

Peripheral group 2 innate lymphoid cells are decreased following nasal allergen challenge in allergic rhinitis

To the Editor,

Allergic rhinitis (AR) is an upper airway inflammatory disorder involving IgE-mediated inflammation of the nasal mucosa. Inflammatory responses are triggered following inhalation of sensitized aeroallergens and are characterized by Th2 inflammation and the rapid migration of eosinophils into the nasal cavity.¹ Group 2 innate lymphoid cells (ILC2s) represent an alternative source of Th2 cytokines, potentially augmenting pre-existing Th2-driven inflammation seen in AR. Lacking a specific antigen receptor and lineage surface markers for B and T cells, ILC2s are activated by IL-25, IL-33, and TSLP and can drive Th2 inflammation via production of IL-5, IL-13, and modest amounts of IL-4.²

In AR patients, elevated levels of peripheral blood ILC2s were reported during the grass pollen season and following a nasal allergen challenge (NAC) with cat allergen extract.³⁻⁵ This suggests a possible role for these cells in driving allergic symptoms. However, participants from these studies may have been primed (reversible increase in reactivity of the nasal tissue) due to repeated low-dose allergenic stimulation during pollen season or at home (ie, owning a cat). Sensitized individuals who are also primed can experience more severe allergic symptoms and changes in systemic immunity after allergen exposure. To date, few studies have assessed the effects of acute allergen exposure on circulating ILC2s in non-primed AR individuals. Thus, the aim of the current study was to evaluate the frequency of peripheral blood ILC2s in AR individuals after a NAC performed outside of the local birch pollen season. We also evaluated ILC2 frequencies in nasal lavage (NL) samples collected pre- and post-challenge.

Eleven individuals with birch pollen-induced AR and eight non-allergics were recruited into the study (Table S1). The study was reviewed and granted ethical clearance by the Queen's University and Affiliated Teaching Hospitals Research Ethics Board, and all participants provided written informed consent. All participants underwent a NAC with a pre-titrated dose of birch pollen extract (ALK-Abello) (Figure S1A). Total nasal symptom score (TNSS; sum of rhinorrhea, nasal congestion, sneezing, and nasal itching) and peak nasal inspiratory flow (PNIF) were recorded during the challenge. Peripheral blood and NL samples were collected at baseline and 4 hours post-challenge. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation and cryopreserved. The 4-hour time point was selected to remain consistent with the cat-NAC study.³ Details of the NAC methodology can be found in the Methods S1 section.

Thawed PBMCs and freshly obtained NL samples were stained with a fixable viability dye (PBMCs only, eBioscience), a lineage

cocktail (CD3, CD14, CD16, CD19, CD20, CD56; BD Biosciences), and antibodies to CD4, CD11b, CD235a, FcεRI, CD45, CRTH2, and CD127 (all from eBioscience). ILC2s were identified as CD45⁺ lymphocytes that were also lineage negative and expressed CRTH2 and CD127 (Figure 1A).

Compared to non-allergics, birch-allergic participants experienced a significantly higher TNSS at 15 minutes ($P < 0.0001$) through 8 hours ($P = 0.0048$) and a larger PNIF reduction at 15 ($P = 0.0016$) and 30 minutes ($P = 0.0004$) post-allergen challenge (Figure S1B,C).

The mean frequency of peripheral blood CD45⁺ cells remained unchanged after allergen challenge in both groups (Figure 1B). The mean frequency ($\pm$ SEM) of peripheral blood ILC2s at baseline ($0.019 \pm 0.003\%$ vs $0.018 \pm 0.006\%$, $P = 0.60$) and post-challenge ($0.014 \pm 0.002\%$ vs $0.015 \pm 0.004\%$, $P = 0.90$) was comparable between birch-allergic and non-allergic participants. Contrasting previous studies, peripheral blood ILC2s were significantly decreased following allergen challenge in birch-allergic participants ($P = 0.0344$) (Figure 1C). This was not detected in non-allergic participants ($P = 0.25$). A reduction in ILC2s was noted in this group; however, it was likely driven by one non-allergic participant.

In birch-allergic participants, percentage changes in peripheral blood ILC2s after challenge significantly correlated with TNSS at 4 hours ($r = 0.73$, $P = 0.01$) (Figure 1D). This was not observed for 4-hour percentage PNIF reduction ($r = -0.28$, $P = 0.41$) (Figure 1E). In contrast, peripheral ILC2s at 4 hours alone did not correlate with 4-hour TNSS ($r = -0.16$, $P = 0.63$) and 4-hour percentage PNIF reduction ($r = -0.13$, $P = 0.70$) (Figure 1F,G). A borderline correlation was observed between percentage changes in peripheral ILC2s and percentage PNIF reduction at 8 hours (late-phase response, $r = 0.61$, $P = 0.0491$) (Table S2). Finally, correlations were not observed for 15-minute (peak symptom severity) symptom scores (Table S2). These findings suggest that in pollen-sensitized asymptomatic individuals, the change in peripheral ILC2s after a high-dose pollen challenge may better reflect symptom severity compared to post-challenge measurements alone, as observed in symptomatic individuals during pollen season.⁵

To investigate local ILC2 responses following allergen challenge, a similar gating strategy was used. We were unable to detect nasal ILC2s in NL samples collected pre- and post-NAC (Figure 2A). Only a very small proportion of NL cells expressed CD45 (median frequency, 3.2%–6.5% of all cells), which remained unchanged after allergen challenge (Figure 2B). The nasal lymphocyte population was also less defined, with 15/19 participants having less than 100

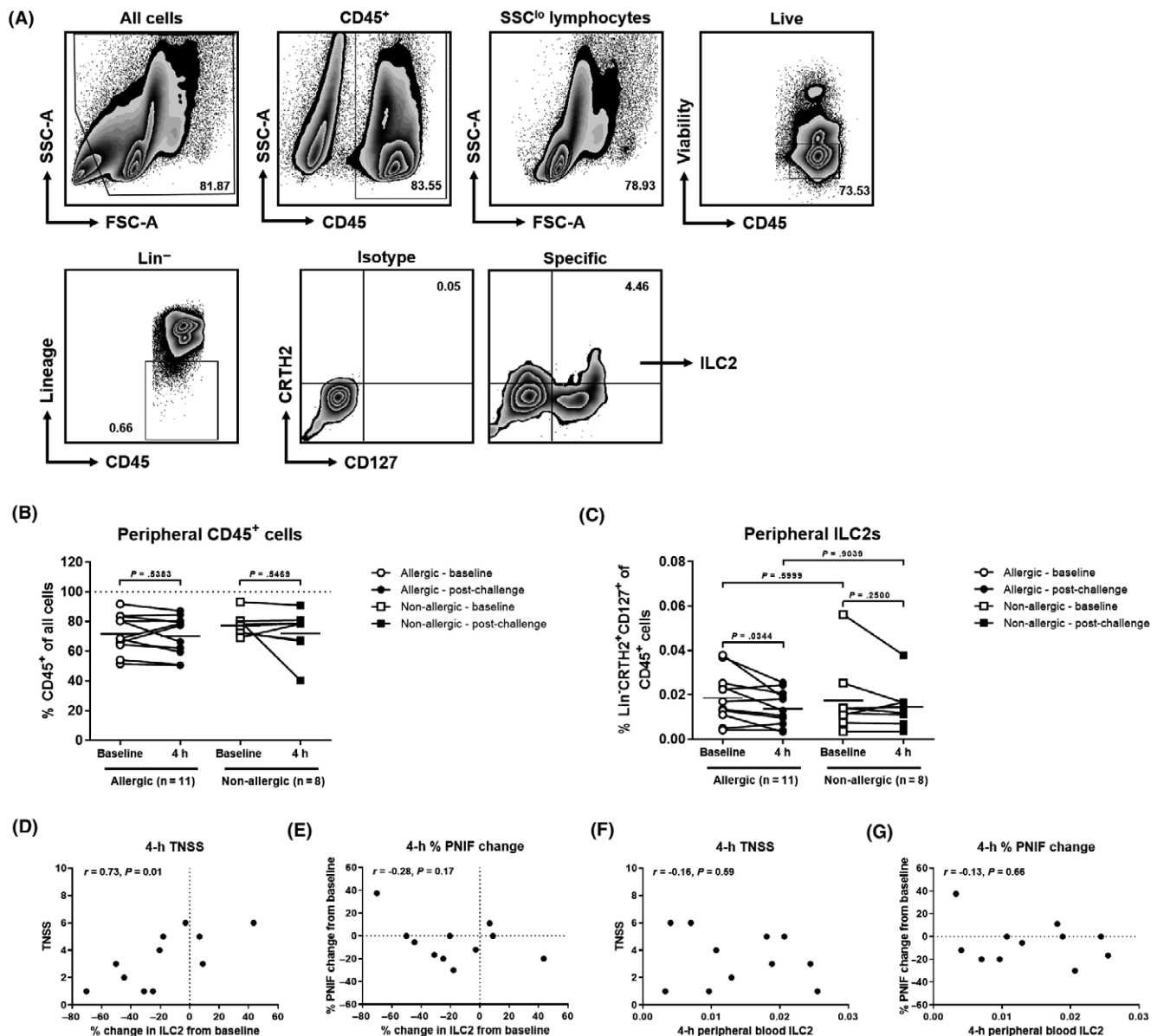


FIGURE 1 Enumeration of peripheral blood ILC2s after nasal allergen challenge and correlation with allergic symptoms. Peripheral ILC2s were identified as live CD45⁺Lin⁻CRTH2⁺CD127⁺ lymphocytes. Plots shown were from one birch-allergic participant at baseline (A). Peripheral CD45⁺ cells were unchanged after allergen challenge in birch-allergic and non-allergic participants (B). Peripheral ILC2s were significantly decreased following a NAC in birch-allergic participants (C). A significant correlation was seen between percentage change in peripheral ILC2s and 4-h TNSS (D), not 4-h PNIF (E). Correlations between peripheral ILC2 frequencies at 4 h and 4-h TNSS (F) and PNIF (G) were not observed. Data are represented as individual data points. Horizontal black bars represent the mean of each dataset (B, C). PNIF, peak nasal inspiratory flow; TNSS, total nasal symptom score

events in the CRTH2 vs CD127 biaxial plots in both pre- and post-challenge samples, rendering the analysis of ILC populations infeasible. Thus, NL may not be the optimal method to assess rare nasal lymphocyte populations as collected leukocytes are primarily CD45⁺ granulocytes, as observed in our FSC-A vs SSC-A plots and a previous study.⁶

Our findings in the blood contrast the observations made during the pollen season, suggesting that possible priming due to low-dose allergen exposure may differentially regulate peripheral ILC2 migration. A recent study illustrated the trafficking of ILC2s out of the

bone marrow and into the blood and lungs of nonsensitized mice following intranasal *A. alternata* challenge.⁷ Thus, without ongoing exposure to allergen, ILC2s may more readily migrate out of circulation than from the bone marrow after an acute high-dose allergen challenge, leading to an overall decrease. However, additional research is needed to investigate this hypothesis.

Collectively, we show that peripheral blood ILC2s were decreased following a NAC and positively correlated with AR symptoms in birch-allergic participants challenged outside of the local birch pollen season. Nasal ILC2s were undetectable in NL samples,

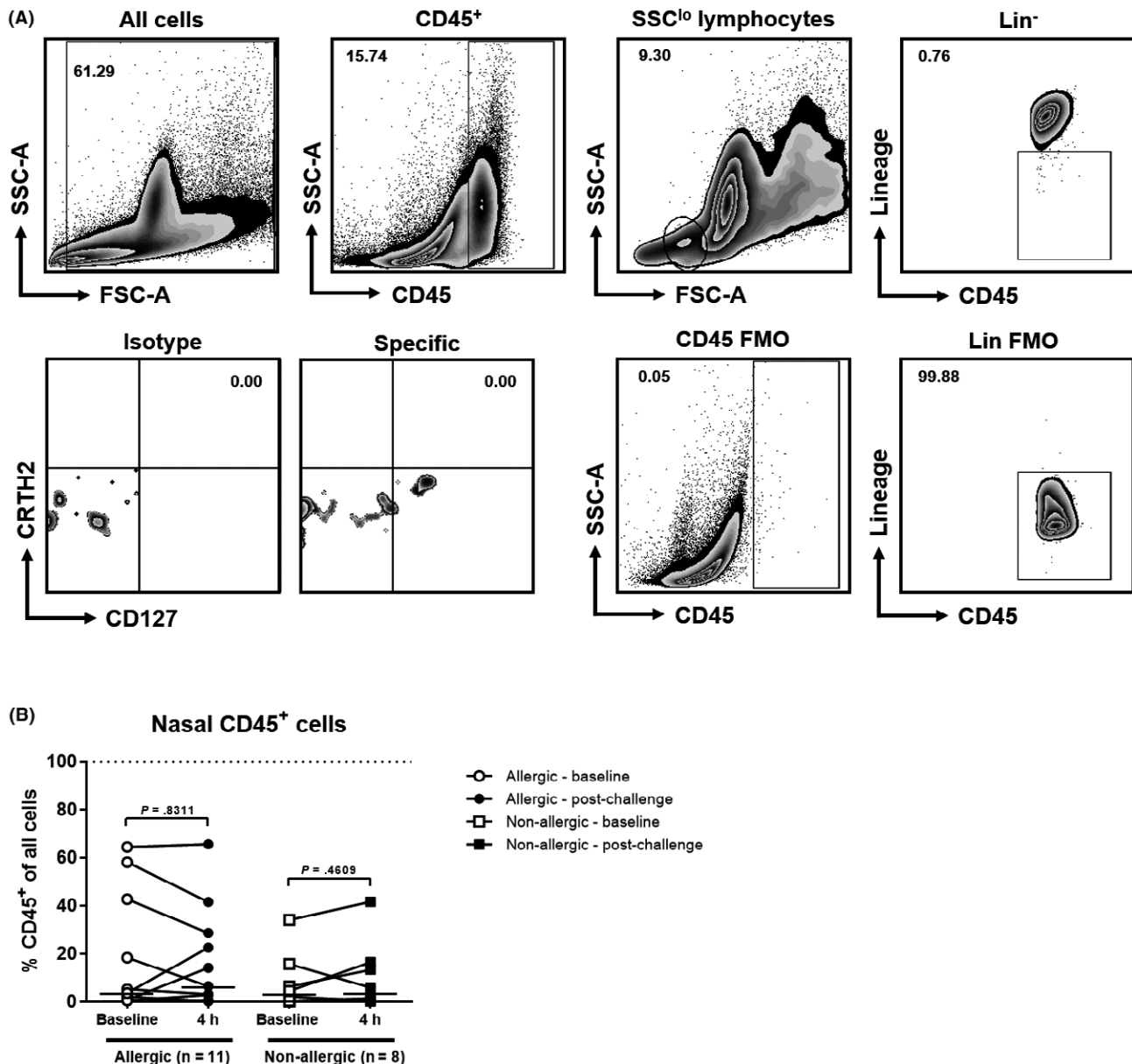


FIGURE 2 Identification of ILC2s in nasal lavage samples. Nasal ILC2s were defined as CD45⁺Lin⁻CRTH2⁺CD127⁺ lymphocytes (A) and could not be identified in nasal lavage samples from birch-allergic and non-allergic participants. Plots shown were from one non-allergic participant at baseline. Frequency of CD45⁺ nasal lavage cells was unchanged after allergen challenge (B). Horizontal black bars represent the median of each dataset (B). PBMC, peripheral blood mononuclear cell

perhaps indicating that these cells reside in the mucosal tissue rather than the nasal lumen. These findings do not directly challenge previous studies performed in-season. Rather, they underscore the importance of considering ongoing chronic allergen exposure as an additional factor when assessing the role of immune cells in AR pathophysiology.

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CONFLICTS OF INTERESTS

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Long-term exposure to neighborhood smoke from household heating and risk of respiratory and dermatological prescription medications—Growing Up in New Zealand child cohort study

To the Editor,

Domestic wood-heating has regained popularity in Europe and Northern America due to policies encouraging wood and other biomass burning for energy security and climate change mitigation.¹ Whether these policies are truly low-carbon has been questioned,² and they also have important implications for air quality. Ambient wood-smoke exposure is associated with childhood respiratory diseases³ but investigation of wood-smoke exposure on childhood skin diseases is limited. We aimed to assess childhood exposure to neighborhood environments with wood/coal heated households and the onset of both respiratory and skin diseases.

This analysis included 3461 children (Data S1) from the *Growing Up in New Zealand* cohort study⁴ and used data collected from face-to-face and telephone interviews with their parents during the third trimester of the mother's pregnancy (2008-2010), when the children

were 9 months (2009-2011), 2 years (2011-2012), and 4½ years (2013-2014) old.

We established data linkage with pharmaceutical (Data S2) and perinatal records from New Zealand national datasets. We assessed first-time dispensing for respiratory and/or dermatological medication prescriptions during the cooler season (April to November) (Data S3) in the first 48 months of life as the outcome group (those with no such prescriptions dispensed as the reference group) after excluding same-date episodes that also contained outcomes other than respiratory and/or dermatological (i.e., multiple outcomes).

Based on the smallest statistical area unit (meshblock), we geocoded the children's residential locations to link up with the 2013 New Zealand Index of Deprivation and the density of wood/coal heated households (number per hectare) derived from census

SUPPLEMENTARY MATERIALS

Peripheral group 2 innate lymphoid cells are decreased following nasal allergen challenge in allergic rhinitis

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Supplementary Methods

Study Participants

Eleven birch-allergic and eight non-allergic individuals aged 18-65 years old were recruited into the study outside of the local birch pollen season. All birch-allergic participants had a ≥ 2 -year history of moderate to severe rhinoconjunctivitis symptoms during the birch pollen season prior to enrollment, and a positive skin prick test to birch pollen extract (defined as a wheal diameter ≥ 3 mm than the negative control). Non-allergic participants had a completely negative skin prick test to a panel of common environmental allergens (*Alternaria*, short ragweed, rye grass, Timothy grass, birch, tree mix, *D. pteronyssinus*, *D. farinae*, cat, dog).

Exclusion criteria included asthma history, any structural nasal abnormalities or nasal polyps detected upon examination, signs/symptoms of active perennial rhinitis or seasonal AR (e.g. baseline screening TNSS > 2), and noncompliance with washout periods for restricted medications (e.g. systemic, topical, or inhaled corticosteroids, cromolyn, leukotriene receptor antagonists, H1 receptor antagonists).

All participants provided written informed consent prior to study involvement. All experimental procedures were reviewed and granted ethical clearance by the Queen's University and Affiliated Teaching Hospitals Research Ethics Board.

Nasal Allergen Challenge

All study participants underwent a nasal allergen challenge (NAC) with diluted birch pollen extract (ALK-Abello) outside of the local birch pollen season. Each NAC was comprised of a screening visit (to determine the challenge dose of birch pollen extract) and a challenge visit as per previously published methodology (Figure S1, A).^{E1} During screening, all participants first underwent a nasal wash with 5 ml sterile 0.9% saline, then a NAC with diluted extract solvent to rule out non-specific nasal hyper-responsiveness towards the glycerin and phenol preservative contained in the birch pollen extract (TNSS > 2). Serial dilutions of birch pollen extract (1:128 up to 1:2) were delivered intranasally to the remaining participants using a four-fold escalating dose protocol. Total nasal symptom score (TNSS; omnibus score comprised of rhinorrhea, nasal congestion, sneezing, and nasal itching) and peak nasal inspiratory flow (PNIF) were recorded ten minutes after each allergen dose. Symptom severity was rated on a 4-point Likert scale of 0 to 3 (0, none; 1, mild; 2, moderate; 3, severe) with a maximum score of 12 for TNSS. Dose escalation stopped after participants met the qualifying criteria (TNSS $\geq$ 8/12 and a percentage PNIF reduction $\geq$ 50%). The sum of all serial dilutions received during screening comprised the challenge dose for each participant. For example, participants qualifying at 1:8 received the 1:6 (1:128 + 1:32 + 1:8) challenge dose during the NAC.

During the challenge visit, each participant received 100 μ l of their qualifying challenge dose of birch pollen extract, delivered to each nostril using a Pfeiffer Bidose Nasal Delivery Device (Aptar Pharma). TNSS and PNIF were recorded at baseline (before challenge), 15 and 30 minutes, 1 hour, and hourly until 12 hours post-challenge. Peripheral blood and NL samples were collected at baseline and 4 hours post-challenge (Figure S1, A).

Peripheral Blood Collection and Processing

Peripheral blood was collected by venipuncture into Heparin-containing vacutainer tubes (BD Biosciences). Blood was diluted in McCoy's 5A modified medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS, Wisent Bioproducts) and layered onto Ficoll-PaqueTM PLUS (GE Healthcare). Peripheral blood mononuclear cells (PBMCs) were collected, washed, re-suspended in freezing media (40% McCoy's 5A, 50% FBS, 10% DMSO), and cryopreserved. Stored PBMCs were rapidly thawed and washed with RPMI 1640 medium (Life Technologies) supplemented with 10% FBS prior to flow cytometry staining.

Nasal Lavage Collection and Processing

NL samples were collected using a modified "nasal pool" method.^{E2} Participants were seated in a forward-flexed neck position (approximately 60° from the upright position) and a tight seal was formed between the left nostril and a nasal olive attached to a sterile 0.9% saline-filled syringe. The nasal cavity was gently flushed with saline over a one-minute period. The resulting fluid was collected and the procedure was repeated for the right nostril. Lavage samples from both nostrils were pooled, treated with 1% dithiothreitol (Sigma-Aldrich) in Dulbecco's PBS

(DPBS, Life Technologies), passed over a 70 μ m cell strainer (VWR International), and washed prior to flow cytometry staining.

Flow Cytometric Analysis for ILC2s

Thawed PBMCs were initially washed twice with DPBS and stained with eFluor® 780-fixable viability dye (eBioscience). Stained PBMCs and freshly filtered NL cells were washed twice with FACS buffer (eBioscience) and blocked with Fc Receptor Binding Inhibitor reagent (eBioscience) to prevent non-specific binding. Blocked cells were then immediately stained with a FITC-lineage (Lin) cocktail (CD3 [SK7], CD14 [M ϕ P9], CD16 [3G8], CD19 [SJ25C1], CD20 [L27], CD56 [NCAM16.2]; BD Biosciences) and antibodies to CD4-FITC (RPA-T4), CD11b-FITC (ICRF44), CD235a-FITC (HIR2), Fc ϵ RI-FITC (AER-37), CD45-APC (HI30), CRTH2-PE (BM16), and CD127-PE-Cy7 (eBioRDR5) or relevant isotype controls (all from eBioscience). Lin cocktail and FITC-conjugated antibodies were used to exclude B and T cells, eosinophils, neutrophils, basophils, monocytes, natural killer cells, dendritic cells, and red blood cells during ILC2 enumeration. Stained cells were washed with FACS buffer and fixed with 4% paraformaldehyde diluted in DPBS. Fixed cells were then washed, resuspended in FACS buffer, and acquired using a Beckman Coulter FC500 flow cytometer or a Becton Dickinson FACS Aria III cell sorter within 24 hours. Data were analyzed using FlowJo Software (TreeStar). ILC2s were defined as CD45⁺Lin⁻CRTH2⁺CD127⁺ lymphocytes and were reported as a percentage of total CD45⁺ cells.

Statistical Analyses

All data were analyzed using GraphPad Prism 7 software (GraphPad Software). Data were tested for normality using D'Agostino's K-squared test. Fisher's exact test was used to analyze categorical outcomes. Two-way repeated measures ANOVA with Bonferroni's corrections were used to analyze symptom outcomes. Independent and paired sample t tests were used to analyze NAC-induced changes. Mann-Whitney U tests and Wilcoxon signed-ranked tests were used for non-normally distributed data. Spearman correlation was used to assess the relationship between ILC2s and symptom outcomes. A *P* value of < .05 was taken as significant.

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Characteristics		Allergic (n=11)	Non-allergic (n=8)	
Mean age in years (SD)		43.2 (8.6)	37.5 (13.0)	$P = 0.26^1$
Female (%)		7 (63.6)	5 (62.5)	$P > 0.99^2$
Race (%)				
	Caucasian	10 (90.9)	7 (87.5)	$P > 0.99^2$
	Black	0	0	
	Asian	1 (9.1)	1 (12.5)	$P > 0.99^2$
	American Indian/Alaska Native	0	0	
	Native Hawaiian/Other Pacific Islander	0	0	
	Other	0	0	
Asthma (%)		0	0	
Mean SPT wheal to birch pollen (in mm, mean [SD])		8.6 (3.4)	0 (0.0)	$P < 0.0001^3$
Specific IgE to birch (in kUA/l, mean [SD])		1.6 (1.3)	0.02 (0.02)	$P < 0.0001^3$
Co-morbid sensitization (%)				
	Alternaria	2 (18.2)	0	

Table S1. Participant Demographics.

Ragweed	10 (90.1)	0
Rye grass	9 (81.8)	0
Timothy grass	9 (81.8)	0
Birch	11 (100)	0
Tree mix	11 (100)	0
House dust mite (<i>D. pteronyssinus</i>)	6 (54.5)	0
House dust mite (<i>D. farinae</i>)	4 (36.4)	0
Cat	3 (27.3)	0
Dog	1 (9.1)	0

¹Unpaired t test

²Fisher's exact test

³Mann-Whitney U test

Table S2. Correlation between percentage changes in blood ILC2s and instantaneous symptom outcomes in birch-allergic participants.

Symptom outcomes (n=11)	r	P value
TNSS		
15 minutes	0.0518	0.8816
4 hours	0.7345	0.0133
8 hours	0.0516	0.8832
% PNIF change from baseline		
15 minutes	0.1455	0.6731
4 hours	-0.2759	0.4085
8 hours	0.6147	0.0491

Significant correlations are bolded.

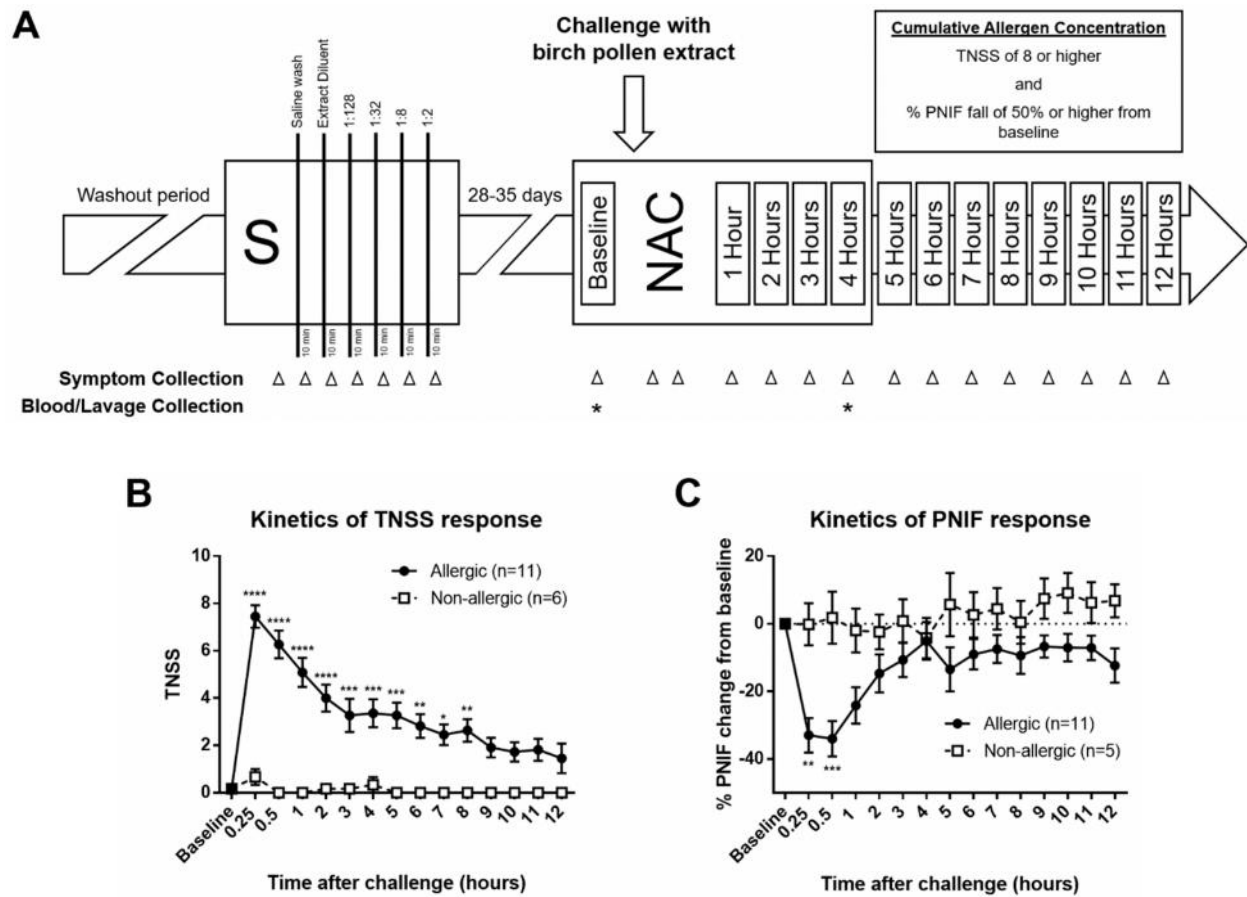


Figure S1. Nasal allergen challenge model and symptom outcomes. Participants were challenged with their pre-titrated dose of birch pollen extract intranasally to both nostrils (**A**). Nasal symptoms were recorded and biological samples were collected at the specified time intervals. Significant changes in TNSS (**B**) and PNIF (**C**) were observed in allergic participants compared to non-allergics after allergen challenge. Two and three non-allergic participants were excluded from the TNSS and PNIF analysis respectively due to missing symptom data. Data are represented as mean $\pm$ SEM. * $P < .05$; ** $P < .01$; *** $P < .001$; **** $P < .0001$ (all vs. non-allergics at indicated time point). PNIF, peak nasal inspiratory flow; TNSS, total nasal symptom score.