids. As a group, children with STRA have reduced lung function, marked eosinophilic airway inflammation and airway remodelling [1]. However, little is known about the molecular phenotype of DA, and whether DA and STRA can be distinguished using molecular as well as clinical phenotypes. If this is possible, it may prevent inappropriate administration of biologicals to children with steroid sensitive disease.

It is recognized that STRA is a heterogeneous disease [7] and the underlying immunological mechanisms are yet to be fully understood. Although traditionally allergic asthma is considered a Th2 mediated disease, emerging evidence, from studies in adults and children, suggests non-Th2 mechanisms may contribute
particularly to severe disease [8, 9]. IL-33 is an innate epithelial cytokine which is elevated in paediatric STRA and is associated with airway remodeling and severe steroid resistant disease [10, 11]. Numerous experimental murine models have underscored the importance of IL-33 in the initiation of allergic airways disease via the induction of type 2 innate lymphoid cells (ILCs) [12-14]. ILCs are a rare population of cells of lymphoid lineage, found predominantly at mucosal surfaces, which can mirror the functions of T helper cell sub-types. Type 2 ILCs are implicated in allergic diseases [15] and are increased in sputum and bronchoalveolar lavage (BAL) from adults [16] and children with severe asthma [17]. Although their importance in severe disease is predicted, little is known regarding their role in milder disease, or how common asthma treatments, such as steroids, impact their function or phenotype.

The Th17 pathway has also been proposed as important in mediating adult, non-type2 severe asthma [18]. Although IL-17 is induced from paediatric STRA PBMCs following in vitro stimulation with steroids [19], nothing is known about IL-17⁺ILCs or the functional importance of IL-17 in paediatric STRA.

Since pulmonary IL-33 remains elevated despite maximal steroid therapy in paediatric STRA [10], we hypothesised that the downstream effector cells, type 2 ILCs remain elevated despite steroids and mediate the pathophysiology of STRA, while they would be lower in DA. We analysed the phenotype and proportion of airway (induced sputum) and peripheral blood CD4⁺ T cells and ILCs in children with STRA compared to DA and age-matched disease controls. To investigate the effect of steroids in true severe disease, proportions of lymphoid cells were compared in induced sputum before and after systemic steroids, and cultured PBMCs were stimulated with allergen and steroids.
Materials and Methods

Subjects

Children (6-16 years) undergoing clinically indicated investigations (blood tests and induced sputum) for severe therapy resistant asthma (STRA), difficult asthma (DA) or recurrent lower respiratory tract infections (LRTI) were included. Clinical characterization, processing of induced sputum and peripheral blood mononuclear cells (PBMCs) were as described previously [17]. STRA (n=16) children had confirmed asthma with poor control despite maximal dose inhaled corticosteroids (≥800mcg/day budesonide equivalent) and underlying modifiable factors, such as adherence, being optimized [2]. DA (n=6) were prescribed maximal dose maintenance therapy, but had evidence of poor adherence as an explanation for poor control [20]. Spirometry and bronchodilator reversibility, fractional exhaled nitric oxide (FeNO), sputum induction and symptom control (Asthma Control Test) were undertaken in all STRA and DA children. Non-asthmatic disease control patients (n=8) had persistent or recurrent cough not responding to antibiotics (n=6), cystic fibrosis (n=1), or primary ciliary dyskinesia (n=1), these were collectively termed chronic inflammation (CI).

Study approval was obtained from the local research ethics committee, parental written informed consent and age-appropriate child assent was obtained.

Flow Cytometry

Cells were incubated for 4hrs with Phorbol-12-myristate 13-acetate [21], Ionomycin and Brefeldin A, stained with a Fixable Viability Stain (Zombie UV, BioLegend, London, UK) and ILC and T cell markers. All ILCs were lineage negative (CD3, CD14, CD16, CD19, CD20, CD56, CD4, FcεR1), CD45+. Type 2 ILCs were CRTH2+ or CD127+ and/or IL-13+ or IL-4+. Th2 cells were CD3+CD4+ expressing CRTH2/IL-
13/IL-4. Th17 cells (CD4+IL-17+) and IL-17+ILCs (lineage negative, IL-17+).

Antibodies used: CD45 (Life Technologies, UK), Lineage cocktail, CD127, CRTH2, CD3, CD4, CD8, IL-13, IL-17A, GATA-3 (BioLegend, UK) FcεR1 (eBioscience, UK).

Data was acquired on BD Fortessa (BD Bioscience, SanJose, CA) and analyzed using Flowjo v10 (Flowjo, Ashland, OR).

**PBMC culture**

PBMCs were cultured with IL-2 (20ng/ml) (T cells), or IL-7 (ILCs) (20ng/ml) with 25μg/ml house dust mite (HDM) extract (Greer Laboratories, USA) and/or budesonide (10⁻⁷ mmol/L). Culture supernatants were collected after 72hrs for cytokine analysis.

**ILC cultures**

ILCs were enriched from whole blood (adult mild asthmatics and healthy controls) using RosetteSep™ Human ILC2 Enrichment Kit (STEMCELL Technologies, UK) and sorted by flow cytometry using CD45⁺ Lineage⁺⁻ [CD1a, CD3, CD4, CD5, CD8, CD11c, CD14, CD16, CD19, CD20, CD34, FcεRI and CD123] (Biolegend), CD161⁺, CD127⁺, CRTH2⁺ and C-Kit⁺. In order to fully differentiate between lineage negative and positive cells after RosetteSep™ antibody cocktail staining, an expanded lineage panel was used. ILCs were cultured in IL-2, IL-7 (5ng/ml) and IL-33 (10ng/ml) (eBioscience). ILC cultures were stimulated as described for PBMC cultures.

**qPCR**

Cultured ILCs were lysed with 350μl RLT buffer (Qiagen, Manchester). Total RNA was extracted using the RNeasy Micro Kit (Qiagen, Manchester) and converted to cDNA. Real-time PCRs were performed using Taqman Fast Advanced Master Mix
with TaqMan primer/probe sets for *IL13* and *NR3C1* and data normalised against the GAPDH and actin to calculate relative expression.

**Immunohistochemistry**

Cytospins of ILC2s stained with anti-Glucocorticoid Receptor (D8H2) XP® Rabbit mAb followed by a biotinylated Goat Anti-Rabbit IgG secondary antibody. Images were acquired on an inverted confocal microscope.

**Statistics**

Sample size was opportunistic as there were no data to inform a power calculation. Non-parametric Kruskal-Wallis, followed by Dunns corrections for multiple comparisons were used to assess between group differences. Wilcoxon matched pairs test was used for paired data. Data presented as median. Correlations were assessed using the Spearman rank correlation test. Graph Pad Prism v5 (GraphPad Software, La Jolla, CA). Statistical significance was accepted as p<0.05.

**Results:**

**Patient characteristics:**

The baseline characteristics of children with STRA, DA and non-asthmatic controls are shown in Table1. Administration of intramuscular triamcinolone was undertaken in STRA as part of our clinical severe asthma investigation protocol and to assess suitability for add-on therapies such as omalizumab [4]. All STRA and DA patients had been prescribed high dose inhaled steroids, long acting beta-agonists and/or leukotriene receptor antagonists (Table 1) [2]. STRA patients had significantly higher
total serum IgE and sputum eosinophils compared to DA and non-asthmatic controls. There was no difference in age, weight, height, forced expired volume in 1 second (FEV$_1$), or forced vital capacity (FVC) between the groups (Table 1).

Table 1. Demographics of paediatric severe therapy-resistant asthma (STRA), difficult asthma (DA) and chronic inflammation (CI) patients.

FEV$_1$: forced expired volume in 1 second, FVC: forced vital capacity, ACT: asthma control test, FeNO: exhaled nitric oxide, ICS: inhaled corticosteroids, OCS: oral corticosteroids

Airway eosinophils were only increased in STRA, while blood eosinophils were higher in STRA and DA compared to controls

Although elevated blood eosinophils are considered a biomarker for severe asthma in adults [22], their role in paediatric severe asthma is less certain [23]. We compared eosinophil numbers in blood and sputum in STRA, DA and non-asthmatic controls. Blood eosinophils were similarly elevated in both STRA and DA compared to controls, while sputum eosinophils were only significantly higher in STRA, and were almost undetectable in DA and controls (figure 1a). There was no correlation between sputum and blood eosinophils in either children with STRA or DA (Supplementary figure 1). Sputum neutrophils were higher in non-asthmatic controls who have recurrent infections (figure 1b), while lymphocytes and macrophages were elevated in STRA and DA (figure 1c & d). Blood neutrophils, lymphocytes and monocytes were similar in all three groups (figure 1b-c).
Increased functional airway type 2 ILCs and T cells in paediatric STRA

We have previously identified type 2 ILCs and T cells in BAL and sputum from STRA patients on the basis of expression of the extra-cellular marker CRTH2⁺ [17]. However, in order to gain greater phenotypic and functional definition we now examined all Lin⁻CD45⁺ innate lymphoid cells (ILCs) for the expression of IL-13, IL-4 and IL-17, the cytokines which may drive disease phenotype (supplementary figure 2). STRA patients had significantly higher frequency of sputum ILCs (Lin⁻CD45⁺) and CD4 T cells expressing CRTH2 than DA and non-asthmatic controls (figure 2a). A higher frequency of sputum ILCs and CD4 T cells from STRA patients also expressed IL-4 (figure 2b) and IL-13 (figure 2c) compared to DA and non-asthmatic controls. However, there was no difference between the three groups in frequency of sputum ILCs or CD4 cells expressing IL-17 (figure 2d). Of note, the non-asthmatic controls with chronic inflammation had elevated sputum neutrophils (figure 1b), but no increase in IL-17⁺ ILCs or CD4 cells (figure 2d). An increased number of ILCs and T cells with the capacity to produce type 2 cytokines defines the patients with STRA.

We therefore measured the levels of type 2 cytokines in the sputum of patients. Levels of IL-13 were elevated in STRA patients compared to controls and the amount of IL-5 was significantly increased in these patients (supplementary figure 3). Peripheral blood ILCs and CD4 T cells expressing CRTH2, IL-13, or IL-17 were not different between STRA and DA (supplementary figure 4 a-c).

Phenotypic features of airway IL-13⁺ ILCs in STRA, DA and controls

Numerous definitions are used for ILCs; based on extracellular expression of CRTH2, or IL-7Rα (CD127), or intra-cellular cytokine expression (IL-13⁺, IL-4⁺, IL-17⁺). We examined both extracellular markers and intra-cellular cytokine expression
to further define the phenotype of airway ILCs in DA and STRA. Furthermore, in order to determine whether only CD127+ ILCs are steroid resistant in patients with severe asthma, as has recently been published [24], we investigated Lin’CD45+ cells that expressed CD127 or CRTH2 or IL-13. Sputum Lin.negCD45+IL-13+ cells expressing the type 2 marker CRTH2 were significantly higher in STRA than DA (figure 3a), while Lin.negCD45*IL-13+ cells expressing the general ILC marker CD127 were similar in both groups (figure 3b). Of all sputum Lin.negCD45*IL-13+ cells in STRA, only 16% were both CD127+ and CRTH2+, the majority (65%) of sputum Lin.negIL13+ cells did not express CD127 and only 33% expressed CRTH2 (figure 3c). We therefore assessed the frequency of Lin.negCD45*IL-13+ that were CRTH2°CD127+ and CRTH2’CD127- in STRA compared to DA and controls. Cells that expressed both CD127 and CRTH2, or neither of the markers were increased in STRA compared to controls (figure 3d & e), suggesting Lin.negCD45*IL-13+ cells may be functionally important in driving STRA. Interestingly, in a similar manner, the majority of sputum Lin.negCD45*IL-17+ cells (79%) also did not express CD127. No Lin.negCD45*IL-17+ cells expressed the type2 marker CRTH2+ (supplementary figure 5).

**Airway type 2 lymphoid cells are reduced in STRA after systemic corticosteroids**

To assess the clinical and immunological response to systemic steroids in children with STRA, sputum induction was performed before and 4 weeks after administration of intramuscular triamcinolone, as part of our clinical protocol [25] (figure 4a). Briefly, children with STRA had an assessment of spirometry, exhaled nitric oxide and symptom score on the morning of receiving triamcinolone and again 4 weeks later (Figure 4a). There was no change in lung function (FEV1 %predicted) up to 12
months after triamcinolone (figure 4b), but there was a significant reduction in exhaled nitric oxide (figure 4c) and an improvement in symptoms assessed using the asthma control test (ACT score) 4 weeks later (figure 4d), and a reduction in asthma attacks (defined as short course of oral corticosteroids prescribed in the year after triamcinolone compared to the year before) (figure 4e), following administration of triamcinolone. The number of sputum eosinophils (figure 4f) and levels of sputum eosinophil peroxidase (a marker of eosinophil activation) were unchanged after triamcinolone (figure 4g). Detailed phenotyping of sputum lymphoid cell populations showed Lin$^{neg}$CD45$^+$ and CD4$^+$T cells expressing either CRTH2$^+$ or IL13$^+$ were reduced, suggesting both of these cell types are steroid sensitive in the airways in vivo (figure 5a - d). In addition, quantification of type2 mediators in sputum supernatants showed a reduction in both IL-13 and IL-5 after triamcinolone, even though eosinophil numbers remained elevated (Figure 5e).

**Functional peripheral blood type 2 lymphoid cells defined using IL-13$^+$ are steroid sensitive in vitro**

To test the hypothesis that ILCs do not just respond to a muted inflammatory environment resulting from steroid suppression in vivo but are themselves steroid sensitive, we cultured PBMCs isolated from 9 STRA patients sensitized to house dust mite (HDM). Cells were stimulated with HDM extract with or without budesonide and then ILC and T cell subsets were analyzed. Stimulation with HDM resulted in a significant increase in Lin$^{neg}$CD45$^+$IL-13$^+$ ILCs and CD4$^+$IL13$^+$ T cells, in PBMC cultures from HDM-sensitized STRA children (figure 6a & b). However, there was a significant reduction in the number of IL-13$^+$ ILCs and IL13$^+$ T cells when the PBMCs were cultured with HDM and budesonide (figure 6a & b). Levels of secreted IL-13
following HDM stimulation in PBMC cultures were also reduced by budesonide (figure 6c).
Circulating IL-17+ ILCs and Th17 cells are steroid refractory

To determine the effect of steroid treatment on IL-17 expressing lymphoid cells, we examined Lin<sup>−</sup>CD45<sup>+</sup> ILCs and CD4<sup>+</sup> cells in PBMC cultures from HDM sensitised patients (n=9) with STRA. Intriguingly, we found that budesonide alone resulted in a significant induction of IL-17+ ILCs, while IL-17<sup>+</sup> T cells remained unchanged (figure 7a & b). However, no difference was noted in levels of IL-17 protein in the supernatant following addition of either HDM and/or budesonide (figure 7c).

IL-13<sup>+</sup> innate lymphoid cells respond directly to steroids

In order to establish whether a pure population of ILCs (CD45<sup>+</sup>, Lin<sup>−</sup>, CD161<sup>+</sup>, CD127<sup>+</sup>, CRTH2<sup>+</sup> and C-Kit<sup>var</sup>, IL-13<sup>+</sup>) responded directly to steroid treatment, peripheral blood cells were sorted and cultured with recombinant IL-2, IL-7 and IL-33 to skew towards a type 2 phenotype [24], for at least 4 weeks (>99% ILC2s). ILCs were sorted from adults because of ethical restrictions preventing large volumes of blood being obtained from children to isolate this rare cell population. Incubation of in vitro cultured cells with budesonide resulted in a reduction in the number of ILCs expressing IL-13, together with reduced IL-13 protein levels in culture supernatants (figure 8a-b). ILCs, which are not antigen specific, but may respond to one of the complex HDM components such as lipids or proteins, did not respond directly to HDM stimulation (figure 8a-b). In the presence of budesonide IL-13 gene expression was also rapidly down regulated (figure 8c). We also looked for expression of the glucocorticoid receptor and showed constitutive expression of NR3C1 by ILCs, with levels of expression increasing in the presence of budesonide (figure 8d). Expression of the glucocorticoid receptor on purified ILCs at the protein level was confirmed by immunofluorescence (figure 8e). These data demonstrate definitively
that in the presence of sufficient doses of steroids, functional Lin$^{\text{neg}}$CD45$^{+}$IL-13$^{+}$ are steroid sensitive.

**Discussion:**

Although paediatric STRA is recognised as being characterised by severe atopy and steroid resistant airway eosinophilia [1, 25], little was known about lymphoid cell populations driving the disease. We have shown increased numbers of ILCs, expressing the type2 markers CRTH2 and IL-13 in the airways from STRA children during stable disease compared to children with DA and disease control patients. Airway type2 ILCs, T cells and eosinophils were elevated in STRA despite prior assessments to ensure optimal adherence to high-dose maintenance inhaled steroid therapy. In contrast, neither lymphoid populations nor eosinophils were elevated in the airways of DA children, suggesting these are clinically and molecularly distinct phenotypes and DA is characterized by more steroid sensitive disease. Contrary to our hypothesis, we found that although airway CRTH2$^{+}$IL-13$^{+}$ ILCs were increased in STRA at baseline, they were reduced by high dose systemic steroids *in vivo* and steroid stimulation *in vitro*. Of note, none of the children were on maintenance oral steroids. In contrast, IL-17$^{+}$ILCs were similar in STRA, DA and controls, and were steroid resistant *in vitro*. There was a reduction in numbers of airway type2 lymphoid cells following high dose systemic steroids and an improvement in symptoms and asthma attacks, but no change in eosinophil numbers in STRA. However, systemic steroids are not a feasible long term therapeutic option and alternative steroid sparing therapies that dampen type2 lymphoid cells are needed.
We acknowledge the numbers of children from whom good quality samples to phenotype sputum lymphoid cells could be obtained is small. We did not select a subgroup for whom data is shown in any of the figures. The reason the numbers in some of the figures is low is because of variable cell numbers in the sputum and limited paired sputum samples before and after triamcinolone, and this is an inevitable weakness of these sorts of studies. We did not select specific children to include in the results. However, we did very carefully clinically phenotype the children and ensure objective assessments of adherence were undertaken prior to defining STRA and DA and the findings show clear distinctions between the groups despite small numbers.

We have previously reported the importance of distinguishing children with DA, who have poor control because of underlying modifiable factors, such as poor adherence, from those with STRA, who remain with poor control despite optimal adherence [4, 5]. We have demonstrated clinical distinctions between STRA and DA, whereby STRA is characterized by persistent poor lung function, frequent attacks and severe and multiple atopic sensitization [6]. However, the molecular phenotype of DA and STRA were unknown. We now demonstrate that children with true severe asthma (STRA) have elevated airway eosinophils, IL-13+ ILCs and T cells compared to children with DA, and importantly these distinctions are only apparent in sputum, not in peripheral blood. Our data have highlighted the importance of investing time and resources to accurately clinically phenotype children with poor asthma control despite maximal prescribed therapy in order to distinguish STRA from DA. If this is not done, as increasing targeted biologics and small molecule therapies become available [26], there is a risk that children may be given these novel drugs inappropriately.
Although airway ILCs are increased in STRA, their role in promoting disease severity, particularly in humans, remains unclear. Since IL-33 is increased in STRA, is a relatively steroid resistant cytokine [10] and induces ILCs [12], we undertook detailed phenotyping of airway ILCs and their response to steroids. We have shown that although the extra-cellular marker CRTH2 is used to denote type2 lymphoid cells, and CD127 is used to denote ILCs, the majority (approx. 65%) of airway IL-13^{+} Lin^{neg}CD45^{+} cells are CD127^{-} and approximately half are CRTH2^{-}. However, the majority of human studies to date that have reported on the prevalence and response of airway ILCs to steroids in the context of allergic disease have included extra-cellular markers as part of the definition [27]. This suggests a large proportion of cells that are lymphoid in origin and are lineage negative, but have the capacity to secrete type 2 cytokines, and are therefore functionally equivalent to an ILC2, have been disregarded. We have shown here, and confirmed our previous findings [17], that in the airways of children with severe asthma, a significant proportion of Lin^{-} CD45^{+} cells do not express the extracellular markers currently used to define ILC2s. It is essential to consider the plasticity of ILCs and the influence of the local environment on their function [28]. Restricting the definition to include the extracellular markers CD127 and/or CRTH2 may therefore be inappropriate for describing ILC2 function, particularly those from tissues in children.

We report that ILCs (Lin^{neg}CD45^{+}CRTH2^{+} and Lin^{neg}CD45^{+}IL-13^{+}) are sensitive to steroids both in vivo and in vitro. These results mirrored the response of Th2 cells to steroids. The data are in agreement with studies in nasal polyps of adults with
chronic rhinosinusitis which have shown that ILC2s are steroid sensitive [29]. However, this is the first demonstration in patients and in airway ILCs following steroids in severe asthma. It has previously been reported that ILC2s in broncho-alveolar lavage fluid (BAL) from adult patients with asthma were resistant to dexamethasone [24]. However, that was demonstrated by \textit{in vitro} stimulation of airway ILCs using a steroid that is not routinely used in clinical practice to treat asthma. We have maximised information obtained from our routine clinical protocol which involves a single dose of systemic steroids in children with STRA [25], and shown that sputum IL-13$^+$ ILCs and CRTH2$^+$ ILCs reduced significantly, in tandem with CD4$^+$ type2 lymphocytes following triamcinolone. In order to confirm the effect of steroids on type2 ILCs alone, and to eliminate the effects of other leukocytes, we demonstrated cultures of peripheral blood type2 ILCs had glucocorticoid receptor expression and also reduced when stimulated with budesonide \textit{in vitro}. It is plausible that although ILC2s are steroid sensitive, delivery of currently available inhaled steroid medications do not reach the airways at optimal concentrations to allow a reduction in the number of these cells, thus explaining elevated numbers in STRA during stable disease. The reduction in exacerbations, coupled with improved symptom score and the associated reduction in type2 lymphoid cells following triamcinolone, suggest it is likely that these cells do play a role in driving symptoms in children with STRA despite high dose treatment with inhaled steroids.

Although we have recently shown no increase in airway Th17 cells in paediatric STRA [30], we had not previously investigated the potential role of IL-17$^+$ ILCs. In agreement with our findings of Th17 cells, airway IL-17$^+$ ILCs were not increased in STRA compared to DA or disease controls. Furthermore, unlike type 2 lymphoid
cells, IL-17+ILCs and Th17 cells were steroid resistant both in vivo and in vitro. We have also demonstrated a significant induction of IL-17+ ILCs with budesonide in vitro. The Th17/IL-17 axis has been linked to severe, steroid resistant adult asthma [31], however, the number of IL-17+ cells in our patients with STRA did not show any correlation with clinical parameters. This is in keeping with our previous report of a lack of correlation between IL-17 levels, Th17 cells and clinical features in paediatric STRA [30]. Even though the disease control children with recurrent infections had an airway neutrophilia, they did not have increased IL-17+ lymphoid cells. This may be because the majority of lymphoid cells in children with neutrophilic airways diseases are in the airway wall, not the lumen [32, 33].

We accept the number of children in the cohort studied before and after triamcinolone was small, this is because not all were able to produce an adequate sputum sample at both time points as this is a technically difficult procedure in young children. However, despite the small numbers, the data demonstrate clear changes in type2 lymphoid cells, and no previous studies have demonstrated longitudinal changes in rare cell populations in airway samples from children. Importantly, all children were clinically stable at the time of sputum induction, with at least 2 weeks since any previous exacerbation. The lack of change in lung function and the relatively high values at baseline for FEV₁ may be explained by the ‘stable’ nature of the patients, but is a consistent clinical finding in children with severe asthma [34]. It would be interesting to assess any change in proportions of ILCs during exacerbation. Murine data suggest ILC3s are important in exacerbations during obese allergic airways disease [35] and ILC2s contribute to influenza induced
episodes [36]. However, their role in viral induced exacerbations in humans remains uncertain.

Significant strengths of our data is the inclusion of carefully clinically characterized patients with STRA and DA, and the utility of non-invasive airway sputum cells to demonstrate changes in immune cells after steroid treatment in vivo and in vitro. We accept our study has limitations. We included children with recurrent lower respiratory tract infections i.e. chronic lower airway inflammation as the best pragmatic control group. Ideally, healthy controls should be recruited, however obtaining airway samples from healthy children is not ethically possible. Of note, previous adult studies have also included similar types of disease controls [24, 37]. In addition, even though significantly more disease controls had neutrophils in sputum, this did not impact numbers of IL-17+ lymphoid cells, even though IL-17 has been associated with neutrophilia [38, 39].

In summary, we have shown the contribution of airway Lin<sup>neg</sup>CD45<sup>+</sup>CRTH2<sup>+</sup> and Lin<sup>neg</sup>CD45<sup>+</sup>IL-13<sup>+</sup> ILCs in children with STRA compared to DA, and the effect of steroids on these cells in paediatric STRA. The reduction in both type2 ILCs and T cells after systemic steroids was associated with a reduction in exacerbations and improvement in symptoms. However, there was no change in numbers of airway eosinophils following steroids. We have also shown that IL-17<sup>+</sup> ILCs and Th17 cells were not increased in STRA compared to DA at baseline and both cell types persisted despite steroids. The clinical improvement despite elevated IL-17<sup>+</sup> cells questions their role in mediating the pathophysiology of paediatric STRA.
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Declarations of interest

none
References


Table 1. Demographics of paediatric severe therapy-resistant asthma (STRA), difficult asthma (DA) and chronic inflammation (CI) patients. ICS: inhaled steroids, OCS: oral steroids, FEV₁: forced expiratory volume in one second, FVC: forced vital capacity, ACT: asthma control test, >19 is normal, FeNO: fractional exhaled nitric oxide. Groups compared by Mann Whitney-U test, significance p<0.05.

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<th>CI (n=8)</th>
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<td>0.05</td>
</tr>
<tr>
<td>FEV₁ (%)</td>
<td>87.5 (66,134)</td>
<td>82.5 (63, 110)</td>
<td>86 (63, 115)</td>
<td>0.06</td>
<td>0.1</td>
</tr>
<tr>
<td>FVC (L)</td>
<td>2.9 (1.2, 4.79)</td>
<td>2.3 (2.13, 3.76)</td>
<td>2.7 (1.5, 4)</td>
<td>0.09</td>
<td>0.1</td>
</tr>
<tr>
<td>FVC (%)</td>
<td>99.8 (89,6,132.1)</td>
<td>95 (68, 106)</td>
<td>102.5 (76, 124)</td>
<td>0.08</td>
<td>0.3</td>
</tr>
<tr>
<td>Total IgE (IU/ml)</td>
<td>321.5 (21, 1938)</td>
<td>161 (81, 801)</td>
<td>38.5 (19, 1252)</td>
<td>0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>Atopic</td>
<td>16 (100%)</td>
<td>5 (83.3%)</td>
<td></td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Aeroallergen sensitisation (number of allergens [median – range])</td>
<td>3 (1-5)</td>
<td>2 (1-3)</td>
<td></td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Sum of specific aeroallergen IgE (IU/ml)</td>
<td>3.3 (0.8-102)</td>
<td>2.1 (0.9-100)</td>
<td></td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>ACT</td>
<td>21 (13, 23)</td>
<td>16.5 (16, 23)</td>
<td>-</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>FeNO (ppb)</td>
<td>12.5 (8, 43)</td>
<td>12 (10, 31)</td>
<td>-</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>ICS (mg/d)</td>
<td>1 (0.8-2)</td>
<td>1 (0.8-2)</td>
<td>0.4 (0-1.6)</td>
<td>0.1</td>
<td>0.04</td>
</tr>
<tr>
<td>OCS (n)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Omalizumab</td>
<td>5</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sputum eosinophil (%)</td>
<td>4.7 (1, 68.1)</td>
<td>0 (0, 1.2)</td>
<td>0.00 (0, 0.5)</td>
<td>0.002</td>
<td>0.002</td>
</tr>
<tr>
<td>Sputum</td>
<td>12.5 (0, 74)</td>
<td>7.7 (0, 77.3)</td>
<td>71.3 (34, 97.5)</td>
<td>0.1</td>
<td>0.001</td>
</tr>
<tr>
<td>Neutrophil (%)</td>
<td>Sputum Lymphocytes (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------</td>
<td>------------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 (0,1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 (0,0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 (0,0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Figure 1. Elevated blood eosinophils in STRA and DA compared to CI, but sputum eosinophils only increased in STRA. a) Frequency of eosinophils in blood and induced sputum from children with severe therapy-resistant asthma (STRA), difficult asthma (DA) and with recurrent lower respiratory tract infections (CI) assessed by morphology. b) Frequency of neutrophils, c) lymphocytes and d) monocytes and macrophages in blood and induced sputum. Kruskall-Wallis test with a Dunns post-test, followed by Mann Whitney test between indicated pairs of groups, **P < 0.01, ***P < 0.001 and ****P < 0.0001. STRA n≥16, DA n≥6 and CI n≥7.

Figure 2. Sputum ILC2s higher in STRA than DA, but IL-17^+ cells similar. a) Induced sputum frequency of CRTH2^+ ILCs (Lin^negCD45^+) and CD4^+ T cells (CD4^+CD3^+) from children with severe therapy-resistant asthma (STRA), difficult asthma (DA) and with recurrent lower respiratory tract infections (CI). b) Frequency of IL-4^+, c) IL-13^+ ILCs and d) IL-17^+ ILCs and CD4^+ T cells. Kruskall-Wallis test with a Dunns post-test, followed by Mann Whitney test between indicated pairs of groups, *P <0.05, and **P < 0.001 STRA n ≥9, DA n≥4 and CI n≥6.

Figure 3. Phenotypic features of IL-13^+ ILCs in STRA, DA and CI. Frequency of ILC2s (IL-13^+Lin^negCD45^+) expressing a) CRTH2 and b) CD127 in STRA, DA and CI patient sputum. c) Pie chart shows frequencies of IL-13^+ ILCs expressing CRTH2 and CD127 in sputum from STRA patients. d-e) Frequency of ILC2s (IL-13^+Lin^negCD45^+) expressing d) both CRTH2 and CD127, e) neither CRTH2 nor CD127 in STRA, DA and CI patient sputum. Kruskall-Wallis test with a Dunns post-test, followed by Mann Whitney test between indicated pairs of groups, *P < 0.05 and **P < 0.01 and ***P < 0.001, STRA n≥13, DA n≥5 and CI n≥8.

Figure 4. Reduction in clinical parameters after high dose systemic steroids. a) Clinical outputs and immune phenotype of STRA patients were assessed before and 4 weeks after intramuscular treatment with the steroid triamcinolone. Clinical assessment of lung function via b) forced expiratory volume in one second (FEV₁), c) fractional exhaled nitric oxide (FeNO), d) asthma control test (ACT) and e) Frequency of clinical exacerbations (per year). Dash lines show FEV₁>80%, ACT>19 and FeNO<25ppb is clinically normal (n=11), f) Induced sputum eosinophil frequency (n=5), g) Levels of eosinophil peroxidase in sputum (n=6). Mann-Whitney U test, **P < 0.01.
Figure 5. Airway ILCs and T cells are steroid sensitive in vivo, after high dose systemic steroids. Induced sputum frequency of ILCs (Lin^{neg}CD45^{+}) and CD4^{+} T cells (CD4^{+}CD3^{+}) expressing a & c) CRTH2 or b & d) IL-13. e) IL-13, IL-4 and IL-5 levels in sputum supernatant. n≥3. Mann-Whitney U test, *P <0.05

Figure 6. Frequency of IL-13^{+} CD4^{+} T cells and IL-13^{+} ILCs from STRA patient peripheral blood are significantly reduced after steroids in vitro. Frequency of IL-13 expressing a) ILC2s (CD127^{+}CD45^{+}Lin^{neg}) and b) CD4^{+} T cells (CD45^{+}CD3^{+}CD4^{+}) after in vitro treatment of PBMC cultures with HDM and/or Budesonide for 72hrs. c) IL-13 protein levels in culture supernatants. STRA n=9. Wilcoxon matched pairs test, *P < 0.05 and **P < 0.01

Figure 7. IL-17^{+} CD4^{+} and IL-17^{+} ILCs are steroid resistant in vitro. Frequency of IL-17^{+} a) ILCs (CD127^{+}CD45^{+}Lin^{neg}) and b) CD4^{+} T cells (CD45^{+}CD3^{+}CD4^{+}) after in vitro treatment of PBMC cultures with HDM and/or Budesonide for 72hrs. c) IL-17 protein levels in culture supernatants. ILC cultures n=8. Wilcoxon matched pairs test, *P < 0.05 and **P < 0.01.

Figure 8. Cultured ILC2s are steroid sensitive in vitro. Expression of a) IL-13 by ILC cultures (Lin^{neg}CD45^{+}CD161^{+}CRTH2^{+}CD127^{+}) treated with HDM and/or Budesonide. b) IL-13 protein levels in ILC culture supernatants. mRNA expression of c) IL13 and d) NR3C1 at baseline and following incubation of cells with budesonide for up to 4hrs, e) ILC2s were enriched and sorted (Lin^{neg}CD45^{+}CD161^{+}CRTH2^{+}CD127^{+}) from peripheral blood of 3 individual donors. mRNA expression of NR3C1 relative to endogenous controls was determined. f) Immunofluorescence staining of the glucocorticoid receptor and overlaid with actin (red) on human ILC cultures. ILC cultures n=8. Wilcoxon matched pairs test, **P < 0.01 and ***P < 0.001.
Lin\textsuperscript{neg} ILCs vs. CD4\textsuperscript{+} T Cells

- **a)** CRTH2
- **b)** IL-4
- **c)** IL-13
- **d)** IL-17

**Legend:**
- STRA: Red squares
- DA: Blue circles
- CI: Black triangles

**Statistical Symbols:**
- ***: p < 0.001
- **: p < 0.01
- *: p < 0.05

**Note:**
- Graphs show the percentage of Lin\textsuperscript{neg} cells among various cell populations.
- CRTH2 expression
- IL-4, IL-13, IL-17 cytokine expression
- Comparisons across STRA, DA, CI treatments.
Visit 1 i.m. triamcinolone
Visit 2 4 weeks later
Child with STRA
Spirometry, exhaled nitric oxide, symptom control score, sputum induction

FeNO
Visit 1 Visit 2
0 10 20 30 40 ppb

ACT
Visit 1 Visit 2
5 10 15 20 25 30 /25

Exacerbations
Previous 1y Subsequent 1yr

Sputum Eosinophils
Visit 1 Visit 2
0 2 4 6 8 % total cells

Eosinophil Peroxidase
Visit 1 Visit 2
0 10 20 30 40 ng/ml/gram
Airway ILCs

a) CRTH2^+Lin^{-}negCD45^{+}

b) IL-13^+Lin^{-}negCD45^{+}

Airway CD4^{+} T cells

c) CRTH2^+CD4^{+}

d) IL-13^+CD4^{+}

Airway Type 2 mediators in sputum supernatants

e) IL-13

f) IL-4

g) IL-5
a) IL-17 Lin^neg

b) IL-17 CD4^+ T cells

c) IL-17 Levels
Supplementary figure 1. No relationship between blood and sputum eosinophils in STRA or DA. The percentage of sputum eosinophils (y-axis) was correlated with the percentage of blood eosinophils (x-axis) in children with severe therapy resistant asthma (STRA) – squares and children with difficult asthma (DA) – triangles. There was no significant relationship in either group. Dotted lines represent normal value cut-offs.

Supplementary figure 2. Sputum cells isolated from STRA patients were stained for extracellular markers that distinguish ILC and T cells. a) Plot shows forward/side scatter and identified lymphoid population. Identification of viable and CD45$^+$ cells that were lineage negative (Lin$^-$ (CD14, CD16, CD19, CD20, CD56, CD4)) and CD3$^-$ and evaluated for extracellular markers CRTH2 and CD127 and intracellular cytokines IL-13 and IL-4. b) shows live lymphocytes that were gated for CD3$^+$CD8$^+$ versus CD3$^+$CD4$^+$ T cell subsets and intracellular cytokines IL-4 and IFN-γ were evaluated in CD3$^+$CD4$^+$ T cells

Supplementary figure 3. Levels of IL-4, IL-5 and IL-13 in sputum supernatants measured by multi-plex assay (MSD).

Supplementary figure 4. PBMC ILC2s and Th2 cells similar in STRA and DA. b) frequency of CRTH2$^+$ ILCs (Lin$^{\text{neg}}$CD45$^+$) and CD4$^+$ T cells (CD45$^+$CD3$^+$) in PBMCs from children with severe therapy-resistant asthma (STRA), difficult asthma (DA) and with recurrent lower respiratory tract infections (CI). c) Frequency of IL-13$^+$ in ILCs and CD4$^+$ T cells. d) Frequency of IL-17$^+$ ILCs and CD4$^+$ T cells. Kruskall-Wallis test with a Dunns post test, followed by Mann Whitney test between indicated pairs of groups, *P <0.05, and **P < 0.01. STRA n =≥11, DA n=≥4 and CI n =≥6.

Supplementary figure 5. Extracellular markers on IL-17$^+$ ILCs. Pie chart shows frequencies of IL-17$^+$ ILCs (Lin$^{\text{neg}}$CD45$^+$) expressing CRTH2 and CD127 in in sputum from STRA patients. n=12.
Linneg ILCs CD4+ T Cells

a) CRTH2

% of Lin^neg cells

b) IL-13

% of Lin^neg cells

c) IL-17

% of Lin^neg cells

% of CD4+ cells

Strain DA CI

**

*
STRA IL-17⁺ Lin⁻neg CD45⁺

CD127⁺

CD127⁻neg

CRTH2⁻neg

21%

79%