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ORIGINAL ARTICLE **Asthma and Rhinitis**

Group 2 innate lymphoid cells (ILC2s) are increased in chronic rhinosinusitis with nasal polyps or eosinophilia

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Clinical & Experimental Allergy

Summary

Background Chronic rhinosinusitis (CRS) is a heterogeneous disease with an uncertain pathogenesis. Group 2 innate lymphoid cells (ILC2s) represent a recently discovered cell population which has been implicated in driving Th2 inflammation in CRS; however, their relationship with clinical disease characteristics has yet to be investigated.

Objective The aim of this study was to identify ILC2s in sinus mucosa in patients with CRS and controls and compare ILC2s across characteristics of disease.

Methods A cross-sectional study of patients with CRS undergoing endoscopic sinus surgery was conducted. Sinus mucosal biopsies were obtained during surgery and control tissue from patients undergoing pituitary tumour resection through transphenoidal approach. ILC2s were identified as $CD45^+Lin^-CD127^+CD4^-CD8^-CRTH2(CD294)^+CD161^+$ cells in single cell suspensions through flow cytometry. ILC2 frequencies, measured as a percentage of $CD45^+$ cells, were compared across CRS phenotype, endotype, inflammatory CRS subtype and other disease characteristics including blood eosinophils, serum IgE, asthma status and nasal symptom score.

Results 35 patients (40% female, age 48 ± 17 years) including 13 with eosinophilic CRS (eCRS), 13 with non-eCRS and 9 controls were recruited. ILC2 frequencies were associated with the presence of nasal polyps ($P = 0.002$) as well as high tissue eosinophilia ($P = 0.004$) and eosinophil-dominant CRS ($P = 0.001$) (Mann–Whitney *U*). They were also associated with increased blood eosinophilia ($P = 0.005$). There were no significant associations found between ILC2s and serum total IgE and allergic disease. In the CRS with nasal polyps (CRSwNP) population, ILC2s were increased in patients with co-existing asthma ($P = 0.03$). ILC2s were also correlated with worsening nasal symptom score in CRS ($P = 0.04$).

Conclusion and clinical relevance As ILC2s are elevated in patients with CRSwNP, they may drive nasal polyp formation in CRS. ILC2s are also linked with high tissue and blood eosinophilia and have a potential role in the activation and survival of eosinophils during the Th2 immune response. The association of innate lymphoid cells in CRS provides insights into its pathogenesis.

Keywords chronic rhinosinusitis, eosinophils, group 2 innate lymphoid cell, human, ILC2, nasal polyp, tissue

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Introduction

Chronic rhinosinusitis (CRS) encompasses a heterogeneous group of disorders defined by the 'inflammation of the paranasal sinuses' and represents a significant health problem worldwide [1]. Importantly, the pathogenesis of CRS is poorly understood, as simple allergic or infective models are not representative of the

pathophysiologic process, which leads to difficulties in disease management and resulting in poor quality of life and substantial financial burden [2].

Chronic rhinosinusitis has been typically categorised based on findings on endoscopic examination: CRS with nasal polyps (CRSwNP) and without nasal polyps (CRSsNP). This diagnosis by phenotype is not always indicative of a skewed mucosal inflammatory response (eosinophilic cf neutrophilic). This can lead to difficulties in medical management of patients with CRS for those judging the appropriateness of interventions (e.g. corticosteroid) purely on the patient phenotype. Emerging histopathological classification, endotyping, is based on tissue eosinophilia and classifies patients into eosinophilic CRS (eCRS) and non-eosinophilic CRS (non-eCRS). This is thought to reflect the underlying inflammatory process more closely and eCRS has been linked with increased disease severity, development of nasal polyps and poorer prognosis [3, 4]. Both CRSwNP and eCRS are thought to be driven by a skewed Th2 immune response driven by a dysregulated sinus mucosa [3], potentially in a similar mechanism to asthma [5].

Group 2 innate lymphoid cells (ILC2s) have been identified as a major innate producer of interleukin (IL)-13 and IL-5, in response to IL-25 and IL-33 expression [6–8]. ILC2s have been identified in Th2 inflammatory diseases and shown to mediate helminth immunity in the intestine, airway hyperreactivity and obstruction in asthma and influenza infection as well as tissue remodelling and epithelial repair after injury through the expression of an epidermal growth factor known as amphiregulin [7, 9–14].

Whilst ILC2s have now been widely researched in murine models, human studies remain limited. ILC2s, defined as $CD45^+$Lineage–$CD127^+CRTH2(CD294)^+CD161^+$, were demonstrated to be significantly raised in nasal polyp tissue ($n = 4$) in patients with CRS as compared to healthy nasal turbinate tissue ($n = 4$) [15]. A second study identified ILC2s as lineage$^-CD127^+$ cells and reported that patients with CRSwNP ($n = 6$) had significantly elevated ILC2s in their sinus mucosa than patients with CRSsNP ($n = 4$) [16]. Importantly, the study demonstrated that in response to concurrent IL-33 and IL-2 stimulation, ILC2s expressed significantly elevated IL-13 levels in patients with CRSwNP. Functionally, human ILC2s have been shown to be regulated by GATA-3, whose expression is enhanced by thymic stromal lymphoprotein (TSLP) released from the nasal polyp epithelium [17]. The study demonstrated that GATA-3 plays an important role in the induction of surface CRTH2 and release of Th2 cytokines in response to TSLP and IL-33.

It is now widely thought that in CRS, and especially eCRS, there is an immune dysregulation of the underlying mucosa [3, 18]. Importantly, IL-25, IL-33 and eotaxin-3 have been shown to be overexpressed from sinus mucosal epithelium in CRSwNP patients compared to CRSsNP, which promotes Th2 inflammation [19]. The expression of Th2-associated cytokines (IL-13, IL-5, IL-4) is responsible for the activation and mediation of eosinophils, mast cells as well as the differentiation of naïve T cells into activated Th2 cells which further drives the Th2 immune response [20].

As ILC2s remain a recent discovery, current research into their role in CRS remains quite limited. However, they represent a significant development for the understanding of the pathogenesis of CRS. Studies so far have been focused on the role of ILC2s as effector cells, characterising their responsiveness to and expression of different cytokines, with little investigation into their relationship to clinical outcomes, not only in CRS but other Th2 diseases.

This study aims to identify and quantify ILC2 populations, defined as $CD45^+$Lineage$^-CD4^-CD8^-CD127^+$ $CRTH2^+CD161^+$ cells in sinus mucosa in patients with CRS and controls, and comparing cell populations across a series of clinical disease characteristics.

Materials and methods

This study was approved by the St Vincent's Hospital Human Research Ethics Committee (LNR/13/SVH/96). Written informed consent was obtained from all patients.

Subjects

Adult patients diagnosed with CRS, according to the European Position Paper on Rhinosinusitis and Nasal Polyps [1] and undergoing functional endoscopic sinus surgery (FESS) as part of the management for their disease, were recruited. These patients had previously failed conservative medical therapy, and all ceased systemic corticosteroid medication at least 4 weeks prior to their surgery. Patients with established immunodeficiency, coagulation disorder, pregnancy, classic allergic fungal sinusitis or cystic fibrosis were excluded from the study.

Normal control tissue was taken from patients undergoing pituitary tumour resection through endoscopic transphenoidal approach, where normal mucosa was present on both endoscopic examination and computed tomography imaging.

Tissue collection

Sinus mucosal biopsies were sampled from the ethmoid sinuses from patients with CRS and from the sphenoid sinuses from control patients. Biopsy tissue was placed

in sterile 60-mL polystyrene containers containing 10 mL of tissue culture medium [Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco Life Technologies, Paisley, Scotland), with 10% Foetal Calf Serum (FCS) (Bovogen, Keilor East, VIC, Australia) and 100 U/mL Penicillin–Streptomycin (Gibco Life Technologies)]. Samples were transported on ice to the laboratory, and all sample processing began within 90 min of tissue collection.

Tissue processing

The tissue culture medium was removed and discarded. 4 mL of wash buffer [75 μL 0.5 M Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, St. Louis, MO, USA), 50 μL 0.1 M Dithiothreitol (DTT) pH 8.0 (Invitrogen Life Technologies, Carlsbad, CA, USA) made to 50 mL with Hank's balanced salt solution (HBSS) (Gibco Life Technologies), and 500 μL 100 U/mL Penicillin–Streptomycin (Invitrogen Life Technologies)] was added to the fresh tissue and incubated at 37°C with shaking at 130 r.p.m. for 20 min. Supernatant from this wash was collected in a 50-mL polypropylene tube, and the wash was repeated.

The tissue was then minced using sterile scissors (Jamar and Hill Surgical Company, Bondi Junction, NSW, Australia) to approximately less than 2-mm pieces and digested with 200 μL of 200 U/mL collagenase type III (Sigma-Aldrich) and 200 μL of 200 μg/mL DNase type IV (Sigma-Aldrich) in 1.6-mL tissue culture medium. This was then incubated at 37°C, shaking at 110 r.p.m. for 1 h, with manual shake every 20 min to dissociate any tissue clumping. Digested tissue was then strained through a 70-μm nylon mesh cell strainer (Becton-Dickinson, San Jose, CA, USA) using a 5-mL syringe plunger into the 50-mL polypropylene tube containing the wash supernatant.

Hundred microlitre of the single cell suspension was stained with the following antibodies to human proteins: Alexa Fluor (AL)-647-conjugated anti-CRTH2; AL-700-conjugated anti-CD4; allophycocyanin (APC)-Cy7-conjugated anti-CD45; fluorescein isothiocyanate (FITC)-conjugated lineage cocktail (consisting of CD3, CD14, CD16, CD19, CD20, CD56); FITC-conjugated anti-Epcam; Brilliant Violet (BV)-421-conjugated anti-CD127 (BioLegend, San Diego, CA, USA); Horizon-V500-conjugated anti-CD8; phycoerythrin (PE)-conjugated anti-CD161 (Miltenyi Biotec, Bergisch Gladbach, Germany); and PE-Cy7-conjugated anti-CD25. All antibodies were from Becton-Dickinson unless otherwise noted. See additional methods information in Fig. S1. Cells were stained for 15 min at room temperature, washed once with phosphate-buffered saline (PBS; Gibco Life Technologies) and fixed with 200 μL of 0.5% paraformaldehyde.

Flow cytometric analysis

Samples were analysed within 6 h of tissue collection using an LSRII flow cytometer (Becton-Dickinson) with FACSDiva software v6.1.2 (Becton-Dickinson), collecting 1 million events per sample. This data were analysed using FlowJo software v8.8.6 (Tree Star, Ashland, OR, USA). Fluorescent minus one (FMO) controls were used in the determination of gating. ILC2s were identified as $CD45^+Lineage^-CD4^-CD8^-CD127^+$ $CRTH2^+CD161^+$ through the gating strategy in Fig. 1. ILC2s were confirmed to not express mast cell or eosinophil markers with secondary staining showing negative CD123 expression (Fig. S2).

Clinical data

Patients were classified as CRSwNP or CRSsNP based on the presence of nasal polyps on pre-surgery endoscopy. Histopathological analysis was performed on tissue taken at the time of surgery and assessed the

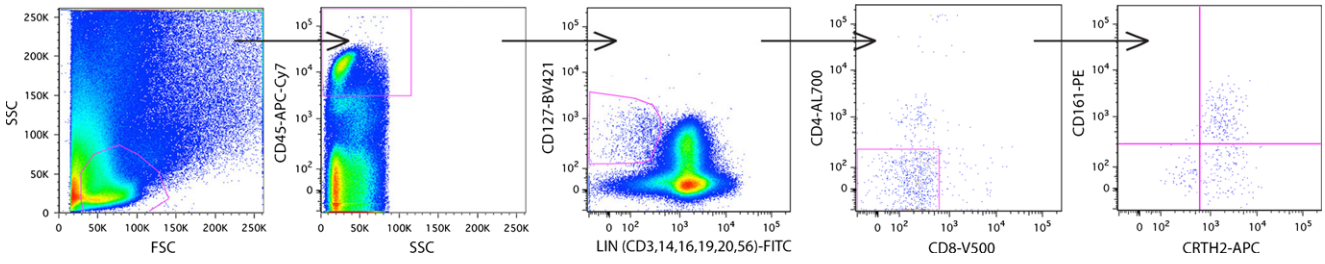

Fig. 1. Gating strategy for the identification of group 2 innate lymphoid cells (ILC2s) in sinus mucosal tissue by flow cytometry. Representative data from one patient with eosinophilic CRS (eCRS) are shown. From 1 million events collected, the sample was initially gated on the lymphocyte population with low forward and side scatter, identifying $CD45^+$ haematopoietic cells. Cells were then gated on positive $CD127^+$ expression and negative expression of lineage markers (CD3, CD14, CD16, CD19, CD20, CD56). Any $CD4^+$ or $CD8^+$ cells were further excluded. ILC2s were gated as cells populations with positive $CRTH2^+$ and $CD161^+$ expression. Results were reported as a percentage of $CD45^+$ cells.

following histopathological parameters: eosinophil count (< 10 eosinophils per high power field (HPF), 10–100 eosinophils/HPF, > 100 eosinophils/HPF) and neutrophil infiltrate (absent, focal, diffuse). eCRS was classified as > 10 eosinophils/HPF and non-eCRS as < 10 eosinophils/HPF. Neutrophilic CRS was classified as < 10 eosinophils/HPF and the presence of a focal or diffuse neutrophil infiltrate.

Peripheral blood was collected at the time of surgery, identifying eosinophil count ($\times 10^9$/L) and total IgE (kU/L). A patient was classified as atopic if they showed a positive (> 1) radioallergosorbent test (RAST) result (in response to either grasses, house dust, moulds or epithelial mixes) or raised total IgE levels defined as > 100 kU/L. Asthma status was determined by either a positive spirometry result on challenge testing or b-agonist use, or if currently using regular inhaled bronchodilator or corticosteroid therapy.

A 5 question nasal symptom score (NSS) subset (need to blow nose, nasal obstruction, loss of smell or taste, thick nasal discharge and facial pain/pressure) assessing the patient's symptom severity over the previous two weeks pre-surgery.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software, version 6.0 for Macintosh (GraphPad, San Diego, CA, USA). Chi-square tests were performed for analyses of baseline characteristics. As ILC2 data were nonparametric, results are reported as median (interquartile range) and analysed with Kruskal–Wallis test with post hoc Dunn or Spearman's rank correlations as appropriate. All *P*-values are two-tailed, and a value of $P < 0.05$ was considered statistically significant.

Results

Thirty-five patients (40% female, age 48.30 ± 16.87 years) were recruited including 9 controls, 13 non-eCRS and 13 eCRS patients. Baseline characteristics are summarised in Table 1. There was no significant difference in age, gender, allergy, asthma and smoking status between the three study groups. Patients with eCRS were more likely to have nasal polyps, due to the Th2-skewed nature of CRS ($P = 0.005$) [4].

CRS phenotype

Previous studies have identified increased frequencies of ILC2s in CRSwNP patients as compared to CRSsNP and control patients [15, 16], and thus, the relationship between ILC2s and CRS phenotype was assessed (Fig. 2). Of 26 patients with CRS, there were 14 CRSwNP and 12 CRSsNP. CRSwNP patients had significantly higher percentages of ILC2s compared with CRSsNP patients [0.09 (0.25) vs. 0.02(0.04); $P = 0.03$] and controls [0.09(0.25) vs. 0.02(0.03); $P = 0.002$].

CRS endotype

As ILC2s are proposed to express Th2-associated cytokines which activate different cells including eosinophils, their relationship with CRS endotype was assessed, with eCRS classified on the basis of > 10 eosinophils/high power field (HPF) (Fig. 3a). Patients with eCRS showed significantly higher ILC2 percentages than found in non-eCRS patients [0.07(0.19) vs. 0.02(0.03); $P = 0.02$] and controls [0.07(0.19) vs. 0.02(0.03); $P = 0.008$].

Tissue eosinophilia

Having identified higher ILC2 frequencies in the eCRS group, patients were further classified based on tissue eosinophilia into 10–100 and > 100 eosinophils/HPF groups (Fig. 3b). There were 13 with < 10 eosinophils/HPF, 6 with 10–100 eosinophils/HPF and 7 with > 100 eosinophils/HPF. Patients with > 100 eosinophils/HPF showed higher ILC2s than controls [0.13(0.35) vs. 0.02 (0.03); $P = 0.001$] and < 10 eosinophils/HPF [0.13(0.35) vs. 0.02(0.03); $P = 0.007$]; however, the difference between the 10–100 and > 100 groups was not significant [0.13(0.35) vs. 0.03(0.04); $P = 0.18$].

Table 1. Baseline characteristics

Patients	Control ($n = 9$)	Non-eCRS ($n = 13$)	eCRS ($n = 13$)	*P* value
Age	46.58 ± 18.19	49.87 ± 19.79	47.92 ± 13.77	0.90
Female (%)	2 (22%)	6 (46%)	6 (46%)	0.45
Nasal polyps (%)	0 (0%)	3 (23%)	11 (85%)	0.005*
Allergy (%)	1 (11%)	6 (46%)	8 (62%)	0.06
Asthma (%)	2 (22%)	3 (23%)	8 (62%)	0.07
History of smoking (%)	1 (11%)	1 (8%)	1 (8%)	0.95

eCRS, eosinophilic CRS.

Chi-squared test.

*$P < 0.05$.

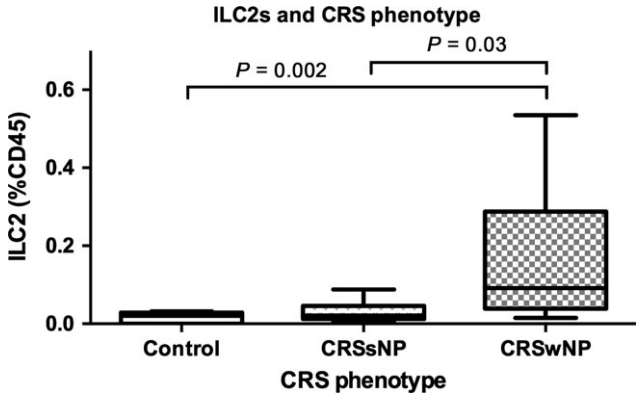

Fig. 2. Group 2 innate lymphoid cells (ILC2s) across CRS phenotype. ILC2 data are presented as a percentage of $CD45^+$ cells. Results are shown as box plots of median and interquartile range, with 5th and 95th percentiles whiskers. Statistical analysis was performed using Kruskal–Wallis test with post hoc Dunn test. CRSsNP, chronic rhinosinusitis without nasal polyps; CRSwNP, chronic rhinosinusitis with nasal polyps.

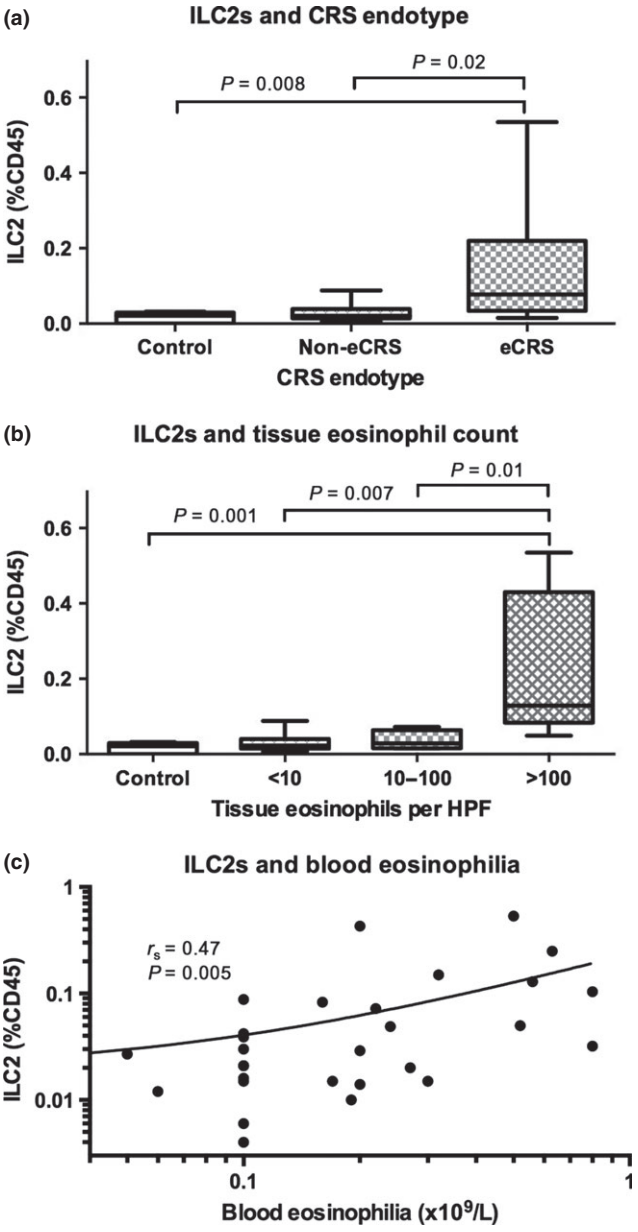

Fig. 3. Group 2 innate lymphoid cells (ILC2s) across eosinophil markers: (a) CRS endotype; (b) tissue eosinophil count; and (c) blood eosinophilia. ILC2 data are presented as a percentage of $CD45^+$ cells. Results are shown as box plots of median and interquartile range, with 5th and 95th percentiles whiskers. Statistical analysis was performed using (a, b) Kruskal–Wallis test with post hoc Dunn test; (c) Spearman's rank correlation. Eosinophilic CRS (eCRS), > 10 eosinophils/high power field; non-eCRS, < 10 eosinophils/high power field; HPF, high power field

Blood eosinophil count

Following on from the significant associations between ILC2s and tissue eosinophilia, the relationship of ILC2s with blood eosinophil count was examined (Fig. 3c). There was a significant positive correlation found between ILC2 frequencies and the eosinophil count in the peripheral blood ($r_s = 0.47$, $P = 0.005$), which was more pronounced in the patients with CRS ($r_s = 0.59$, $P = 0.002$).

Neutrophil infiltrate

Whilst eosinophils have been thought to drive Th2 inflammation, conversely, neutrophils are thought to drive a mixed Th1/Th17 inflammatory process in CRS. When classified according to neutrophil infiltrate, there were 12 with absent, 10 with focal and 4 with diffuse neutrophil infiltrates (Fig. 4a). Patients with absent neutrophils showed significantly higher ILC2 populations than those with diffuse neutrophil infiltrates [0.07(0.09) vs. 0.01(0.01); $P = 0.046$] and controls [0.07(0.09) vs. 0.02(0.03); $P = 0.007$ but no significant difference when compared to focal neutrophil infiltrates [0.07(0.09) vs. 0.02(0.03); $P = 0.22$].

Eosinophilic vs. neutrophilic CRS subtypes

Having demonstrated significant positive association of ILC2s with eosinophil count and significant negative association with neutrophil infiltrate, a comparison between eosinophilic and neutrophilic CRS subtypes was performed (Fig. 4b). There were 13 patients classified with eosinophilic CRS and 7 classified with neutrophilic CRS. Patients with eosinophilic mediated disease showed significantly higher ILC2 frequencies than neutrophilic predominant disease [0.09(0.25) vs. 0.02(0.02); $P = 0.01$] and controls [0.09(0.25) vs. 0.02(0.03); $P = 0.004$].

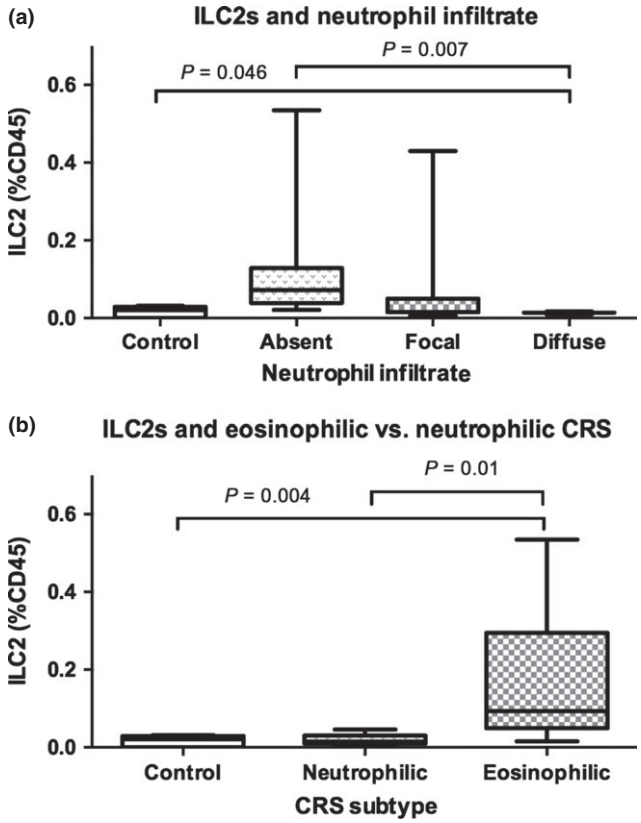

Fig. 4. Group 2 innate lymphoid cells (ILC2s) across (a) neutrophil infiltrates; and (b) across eosinophilic and neutrophilic CRS subtypes. ILC2 data are presented as a percentage of $CD45^+$ cells. Results are shown as box plots of median and interquartile range, with 5th and 95th percentiles whiskers. Statistical analysis was performed using Kruskal–Wallis test with post hoc Dunn test.

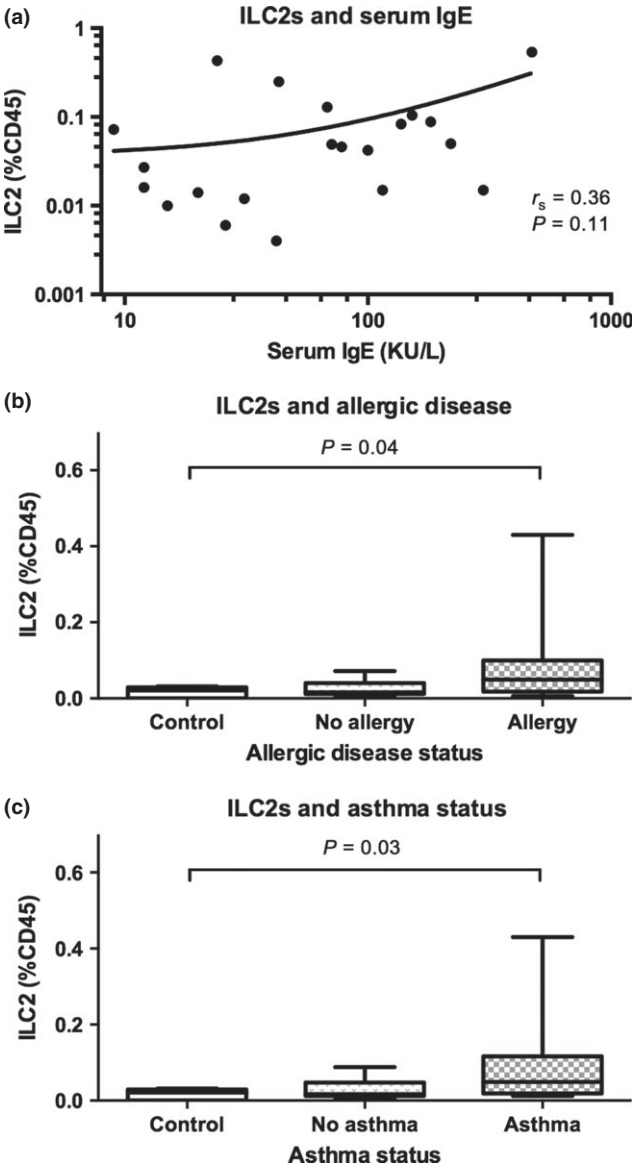

Fig. 5. Group 2 innate lymphoid cells (ILC2s) across: (a) serum IgE; (b) allergic disease; and (c) asthma status. ILC2 data are presented as a percentage of $CD45^+$ cells. Results are shown as box plots of median and interquartile range, with 5th and 95th percentiles whiskers. Statistical analysis was performed using (a) Spearman's rank correlation; (b, c) Kruskal–Wallis test with post hoc Dunn test.

Serum total IgE

IgE has been implicated in the pathogenesis of various allergic diseases, including asthma and allergic rhinitis, usually mediated through Th2 inflammation. As ILC2s have been identified in a number of allergic disease models in mice, the association between ILC2s and serum total IgE was assessed (Fig. 5a). No significant correlation was found between the two measures (r_s = 0.36, P = 0.11).

Allergic disease

wFurther evaluation of the relationship between ILC2s and allergic disease, mediated through the production of Th2-associated cytokines and IgE, was performed (Fig. 5b). There were 15 with positive allergy status and 11 with no allergy. Patients with positive allergic status showed significantly higher ILC2s percentages than controls [0.05(0.08) vs. 0.02(0.03); P = 0.04]. Whilst patients with positive allergic status also showed higher ILC2s than patients with no allergy, this difference was not statistically significant [0.05(0.08) vs. 0.02(0.03); P = 0.08]. There was no association with any particular allergic sensitivity. Further subgroup analysis did not show significant elevation of ILC2 frequencies in allergic individuals with comorbid CRSwNP (P = 0.69) or eCRS (P = 0.80) compared to the non-allergic population.

Asthma

Multiple murine studies into ILC2s have identified a significant role of these cells in mediating airway

hyper-reactivity in asthma in the absence of T and B cells. To determine whether asthma comorbidity influenced ILC2 frequencies in CRS, analysis of these two variables was performed (Fig. 5c). There were 11 patients with CRS with asthma and 15 without asthma. Patients with CRS who reported having asthma showed significantly higher ILC2s than control patients [0.05 (0.10) vs. 0.02(0.03); $P = 0.03$]; however, there was no significant difference between the asthma and no asthma populations [0.05(0.10) vs. 0.02(0.04); $P = 0.19$], and between no asthma and control patients [0.02(0.04) vs. 0.02(0.03); $P > 0.99$]. Further examination of the CRSwNP population revealed that patients with asthma ($n = 8$) showed significantly higher frequencies of ILC2s than those without asthma ($n = 6$), [0.12(0.33) vs.0.03 (0.06); $P = 0.03$].

Patient reported outcome measures

There was a significant moderate correlation found between ILC2s and nasal symptom score ($r_s = 0.30$, $P = 0.04$) (Fig. 6).

Correlations with other cells

ILC2s are part of the innate lymphoid cell (ILC) family, which have morphologic features of lymphoid cells without the rearrangement of antigen receptors [21]. The proportion of ILC2s out of total ILCs was increased in the eCRS population compared with the non-eCRS population; however, this was not significant [0.71 (0.22) vs. 0.44(0.30); $P = 0.32$].

Th2 cells were not measured in this study; however, surrogate correlations of CD4 T-cell subsets showed significant correlation with activated CD4 T cells ($Epcam^-CD45^+CD3^+CD4^+CD45RO^+CD38^+$) ($r_s = 0.385$, $P = 0.03$) as well as TFH cells ($Epcam^-CD45^+CD3^+CD4^+CD45RO^+PD1^{hi}CD127^{lo}CXCR5^+$) ($r_s = 0.425$, $P = 0.02$).

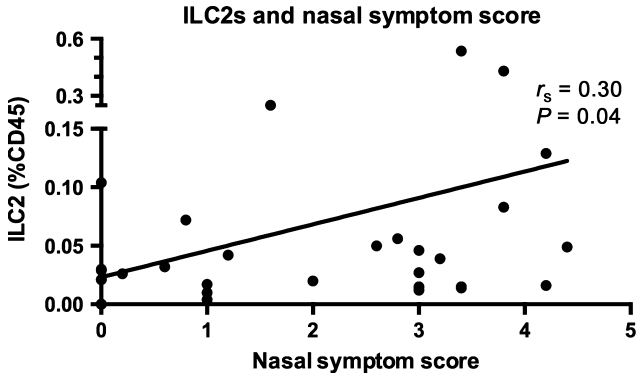

Fig. 6. Correlation of group 2 innate lymphoid cells (ILC2s) and nasal symptom score. ILC2 data are presented as a percentage of $CD45^+$ cells. Statistical analysis was performed using Spearman's rank correlations.

Discussion

One current model of CRS research focuses on an underlying immune dysfunction of the sinus mucosa, as a disordered interface between the host and the environment, with contributions from environmental damage and foreign antigens [18]. CRSwNP and eCRS have been linked with a skewed Th2 immune response through eosinophilic inflammation mainly mediated through IL-5 and IL-13 cytokines [22]. The discovery of ILC2s which promote Th2 inflammation, even in the absence of Th2 cells [7, 8], represents an important finding in immunology and highlights a potential role within CRS. This study examined the role of ILC2s in the pathogenesis of CRS and their association with different clinical disease outcomes.

The data presented reflect previous studies which showed significantly elevated ILC2 frequencies in patients with CRS compared with controls [15], as well as in patients with CRSwNP compared with patients with CRSsNP [16], pointing to a role of ILC2s in nasal polyp development. Phenotyping based on the presence of nasal polyps remains the standard method of classifying CRS; however, factors driving the development and recurrence of nasal polyps remain unknown.

This study links elevated ILC2 frequencies with increased eosinophils in the tissue and blood, showing an important association between ILC2s in the activation and mediation of eosinophil activity in Th2 inflammatory disease in humans and specifically in CRS. These results are complemented by the inverse association between ILC2 frequencies and tissue neutrophil infiltrate as well as ILC2s and neutrophilic CRS subtype. In addition, the varied presentations of CRS clinically have been thought to represent opposite ends of the disease spectrum [22]. CRSsNP is associated with mixed Th1/Th17-skewed inflammation mediated by neutrophils, whereas CRSwNP is linked with a Th2-skewed inflammatory process mediated by eosinophils, which is reflected in these results [23]. The association of ILC2s with tissue and blood eosinophilia further strengthen the argument for eosinophilic endotyping of CRS as part of routine practice as this more accurately reflects the underlying inflammatory disease process [4]. The study was limited due to the measurement of tissue eosinophilia in ordinal groups and future investigations comparing ILC2 frequencies with scale eosinophil data would be of value to further assess the relationship.

ILC2s are thought to play a similar role to Th2 cells, working in conjunction in promoting and mediating the Th2 immune response. Whilst this study did not directly measure Th2 cell frequencies, surrogate correlations of CD4 T-cell subsets showed significant correlation with activated CD4 T cells ($Epcam^-CD45^+CD3^+CD4^+CD45RO^+$

$CD38^+$) ($r_s = 0.385$, $P = 0.03$) as well as TFH cells ($Epcam^-CD45^+CD3^+CD4^+CD45RO^+PD1^{hi}CD127^{lo}CXCR5^+$) ($r_s = 0.425$, $P = 0.02$). As it is theorised that ILC2s activate naïve T cells into Th2 cells [24], these results may be reflective of the dual relationship that ILC2s and Th2 cells play in driving inflammation in CRS.

This association with eosinophil markers reflects the current understanding of ILC2s which have been shown to constitutively express IL-5 and induce IL-13 expression in mice [8, 25, 26]. These cytokines have been shown to play an important role both independently and in conjunction in the recruitment, differentiation and survival of eosinophils [27, 28]. Through a VPAC2 receptor, murine intestinal and lung ILC2s have been shown to stimulate the circadian cycling of blood eosinophils and control eosinophil homeostasis [25]. In addition, Shaw et al. [16]. showed that ILC2s were responsive to IL-33 and IL-2 costimulation, leading to a significant expression of IL-13 in patients with CRSwNP when compared to patients with CRSsNP and no stimulation.

Asthma and allergic disease were not significantly associated with increased ILC2s in CRS. However, analysis of the CRSwNP population showed significantly elevated ILC2 frequencies in the asthmatic group compared with non-asthmatic group. Whilst ILC2s have been identified previously in murine models of asthma and allergic disease [29], it is likely that these cells play a disease-modifying rather than pathogenic role in CRS inflammation. ILC2s in asthma may be upregulated during inflammatory exacerbations and identification of raised ILC2 percentages in human asthma would support a united airways disease hypothesis [30]. There was no significant correlation found between ILC2s and serum IgE; however, serum IgE may not be reflective of IgE levels at the local tissue mucosa where it has been shown to be significantly elevated in CRSwNP [31]. Thus, future studies investigating the relationship between ILC2s and IgE at the local level would be of interest in CRS.

The nasal symptom score covering key symptoms in CRS showed significant correlation with ILC2 frequencies, indicating that these cells may be responsible for differences in symptom severity in patients with CRS and in keeping with the relationship to eosinophilia and the potential for airway damage that activated eosinophils cause [3]. Importantly, disease severity did not correlate with tissue or blood eosinophilia, with non-eCRS patients showing poorer quality of life scores compared with CRS patients, however, this was not significant ($P = 0.09$).

Future studies of ILC2s will include immunohistochemistry, as well as the isolation of these cells from sinus mucosa through flow cytometric sorting to allow further characterisation of these cells through functional or cytokine studies. In this study, the expression of Th2 cytokines from identified ILC2s was not examined. Previous studies have shown functional expression of Th2 cytokines from ILC2s identified as $Lin^-CD127^+CRTH2^+$ (as has been utilised in this study) and confirms that this staining represents an accurate method of identifying true IL-13/IL-5/IL-4 producing ILC2s [16, 17].

Limitations in this study include the exclusion of a live/dead discriminator in flow cytometry analysis; however, this was accounted for using forward and side scatter characteristics as well as exclusion of dying cells through the use of a number of negative markers in identifying ILC2s, which would exclude non-specifically bound monoclonal antibodies. In addition, investigations with a larger population would be of value, including assessments regarding steroid responsiveness and the role of other ILC subsets in CRS.

Whilst still a recent discovery, targeted therapeutics against ILC2s may be an option for the management and treatment of CRS as well as other Th2 diseases in the future. Already, a study has shown that ILC2 activity is induced through the activation of the mammalian target of rapamycin (mTOR) and that rapamycin administration in mice led to reductions in ILC2 frequencies, cytokine expression, eosinophil accumulation and airway mucus production [32]. However, ILC2s have been demonstrated to have both positive and negative effects on respiratory tissue, as the depletion of ILC2s in mice has resulted in impaired airway homeostasis and epithelial airway repair [33]. Thus, it is important to consider any negative side effects of inhibition of ILC2 activity at both a local and systemic level.

Conclusion

Overall, ILC2s represent a significant new area of research for the understanding of the pathogenesis of CRS, especially CRSwNP and eCRS. This study identified significant associations with nasal polyps and eosinophils, reflecting the proposed role of ILC2s in driving Th2 inflammation. Further investigations into the properties and effects of this cell population will provide great insights into the role of ILC2s in CRS and may become a useful target in the management of CRS in the future.

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J.H. was responsible for all experimental work and was the main author of this study. R.H. and W.S. provided the study concept. R.H. and R.S. assisted in sample collection and data entry. M.B. and J.Z. devised the tissue disruption and flow cytometry protocols used in this

study. N.M. and staff at the Sydney ENT Clinic contributed to the administrative aspects of the study.

Conflict of interest

Raymond Sacks is consultant for Medtronic and Nycomed, speakers' bureau for Merek Sharp Dolme and Arthrocare. Richard J Harvey has served on an advisory board for Schering Plough and Glaxo-Smith-Kline, previous consultant with Medtronic, Olympus and Stallergenes, speakers' bureau for Merek Sharp Dolme and Arthrocare and has received grant support from NeliMed. Jacqueline Ho, Michelle Bailey, John Zaunders, Nadine Mrad and William Sewell: no financial disclosures and no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Antibody panel used for the staining for type 2 innate lymphoid cells.

Figure S2. CD123 staining on identified ILC2 cell.