Mucosal type 2 innate lymphoid cells are a key component of the allergic response to aeroallergen

Jaideep Dhariwal$^{1,2,*}$, Aoife Cameron$^{1,2,*}$, Maria-Belen Trujillo-Torralbo$^{1,2}$, Ajerico del Rosario$^{1,2}$, Eteri Bakhsoliani$^{1,2}$, Malte Paulsen$^{3}$, David J Jackson$^{1,2,6}$, Michael R Edwards$^{1,2}$, Batika M J Rana$^{4}$, David J Cousins$^{2,4,5}$, Trevor T Hansel$^{1,2}$, Sebastian L Johnston$^{1,2,*}$ and Ross P Walton$^{1,2,*}$ on behalf of the MRC-GSK strategic alliance consortium

$^1$ Airway Disease Infection Section, National Heart and Lung Institute, London, UK
$^2$ MRC Asthma UK Centre in Allergic Mechanisms of Asthma, London, UK
$^3$ St. Mary’s Flow Cytometry Core Facility, London, UK
$^4$ Department of Respiratory Medicine & Allergy, King’s College London, London, UK
$^5$ NIHR Respiratory Biomedical Research Unit, Department of Infection, Immunity & Inflammation, Leicester Institute for Lung Health, University of Leicester, Leicester, UK
$^6$. Guy's and St Thomas' NHS Trust, London, UK

*These authors contributed equally to this work

Corresponding Author: Dr Ross P Walton, Airway Disease Infection Section, National Heart and Lung Institute, St. Mary’s Campus, Norfolk Place, London, W2 1PG

Sources of Support: This work was supported by a Medical Research Council (MRC) and GlaxoSmithKline Strategic Alliance Programme Grant number G1100238, the National Institute of Health Research (NIHR) Biomedical Research Centre funding scheme and the MRC and Asthma UK Centre Grant G1000758. DJC also acknowledges financial support from NIHR Biomedical Research Centre based at Guy's and St Thomas' NHS Foundation Trust and King's College London and from the NIHR Leicester Respiratory Biomedical Research Unit. SLJ is the Asthma UK Clinical Chair (grant CH11SJ) and is an NIHR Senior Investigator.

Running Title: ILC2s key to allergen response in atopic rhinitis

Descriptor Number: 1.09, 3.33, 7.13, 7.18

Word Count: 3,238

At a Glance Commentary:

Scientific knowledge on the subject: Elevated numbers of type 2 innate lymphoid cells (ILC2s) have been identified at mucosal barrier surfaces in several atopic diseases, including the sputum of severe atopic asthmatic patients. However, the contribution of ILC2s to allergic airways inflammation and asthma and their kinetics following allergen exposure are poorly understood, partly due to current upper airway sampling techniques which don’t allow serial sampling over the course of an allergic response.

What this study adds to the field: Here we demonstrate, for the first time, that ILC2s are recruited to the upper airways of atopic asthmatic subjects in response to nasal allergen challenge, which is associated with increased symptom severity and markers of type 2 inflammation, suggesting a central role for ILC2s in orchestrating
type 2 responses. The novel methodology of nasal curettage, followed by flow cytometric analysis, enables the detailed identification of mucosal cell infiltrates during the allergic response, and presents wide applications in drug development and clinical diagnosis.

**Word count: 166**

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org.
Abstract

**Rationale:** Newly characterised type 2 innate lymphoid cells display potent type 2 effector functionality, however their contribution to allergic airways inflammation and asthma is poorly understood. Mucosal biopsy used to characterise the airway mucosa is invasive, poorly tolerated and does not allow for sequential sampling.

**Objectives:** To assess the role of type 2 innate lymphoid cells during nasal allergen challenge in subjects with allergic rhinitis, using novel non-invasive methodology.

**Methods:** We used a human experimental allergen challenge model, with flow cytometric analysis of nasal curettage samples, to assess the recruitment of type 2 innate lymphoid cells and granulocytes to the upper airways of atopic and healthy subjects following allergen provocation. Soluble mediators in the nasal lining fluid were measured using nasosorption.

**Measurements and Main Results:** Following allergen challenge, atopic subjects displayed rapid induction of upper airway symptoms, an enrichment of type 2 innate lymphoid cells, eosinophils and neutrophils, along with increased production of interleukin-5, prostaglandin D₂, and eosinophil and T-helper type 2 cell chemokines compared to healthy subjects. The most pronounced type 2 innate lymphoid cell recruitment was observed in patients with elevated serum IgE and airway eosinophilia.

**Conclusions:** The rapid recruitment of type 2 innate lymphoid cells to the upper airways of allergic rhinitis patients, and their association with key type 2 mediators, highlights their likely important role in the early allergic response to aeroallergen in the airways. The novel methodology described herein enables the analysis of rare cell populations from non-invasive, serial tissue sampling.
Word Count: 245

Keywords: ILC2, Th2, Allergic Airway Inflammation, Asthma
Introduction

Type 2 immune responses are characterised by the secretion of the cytokines IL-4, IL-5 and IL-13 in response to the epithelial derived cytokines IL-25, IL-33 and thymic stromal lymphopoeitin (TSLP), which are released by airway epithelial cells following exposure to allergen or virus (1-5). Exaggerated type 2 responses drive the pathogenesis of human allergic disease, including atopic asthma and allergic rhinitis (6-8).

In the context of allergen exposure, dendritic cells sense and subsequently present allergen to naïve T cells and promote their differentiation towards T-helper type 2 (Th2) cells, which direct down-stream allergic inflammatory responses and secrete type 2 cytokines. The release of these cytokines leads to IgE production, the recruitment and activation of eosinophils, in addition to directly affecting airways hyperresponsiveness and mucous production by goblet cells, which together promote the pathological features seen in allergic disease.

Type 2 innate lymphoid cells (ILC2s) are a recently characterised group of effector cells of the innate immune response, which also have the capacity to produce large quantities of the type 2 cytokines, especially IL-5 and IL-13 (9-12). An increasing number of animal studies suggest that ILC2s may play an important early role in the initiation of Th2 responses to aeroallergens during allergic lung inflammation, bridging the innate and adaptive immune responses (13, 14). ILC2s have been identified in man (6, 15), where they express the prostaglandin D$_2$ (PGD$_2$) chemo-attractant receptor also found on Th2 cells (CRT$_{th}$2) (15). PGD$_2$ has recently been shown to be important in both ILC2 recruitment and activation, in addition to cysteinyll leukotrienes, and innate cytokines IL-25, IL-33 and TSLP (15-17).
Elevated numbers of ILC2s have been identified across a number of mucosal tissues, in a variety of human type 2 mediated diseases (18-21), notably in nasal polyps from patients with chronic rhino-sinusitis, suggesting a specific role for ILC2s in type 2 mediated disease (15, 22). However, the exact contribution of ILC2s during the orchestration of the allergic response in man is unclear. One challenge in achieving this has been a lack of repeatable, minimally invasive tissue sampling strategies which allow the analysis of inflammatory cell kinetics. We have investigated the localised cellular responses of the nasal mucosa to topical nasal allergen challenge (NAC), in a group of allergic rhinitis subjects, utilising the novel method of applying flow cytometric analysis to nasal curettage samples.

Nasal curettage samples are minimally invasive and well tolerated, collected without necessity for analgesia, which lends itself to routine and sequential sampling. Using this novel technique we have identified the presence of a population of human ILC2s in the localised response to allergen provocation, enhancing our knowledge of the role of these recently characterised cells in human type 2 disease. Some of the results of these studies have been previously reported in the form of an abstract(23).

Methods

Ethics and Consent

This study was approved by the London Bridge Research Ethics Committee (reference 12/LO/1278) and was carried out in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. Informed consent was obtained from all subjects prior to their participation.
**Study Participants**

Non-smoking, moderately severe asthmatic volunteers with allergic rhinitis were recruited. Study volunteers had an Asthma Control Questionnaire (ACQ) score greater than 0.75 (24), were on treatment comprising of inhaled corticosteroids (ICS) or a combination inhaler (long acting beta agonist (LABA)+ICS). Following histamine challenge, asthmatic subjects had objective airway hyperresponsiveness (AHR) with a (PC)_{20} histamine < 8 μg/mL, and evidence of atopy on skin prick testing (≥ 1 positive skin prick test on a panel of 10 aeroallergens to include grass). Histamine challenge and skin prick tests were carried out following standard protocols, as previously described (6). Non-smoking, non-atopic, healthy volunteers were also recruited. Full inclusion and exclusion criteria are displayed in Table 1, which included the use of anti-histamines and oral or nasal steroids as a factor for exclusion. Baseline characteristics of study volunteers are shown in Table 2.

**Study Design**

Study volunteers meeting the inclusion criteria underwent a single nasal allergen challenge, during a period between October to March outside the UK grass pollen season, using Timothy grass pollen (Aquagen SQ (ALK 225), *Phleum pratense*, Number 4, freeze dried extract (ALK Abello, Reading, UK). A working concentration of 1μg of allergen in 100μl of solution was administered by nasal spray into each nostril. Nasosorption of nasal mucosal lining fluid was performed, as previously described (6), pre-challenge and at 2 h intervals up to 8 h post challenge. Nasal curettage sampling was performed following nasosorption at baseline and at 6 h post challenge. Total Nasal Symptom Scores (TNSS) and clinical observations were assessed at serial intervals pre and post-challenge in all study subjects, see Figure
E1 in the online data supplement. TNSS represents the cumulative score of patient assessed runny nose, nasal blockage, itching and sneezing, each scored from 0-3 using a questionnaire. Additional details on clinical methodology are provided in the online data supplement.

**Nasal Curettage Sampling**

Nasal curettage samples were collected using a plastic Rhino-probe™ curette (Arlington Scientific, Springville, UT). Two samples were taken from the surface of the nasal inferior turbinate using a gentle scraping motion and collected into FACS buffer prior to processing into a single cell suspension, using a 21 gauge needle. Nasal curettage samples are smaller than nasal biopsies, up to 60µm in depth (determined by electron microscopy, unpublished data).

**Flow Cytometry and FACS Analysis of Nasal Mucosa Tissue**

Nasal cells were incubated in normal human serum (Sigma-Aldrich, Shaftesbury, UK), to prevent non-specific binding, prior to surface staining for the following populations: eosinophils (HLA-DR<sup>−</sup>, CD9<sup>+</sup> CD16<sup>−</sup>), neutrophils (HLA-DR<sup>−</sup>, CD16<sup>+</sup> CD9<sup>+</sup>) and ILC2s (lineage<sup>−</sup> (CD2, CD3, CD14, CD16, CD19, CD56, CD235a) CD123<sup>−</sup> FcεRIα<sup>−</sup>, CD127<sup>−</sup> CRTh2<sup>+</sup>). CD45 positive selection was used to identify ILC2s in a subgroup of study volunteers, which did not affect ILC2 enumeration. Numbers of ILC2s, eosinophils and neutrophils were calculated for each patient by multiplying their percentage of total live cells, following flow cytometric analysis, by the total number of nasal cells stained. ILC2 cells were sorted and analysed using a Becton Dickinson AriaIIIU Cell Sorter. Granulocytes were analysed using a Becton Dickinson Fortessa LSR-SORP. Further details on the cytometer settings, and
antibodies and buffers used are provided in the online data supplement (Table E1, E2).

**Immunoassays**

IL-2, IL-4, IL-5, IL-6, CXCL8/IL-8, IL-12p40, IL-13, IL-17, IFN-γ, CCL11/eotaxin-1, CCL17/TARC, CCL22/MDC and CCL26/eotaxin-3 levels were analysed using Meso-Scale Discovery (MSD) (Rockville, MD) V-Plex array platform and a Sector Imager 2400 (MSD). PGD₂ levels were analysed using a PGD₂-MOX enzyme immunoassay (Cayman Chemicals, Ann Arbor, MI) and run on a Spectra Max Plus 384 plate reader (Molecular Devices, Wokingham, UK) with SoftMax Pro 5.4.5 software (Molecular Devices).

**Statistical Analysis**

Statistical analysis was performed using Prism 6 (GraphPad Software, La Jolla, CA). Data is presented as median (25th, 27th) with statistical differences within groups determined using Wilcoxon’s signed rank test and between groups using Mann-Whitney U tests. Kruskal-Wallis tests were used to assess differences between groups where appropriate. Correlations were investigated using Spearman’s correlation coefficient. Differences were considered statistically significant at P values <0.05. All P values are two-sided.

**Results**

17 volunteers (9 atopic asthmatic and 8 healthy) underwent a single NAC with Timothy grass pollen. There were no subject withdrawals. One asthmatic volunteer required a single dose of short acting bronchodilator following challenge.
Atopic Patients Experience Rapid Onset of Nasal Symptoms Following NAC

Following NAC atopic volunteers displayed a rapid and significant increase in their Total Nasal Symptom Scores, peaking within the first 30 minutes. This increase in symptoms persisted for up to 6 h before returning to baseline (Figure 1). Healthy subjects experienced minimal symptoms throughout the course of the study duration.

Allergen-induced Granulocyte Recruitment to the Nasal Mucosa is increased in Atopic Individuals

We characterised the granulocytic response in the nasal submucosa by flow cytometric analysis. Single cells were gated on the basis of size and granularity, live cells selected, and neutrophils and eosinophils were identified based on their expression of the markers HLA-DR, CD9 and CD16 (Figure 2A). Atopic volunteers had significantly increased numbers of eosinophils present in the nasal mucosa at baseline compared to healthy controls, this was further increased following allergen challenge, with a trend towards increased eosinophil recruitment to the upper airways following NAC in atopy, which was not present in healthy subjects (Figure 2B, 2C). We also observed significantly higher neutrophil numbers in subjects with allergic rhinitis compared to controls at baseline, along with significant recruitment following NAC (Figure 2B, 2D).

Type 2 Cytokines and Chemokines are Induced Following Allergen Challenge in Atopy

We next sought to determine the nasal levels of mediators associated with the type 2 response. Levels of each mediator are shown at 6 h post-NAC, with full time courses displayed in the online supplement, see Figure E2. NAC evoked a trend towards
induction of IL-5 in the upper airways of atopic subjects, with levels significantly elevated in allergic patients following allergen challenge compared to healthy controls (Figure 3A). Similar trends were observed when we measured PGD$_2$, which is associated broadly with allergic inflammatory responses, but more recently indicated as being the major chemotactic factor for ILC2s (25). Atopic patients had a highly significant increase in PGD$_2$ following NAC, which was not observed in healthy controls (Figure 3B). The Th2 cell chemokine CCL17/TARC was also significantly induced in the airways of atopic patients following allergen challenge, with levels significantly increased in the upper airways compared to healthy controls 6 h post-NAC (Figure 3C). There was a trend towards the induction of the eosinophil chemokine CCL26/eotaxin-3 (Figure 3E) at 6 h post-NAC in atopic subjects, consistent with the increase in eosinophil number observed in this group post-NAC (Figure 2C). This pattern of induction was not seen in healthy subjects. In contrast there was no induction of type 1 cytokine interferon (IFN)-γ following challenge in either group (Figure 3F). The pro-inflammatory mediator IL-6 was elevated 6 h post-NAC in atopic subjects (Figure 3G). Median levels of IL-13, CCL22/MDC, CXCL8/IL-8 and IL-12p40 and are displayed in Table E3 in the online data supplement. No differences were detected in these mediators between healthy and atopic subjects, at baseline or 6 h post-NAC, nor were levels altered following challenge. IL-2, IL-4 and IL-17 were below the assay limit of detection.

**Allergen Challenge Causes Enhanced ILC2 Recruitment to the Nasal Mucosa in Atopic Patients**

We quantified ILC2s within the nasal mucosa, by flow cytometry, identifying them as live, single cells which were negative for lineage markers (CD2, CD3, CD14, CD16, CD19, CD56 and CD235a), CD123 and FcεR1α, and were CD127$^+$ CRTh2$^+$ cells
At baseline atopic subjects had enriched ILC2 numbers compared to healthy controls (Figure 4B, C). In support of a role for ILC2s in the allergic response, atopic patients demonstrated a significant induction of ILC2s 6 h post-challenge, while no change was observed in the healthy controls. Following allergen challenge, ILC2 numbers where significantly higher in atopic individuals compared to healthy volunteers (Figure 4B, C).

**ILC2 Recruitment Correlates with Markers of Type 2 Responses**

Having established that ILC2s were recruited to the upper airway during NAC, we then assessed their association with other parameters of type 2 responses. Nasal ILC2 numbers 6 h post-allergen challenge positively correlated with nasal eosinophil numbers 6 h post-NAC, baseline serum IgE and IL-5 levels 6 h post-NAC (Figure 5A-C), suggesting that ILC2 infiltration is increased following allergen challenge in atopic patients with Th2 high, eosinophil mediated disease. Table 3 displays the statistical analysis for all the parameters correlated with ILC2s, for the total study population, and atopic patients alone.

**Discussion**

Increased understanding of the pathophysiology underlying allergic responses in the airway has led to greater characterisation of disease subsets and ultimately the development of new therapeutic approaches. The recent characterisation of a novel immune cell family, the innate lymphoid cells, and the subsequent identification of a role for ILC2s in executing type 2 effector functionality places them as a focal point for investigation in atopic disease (10, 21). Herein, we utilised a clinical model of
allergen challenge and novel sampling methodology to identify ILC2s as major responders during the acute phase allergic response in the upper airways.

Our study is the first, to our knowledge, to demonstrate that atopic asthmatic patients have a strong trend towards enriched accumulation of ILC2s in the nasal mucosa outside of allergy season compared to healthy control subjects at baseline. Furthermore, following allergen provocation, only atopic subjects have a significant increase in the number of ILC2s in the upper airways, resulting in significantly greater number being present compared with healthy controls. This enhanced recruitment occurs rapidly in the first 6 h after allergen exposure.

We identified increased numbers of eosinophils in the nasal mucosa of allergic rhinitis patients, both pre- and post-allergen challenge, which have classically been associated with the allergic immune response in asthma. We also identified enriched neutrophils in the upper airways in atopic asthmatic patients. While neutrophil influx to the lower airways in asthma has been demonstrated previously, thought to reflect the response to the pro-inflammatory milieu induced after allergen provocation (26, 27), we demonstrated influx to the upper airways following allergen challenge. These observations are consistent with previous grass pollen studies demonstrating presence of a nasal neutrophil RNA signature following allergen provocation (28). We also observed increased levels of the pro-inflammatory mediator IL-6 in our atopic patients, which has been shown to play a crucial role in neutrophil trafficking (29). Interestingly, ILC2s have been demonstrated to be a possible source of IL-6 production (17), although in this study nasal levels did not correlate with ILC2 numbers and would require further interrogation.
Smith et al. previously identified elevated ILC2 numbers in the sputum of patients with eosinophilic asthma (21), and numbers of IL-13+ ILC2s in the peripheral blood of asthmatic patients have been shown to be associated with level of asthma control (30). Here we demonstrate that the recruitment of ILC2s correlates with known features of type 2 inflammation such as baseline total IgE and IL-5 secretion post challenge, in addition to eosinophil number. This suggests that ILC2s comprise a major component of the localised mucosal cellular immune response to allergen provocation in allergic rhinitis patients with asthma.

The positive association of post-NAC ILC2 numbers with both post-NAC eosinophil numbers and IL-5 levels, as well as baseline IgE, suggests that ILC2 responses are associated with a “Th2 high” clinical endotype. In particular, the correlation with baseline IgE highlights a novel potential use of serum IgE as a surrogate marker for ILC2 responses in this subset of patients with chronic lung disease. This is of specific interest in the context of clinical trials as it would provide a rapid and easily performed test to help stratify asthmatic patients into groups based on the likelihood of their ILC responsiveness. A number of CRTh2 antagonists have undergone clinical investigation (31-35). Given the importance of CRTh2 in identifying human ILC2s, combined with recent evidence that PGD₂ engagement of this receptor on ILC2s induces their migration and activation (25), would make CRTh2 antagonists an attractive therapeutic approach against ILC2 mediated allergic inflammation.

Although the mechanisms by which ILC2s are recruited to sites of inflammation remain unclear, following NAC in this study we report significantly increased PGD₂ levels in the nasal lining fluid of allergic rhinitis subjects, which could act to recruit ILC2s to the upper airways. Mast cell degranulation during the allergic response is thought to be the major source of PGD₂, with eosinophils also contributing (36, 37).
In addition, ILC2s are also known to express CCR4, the receptor for the chemokines CCL22 (MDC) and CCL17 (TARC), which are necessary for T-cell trafficking (20). We observed a significant increase in nasal levels of CCL17 in atopic patients following NAC, suggesting this chemokine could also be involved in chemotaxis of ILC2s from the periphery.

One of the key features of ILC2s is their capacity to produce large quantities of the type 2 cytokines, particularly IL-5 and IL-13. We observed increased levels of IL-5 in the upper airways of atopic subjects following NAC compared to healthy non-atopic individuals. Given the short duration of the sampling period, it is unlikely that this would be due to CD4+ T helper cells alone, however mast cells are another likely source (38). Further investigation would be required to determine the absolute relative contribution for each cell population in this setting.

It has previously been shown that ILC2s can represent as many as 50% of the total IL-5 producing cells following allergen provocation in mouse models (39), and together this would suggest that ILC2s play a key role in orchestrating the recruitment of the effector cells of the allergic response, via the early production of the type 2 mediators. The strong correlation observed between ILC2s and eosinophil recruitment following NAC strengthens this observation.

Whilst the significant recruitment of ILC2 to the nasal mucosa in allergic volunteers undoubtedly represents a pathological mechanism of their allergic rhinitis, it remains difficult to ascertain whether the herein observed trend for increased ILC2 numbers present in the upper airways at baseline are directly related to the subject’s allergic rhinitis or their underlying asthma. Interestingly, recent work by Bartemes et al. (40), looking at the capability of ILC2s isolated from peripheral blood to generate type 2
responses, demonstrated that this process was enhanced in asthma alone and not allergic rhinitis. Furthermore, ILC2s are increased in the sputum of severe asthmatic patients, compared to those with mild disease (21). Whilst these studies suggest that the ILC2 responses observed are related to patients’ asthma phenotype and that ILC2s are involved in the pathogenesis of asthma, it is clear that further work is required to distinguish differences in ILC2 responses in relation to specific disease states. These could include a direct comparison of upper airway responses between atopic asthmatic and atopic, non-asthmatic patients following allergen provocation, directly addressing the relative contribution of atopic and asthmatic status to ILC2 biology. We believe it likely that substantial shared mechanisms of cellular inflammation exist between atopy and asthma.

Our atopic asthmatic subjects were all on inhaled corticosteroids, which may have suppressed the degree of ILC2 responses observed, with some reports suggesting that systemic steroid use can result in an approximately 50% reduction in ILC2 number (41). It would therefore be interesting to investigate whether greater ILC2 responses are seen in steroid naïve atopic asthmatic individuals.

In this study we have established a novel technique for the analysis of the local nasal mucosal immune response, using flow cytometric analysis of nasal curettage samples. This has allowed for direct and accurate quantification of discrete cell populations, alongside their in-depth phenotypic analysis within the nasal mucosa. In the context of a NAC model, this has allowed for the careful and complex assessment of the underlying mechanisms of allergic inflammation in a safe, reproducible and non-invasive fashion that permits serial sampling. It could also provide a useful clinical tool for stratifying patients with active symptoms of rhinitis, and monitoring responses to both local and/or systemic treatment.
In summary, this study provides evidence for a population of ILC2s in the nasal mucosa of atopic asthmatic patients, which is significantly increased following allergen provocation and is associated with cardinal features of upper airways allergic inflammation. These changes suggest that ILC2s may play an important role in the pathogenesis of allergen-mediated type 2 disease in upper airways, highlighting a need for greater understanding of their role in the lower airways in asthma and further identifies them as a target for future therapeutic interventions.
Acknowledgements

The authors gratefully acknowledge the St Mary’s Flow Cytometry Core Facility, National Heart and Lung Institute, Imperial College London for support and access to equipment.
References


type 2 inflammation during rhinovirus-induced asthma exacerbations in vivo.

*American journal of respiratory and critical care medicine* 2014;190:1373-1382.


37. Feng X, Ramsden MK, Negri J, Baker MG, Payne SC, Borish L, Steinke JW. Eosinophil production of prostaglandin d2 in patients with aspirin-exacerbated...


Figure Legends

**Figure 1. Increased nasal symptom scores in atopic patients following allergen challenge.** Nasal symptoms were assessed at baseline (30min prior to NAC) and at the time-points show post-NAC (0hr) in atopic patients (N=9) and healthy controls (N=8) using a nasal symptom questionnaire. Patients scored the severity of their nasal running, blockage, itching and sneezing from 0-3, Total Nasal Symptom Score represents the cumulative score. Statistical analysis was carried out using a Kruskal-Wallis non-parametric ANOVA and Mann-Whitney U tests, data is displayed as median (25th, 75th). P values ** < 0.01 *** < 0.001. Abbreviations: NAC nasal allergen challenge.

**Figure 2. Increased recruitment of granulocytes to the upper airways of atopic patients following allergen challenge.** Atopic patients (n=9) and healthy controls (n=8) were challenged intranasally with timothy grass pollen. Nasal curettage samples were collected from the inferior turbinate and granulocytes enumerated via flow cytometric analysis. A) Eosinophils (CD9+ CD16−) and neutrophils (CD9− CD16+) were determined within the single, live, HLA-DR− gate. B) Representative flow cytometric plots display eosinophils and neutrophils from two healthy and two atopic patients at baseline and 6 h post-NAC. C) Eosinophil and D) neutrophil numbers for healthy and atopic subjects at baseline at 6 h post-NAC are shown. Bars represent median values. Kruskal-Wallis tests were used to assess differences between groups and further statistical analysis for paired data was calculated using Wilcoxon matched pairs test and unpaired data using Mann Whitney U test. P values * < 0.05 ** < 0.01, P values < 0.1 displayed. Abbreviations: NAC nasal allergen challenge.
Figure 3. Induction of type 2 mediators in the nasal lining fluid of atopic patients following nasal allergen challenge. Nasal lining fluid samples were collected using nasosorption, at baseline (30 min prior to NAC) and at 2 h intervals up to 8 h post-NAC. Levels of A) IL-5, B) PGD₂, C) CCL17/TARC, D) CCL11/eotaxin-1, E) CCL26/eotaxin-3, F) IFN-γ and G) IL-6 were determined in atopic patients and healthy controls, using MSD and a PGD₂-methoxime enzyme immunoassay. Levels 6 h post-NAC are displayed. Bars represent median values and broken lines represent the limit of detection. Statistical analysis for paired data was calculated using Wilcoxon matched pairs test and unpaired data using Mann Whitney U test. P values * < 0.05 ** < 0.01, P values < 0.1 displayed. Abbreviations: NAC nasal allergen challenge, MSD meso-scale discovery.

Figure 4. Atopic individuals have increased recruitment of ILC2s to the upper airways following allergen challenge. Atopic patients (n=9) and healthy controls (n=8) were challenged intranasally with timothy grass pollen. Nasal curettage samples were collected from the inferior turbinate. A) ILC2s were enumerated via flow cytometry analysis using the following gating strategy: live, single, lineage⁻ (CD2, CD3, CD14, CD16, CD19, CD56, CD235a) CD123⁻ FcεR1α⁻, CD127⁺ CRTh2⁺ cells. B) Representative flow cytometric plots of CD127⁺ CRTh2⁺ ILC2s from two healthy and two atopic patients are shown at baseline and 6 h post-NAC. C) ILC2 numbers from healthy and atopic patients pre- and post-NAC are displayed, bars represent median values. Kruskal-Wallis tests were used to assess differences between groups and further statistical analysis for paired data was calculated using Wilcoxon matched pairs test and unpaired data using Mann Whitney U test. P values * < 0.05 ** < 0.01, P values < 0.1 displayed. Abbreviations: NAC nasal allergen challenge.
Figure 5 Markers of type 2 responses correlate with ILC2 recruitment. Atopic patients (n=9) and healthy controls (n=8) were challenged intranasally with timothy grass pollen. The number of ILC2s in the upper airways 6 h post-NAC correlated with A) eosinophil number 6 h post-NAC, B) baseline serum IgE levels (n=7 healthy controls) and C) IL-5 levels 6 h post-NAC, using Spearman’s correlation coefficient. Abbreviations: NAC nasal allergen challenge.
### Allergic Rhinitis Subject with Asthma: Inclusion Criteria

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age 18-55 years</td>
<td>Histamine PC20 &lt;8ug/ml (or &lt;12ug/ml and bronchodilator response ≥12%)</td>
</tr>
<tr>
<td>Clinical diagnosis of asthma</td>
<td>Daily ICS (daily dose ≥400mcg fluticasone or equivalent) or ICS and LABA</td>
</tr>
<tr>
<td>Positive skin prick test to Timothy grass pollen (in a panel of ten aeroallergens)</td>
<td>ACQ score &gt; 0.75</td>
</tr>
</tbody>
</table>

### Allergic Rhinitis Subject with Asthma: Exclusion Criteria

<table>
<thead>
<tr>
<th>Exclusion Criteria</th>
<th>Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current use of anti-histamines, nasal steroids, LTRA or tiotropium</td>
<td>History of clinically relevant systemic disease or respiratory disease (other than asthma)</td>
</tr>
<tr>
<td>Current symptoms of rhinitis</td>
<td>OCS treatment in the previous 3 months</td>
</tr>
<tr>
<td>Smoking history in the past 6 months</td>
<td>Pregnant or breastfeeding women</td>
</tr>
</tbody>
</table>

### Healthy Subject: Inclusion Criteria

<table>
<thead>
<tr>
<th>Inclusion Criteria</th>
<th>Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age 18-55 years</td>
<td>PC20 &gt;8ug/ml and bronchodilator response &lt;12%</td>
</tr>
</tbody>
</table>

### Healthy Subject: Exclusion Criteria

<table>
<thead>
<tr>
<th>Exclusion Criteria</th>
<th>Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoking history in the past 6 months</td>
<td>History of respiratory or significant systemic disease</td>
</tr>
<tr>
<td>Positive skin prick test</td>
<td>Use of ICS or OCS in the previous 3 months</td>
</tr>
<tr>
<td>History or current symptoms of atopic disease such as allergic rhinitis, asthma</td>
<td>Current use of LABA, nasal spray, anti-histamine, LTRA or tiotropium</td>
</tr>
<tr>
<td>or eczema</td>
<td></td>
</tr>
<tr>
<td>Shortness of breath at screening</td>
<td>Pregnant or breastfeeding women</td>
</tr>
</tbody>
</table>

**Table 1: Study inclusion and exclusion criteria.** Abbreviations: ACQ asthma control questionnaire, ICS inhaled corticosteroid, LABA long-acting β2 agonist LTRA leukotriene receptor antagonist OCS oral corticosteroid, PC$_{20}$ concentration of histamine required to reduce FEV$_1$ by 20%.
<table>
<thead>
<tr>
<th></th>
<th>Allergic Rhinitis (n=9)</th>
<th>Healthy (n=8)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>28 (25, 37.5)</td>
<td>35 (25.3, 40)</td>
<td>NS</td>
</tr>
<tr>
<td>Gender</td>
<td>4F:5M</td>
<td>5F:3M</td>
<td>NS</td>
</tr>
<tr>
<td>Baseline FEV₁ % predicted</td>
<td>87 (77.5, 94.5)</td>
<td>106.5 (92, 115)</td>
<td>0.06</td>
</tr>
<tr>
<td>PC₂₀ (mg/ml)</td>
<td>0.48 (0.08, 1.5)</td>
<td>&gt;8</td>
<td>-</td>
</tr>
<tr>
<td>ICS daily dose beclomethasone/equivalent (µg)</td>
<td>400 (250, 650)</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Number of positive skin pricks</td>
<td>4 (2.5, 5)</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>IgE (IU/ml)</td>
<td>196 (76.05, 906)</td>
<td>23.2* (11.4, 41.3)</td>
<td>0.005</td>
</tr>
<tr>
<td>ACQ</td>
<td>1.67 (1.3, 2.75)</td>
<td>N/A</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 2. Baseline Characteristics of Study Subjects.** All atopic patients were skin prick positive to Timothy grass, *Phleum pratense*. Data is displayed as median (25th, 75th) values and statistical analysis was carried out using Mann Whitney U tests. Abbreviations: ACQ asthma control questionnaire, FEV₁ forced expiratory volume in 1 second, PC₂₀ concentration of histamine required to reduce FEV₁ by 20%, ICS inhaled corticosteroid, N/A not applicable, NS non-significant. *n=7 healthy patients
<table>
<thead>
<tr>
<th>P value:</th>
<th>Allergic Rhinitis and Healthy</th>
<th>Allergic Rhinitis alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eosinophils 6 h</td>
<td>0.0003</td>
<td>0.1475</td>
</tr>
<tr>
<td>Neutrophils 6 h</td>
<td>0.0104</td>
<td>0.0503</td>
</tr>
<tr>
<td>IL-5 6 h</td>
<td>0.0179</td>
<td>0.2125</td>
</tr>
<tr>
<td>PGD$_2$ 6 h</td>
<td>0.2942</td>
<td>0.3125</td>
</tr>
<tr>
<td>CCL17 6 h</td>
<td>0.0854</td>
<td>0.9484</td>
</tr>
<tr>
<td>CCL11 6 h</td>
<td>0.1589</td>
<td>0.9816</td>
</tr>
<tr>
<td>CCL26 6 h</td>
<td>0.4609</td>
<td>0.8432</td>
</tr>
<tr>
<td>IFN-γ 6 h</td>
<td>0.6458</td>
<td>0.6436</td>
</tr>
<tr>
<td>IL-6 8 h</td>
<td>0.744</td>
<td>0.81</td>
</tr>
<tr>
<td>Number of positive skin pricks</td>
<td>n/a</td>
<td>0.2307</td>
</tr>
<tr>
<td>SPT area</td>
<td>n/a</td>
<td>0.6357</td>
</tr>
<tr>
<td>Baseline Serum IgE</td>
<td>0.0007</td>
<td>0.002</td>
</tr>
<tr>
<td>AUC TNSS</td>
<td>0.0025</td>
<td>0.7847</td>
</tr>
</tbody>
</table>

Table 3. Spearman’s correlation coefficient analysis of upper airway ILC2 numbers at 6 h post NAC with study parameters. Correlative analysis was carried out with ILC2 number in the upper airway at 6 h post challenge with the above parameters, analysing both allergic rhinitis and healthy subjects, and allergic rhinitis alone using Spearman’s correlation coefficient. Abbreviations: AUC area under the curve, NAC nasal allergen challenge SPT skin prick test, TNSS total nasal symptom score.
Figure 1
Figure 3

A

IL-6 pg/ml

1000

100

10

1

0.1

Baseline 6 h Baseline 6 h

** 0.07

B

PGD2 pg/ml

10000

1000

100

10

0.1

Baseline 6 h Baseline 6 h

**

C

CCL17 pg/ml

100

10

1

0.1

Baseline 6 h Baseline 6 h

*

D

CCL11 pg/ml

100

10

1

0.1

Baseline 6 h Baseline 6 h

*

E

CCL26 pg/ml

100

10

1

1

Baseline 6 h Baseline 6 h

0.07

F

IFN-γ pg/ml

100

10

1

0.1

Baseline 6 h Baseline 6 h

G

IL-6 pg/ml

100

10

1

0.1

Baseline 6 h Baseline 6 h

*

Legend:
- Healthy
- Allergic Rhinitis
Figure 4

A

B

C

Healthy

Atopy

Baseline

Post-NAC

CD127

CRTH2

ILC2

Healthy

Allergic Rhinitis

Baseline 6 h
Figure 5

(A) Eosinophil number vs. ILC2 number

(B) Serum IgE (IU/ml) vs. ILC2 number

(C) IL-5 pg/ml vs. ILC2 number

- Healthy
- Allergic Rhinitis

Statistics:
- A: p = 0.0003, r = 0.789
- B: p = 0.0007, r = 0.774
- C: p = 0.018, r = 0.574
Mucosal type 2 innate lymphoid cells identified as central to the allergic response in asthma

Jaideep Dhariwal*, Aoife Cameron*, Maria-Belen Trujillo-Torralbo, Ajerico del Rosario, Eteri Bakhsoliani, Malte Paulsen, David J Jackson, Michael R Edwards, Batika M J Rana, David J Cousins, Trevor T Hansel, Sebastian L Johnston*, Ross P Walton* and on behalf of the MRC-GSK strategic alliance consortium

*These authors contributed equally to this work

Online Data Supplement
**Supplementary Methods**

**Nasosorption**

Soluble mediators in the nasal lining fluid were sampled using nasosorption, as previously described (E2). Briefly, synthetic absorptive matrix (SAM) strips, made of leukosorb fibrous matrix, were inserted into the nasal cavity for 2 minutes, held in place by a nasal clip. Soluble mediators were eluted from SAM strips, prior to measurement using MSD or immunoassay.

**Flow Cytometer Specifications**

PMT voltages were adjusted after standardized CST checks, minimizing the spectral overlap to increase data precision. Eosinophils and neutrophils were measured on a Becton Dickinson Fortessa LSR-SORP equipped with 20mW 355nm, 50mW 405nm, 50mW 488nm, 50mW 561nm, 20mW 633nm lasers and a ND1.0 filter in front of the FSC photodiode. ILC2s were sorted using a Becton Dickinson AriaIIIu equipped with 50mW 405nm, 50mW 488nm, 50mW 561nm, 20mW 633nm lasers and a ND1.5 filter in front of the FSC photodiode. A nozzle size of 100um was used and the corresponding BD FACSFlow sheath pressure of 20psi, matched with a transducer frequency of 29.1kHz. Input pressure was adjusted to ensure that every 5th to 6th drop was populated by an event.
Buffer/Media | Composition
---|---
RPMI 10%FCS | Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, Shaftesbury, UK), 10% foetal calf serum (FCS) (PAA, Yeovil, UK), 100U/ml penicillin (Invitrogen, Paisley, UK), 100μg/ml streptomycin (Invitrogen, Paisley, UK), 1% L-glutamine (Gibco, Paisley, UK)
FACS buffer | Dulbecco’s phosphate buffered saline (PBS) without calcium or magnesium (Sigma-Aldrich), 1% FCS (PAA), 2mM ethylenediaminetetraacetic acid (EDTA) (Gibco)
0.1% Human serum | PBS, 0.1% normal human serum (Sigma-Aldrich)

Table E1. Composition of Media and Buffers.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorochrome</th>
<th>Supplier</th>
<th>Clone number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lineage Cocktail</td>
<td>FITC</td>
<td>eBioscience</td>
<td>*</td>
</tr>
<tr>
<td>FcεRIα</td>
<td>FITC</td>
<td>Biolegend</td>
<td>AER-37 (CRA-1)</td>
</tr>
<tr>
<td>CD123</td>
<td>FITC</td>
<td>eBioscience</td>
<td>6H6</td>
</tr>
<tr>
<td>CD45</td>
<td>QDOT605</td>
<td>Invitrogen</td>
<td>HI30</td>
</tr>
<tr>
<td>CD127</td>
<td>Brilliant Violet 421</td>
<td>Biolegend</td>
<td>A019D5</td>
</tr>
<tr>
<td>CRTh2</td>
<td>Alexa Fluor 647</td>
<td>Biolegend</td>
<td>BM16</td>
</tr>
<tr>
<td>CD9</td>
<td>V450</td>
<td>BD Biosciences</td>
<td>M-L13</td>
</tr>
<tr>
<td>CD16</td>
<td>APC</td>
<td>eBioscience</td>
<td>CB16</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>QDOT605</td>
<td>Invitrogen</td>
<td>TÜ36</td>
</tr>
</tbody>
</table>

Table E2. Antibodies Used for Flow Cytometry and FACS. *Lineage cocktail contained the following antibodies: CD2 (RPA-2.10), CD3 (OKT3), CD14 (61D3), CD16 (CB16), CD19 (HIB19), CD56 (CB56), CD235a (HIR2).
**Figure E1. Overview of nasal allergen challenge clinical protocol.** Atopic asthmatic patients and healthy controls were challenged intranasally with Timothy grass pollen. Nasal lining fluid samples were collected using nasosorption at baseline (30 min prior to NAC) and 2, 4, 6 and 8 h post-NAC. Nasal curettage scrapes were taken 30 min prior to and 6 h post-NAC for flow cytometric cellular analysis. Total nasal symptom scores were assessed 30 min pre- and 30 min post-NAC, and every hour post-NAC up to 8 h. Abbreviations NAC nasal allergen challenge.
Supplementary Results

A

B

C

D

E

F

G

IL-5 pg/ml

β

γ

δ

ε

ɛ

Baseline

Healthy

Allergic Rhinitis
Figure E2. Induction of type 2 mediators in the nasal lining fluid of atopic asthmatics following nasal allergen challenge. Nasal lining fluid samples were collected using nasosorption, at baseline (30 min pre-NAC) and 2, 4, 6 and 8 h post-NAC. Levels of A) IL-5, B) PGD₂, C) CCL17, D) CCL11, E) CCL26, F) IFN-γ and G) IL-6 were determined in atopic asthmatics (N=9) and healthy controls (N=8), using MSD and a PGD₂ enzyme immunoassay. Broken lines represent the limit of detection. Abbreviations NAC nasal allergen challenge, MSD meso-scale discovery.

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Healthy Baseline</th>
<th>Healthy 6 h</th>
<th>Asthma Baseline</th>
<th>Asthma 6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-13</td>
<td>6.42 (3.61, 9.96)</td>
<td>5.33 (3.93, 7.97)</td>
<td>6.4 (3.26, 8.41)</td>
<td>6.44 (5.40, 8.59)</td>
</tr>
<tr>
<td>CCL22/MDC</td>
<td>20.61 (14.13, 25.41)</td>
<td>20.8 (14.68, 26.05)</td>
<td>16.96 (12.57, 27.65)</td>
<td>22.02 (15.96, 26.94)</td>
</tr>
<tr>
<td>CXCL8/IL-8</td>
<td>1324 (956, 2488)</td>
<td>1241 (502.8, 1529)</td>
<td>1698 (935, 3207)</td>
<td>1456 (701, 1703)</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>3.76 (2.68, 4.44)</td>
<td>3.685 (2.29, 5.58)</td>
<td>2.92 (1.65, 11.89)</td>
<td>4.29 (2.68, 7.22)</td>
</tr>
</tbody>
</table>

Table E3 Additional soluble mediators measured during allergen challenge.

Nasal lining fluid samples were collected using nasosorption, at baseline (30 min prior to NAC) and 6 h post-NAC. Soluble mediators were assessed using MSD. Median (25th, 75th) levels are displayed. No statistical differences were observed between baseline and 6 h measurements using Wilcoxon matched pairs test, and between patient groups using Mann Whitney U test. IL-2, IL-4, and IL-17 were below assay detection limits. Abbreviations: NAC nasal allergen challenge, MSD meso-scale discovery.
Author Appendix

MRC-GSK strategic alliance consortium list:

Sebastian L Johnston\textsuperscript{1,2}, Roberto Solari\textsuperscript{1,2}, Michael R Edwards\textsuperscript{1,2}, Paul Lavender\textsuperscript{2,4}, Ross P Walton\textsuperscript{1,2}, Hannah Gould\textsuperscript{2,4}, David Cousins\textsuperscript{2,4,5}, Antoon J. van Oosterhout\textsuperscript{3}, Jaideep Dhariwal\textsuperscript{1,2}, Aoife Cameron\textsuperscript{1,2}, Nathan W Bartlett\textsuperscript{1,2}, Patrick Mallia\textsuperscript{1,2}, David J Jackson\textsuperscript{1,2,6}, Maria-Belen Trujillo-Torralbo\textsuperscript{1,2}, Jerico del Rosario\textsuperscript{1,2}, Janet L. Smith\textsuperscript{3}, Matthew J. Edwards\textsuperscript{3}, Karen Affleck\textsuperscript{3}, Nil Turan Jurdzinski\textsuperscript{3}, Veronique Birault\textsuperscript{3}, Peter McErlean\textsuperscript{2,4}, Yu-Chang Wu\textsuperscript{2,4}, Nadine Upton\textsuperscript{2,4}, Ismael Ranz Jimenez\textsuperscript{2,4}.

Author affiliations:

1. Airway Disease Infection Section, National Heart and Lung Institute, Imperial College London, London, UK

2. MRC and Asthma UK Centre in Allergic Mechanism of Asthma, London, UK

3. GlaxoSmithKline, Allergic Inflammation Discovery Performance Unit, Respiratory Therapy Area, Stevenage, UK

4. Department of Respiratory Medicine & Allergy, King’s College London, London, UK

5. NIHR Respiratory Biomedical Research Unit, Department of Infection, Immunity & Inflammation, Leicester Institute for Lung Health, University of Leicester, Leicester, UK

6. Guy's and St Thomas' NHS Trust, London, UK
References
