

# Tropical urban environments reveal a strong association of CD45RB<sup>lo</sup> TH2A subset to allergic rhinitis

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## Abstract

**Background:** Allergic rhinitis (AR) is strongly associated with a type 2 response, characterized by the cytokines IL-5, IL-4 and IL-13. Several studies have implicated ILC2 and TH2A (CD161+ TH2) but it is not yet entirely clear which subsets are driving the common allergic reactions underlying AR. The objective of this study aims to identify critical pathogenic cell populations associated with AR and to determine their phenotype and functional contribution to disease progression. **Methods:** We identified integral allergic-specific cell types by transcriptomic sequencing. Whole blood, PBMCs and plasma from a cross-sectional cohort of 216 individuals were analysed by 9-colour flow cytometry and ultra-sensitive cytokine bead arrays using unsupervised clustering algorithms. Clinically active AR cases were further analysed by functional mass cytometry to define phenotype and cytokine secretion (IL-2, IL-3, IL-4, IL-5, IL-9, IL13, IL-17A and IL-22) as well as the expression of the hematopoietic prostaglandin D synthetase (HPGDS). **Results:** The unbiased analysis revealed that atopy and AR manifestation correlated only with eosinophils, plasma IL-5 and CD161+ TH2 cells. In-depth characterization further revealed that the CD45RB<sup>lo</sup> CD161+ TH2 subset were most closely associated with severity. This subset is able to concomitantly secrete multiple cytokines including IL-5, IL-13 and IL-4 and has been previously reported to be associated with other eosinophilic allergies. **Conclusion:** CD45RB<sup>lo</sup> CD161+ TH2 have key roles in driving the allergic response in AR. Neutralizing the CD45RB<sup>lo</sup> CD161+ TH2 subset should disrupt the allergic response pathway, thus providing a target for lasting therapeutic interventions.

## INTRODUCTION

Allergic airway diseases such as allergic rhinitis (AR) and asthma are a group of chronic immunopathological conditions affecting more than 20% of the world's population. As the most common clinical presentation of allergy, AR affects up to 400 million individuals worldwide and afflicts substantial health and economic morbidity. In many regions, AR and other allergic diseases are still increasing. [1]

The immunopathogenesis of eosinophilic AR and asthma is typically associated with a type 2 response, involving cytokines such as interleukin-5 (IL-5), IL-4 and IL-13. Importantly, IL-5 plays a key role in promoting the differentiation and survival of eosinophils. The main producers of IL-5 are type 2 helper T (TH2) cells and mast cells. In RAG-/- mice lacking both T and B lymphocytes, IL-5 is released by tissue resident Innate Lymphoid Cells type 2 cells (ILC2), which is associated with eosinophilia in the pancreas,

lung, and the spleen after IL-2 treatment. [2] In patients with eosinophilic gastrointestinal disease (EGID), IL-5 is preferentially produced by a subset of TH2 cells which expresses CRTH2, CD161 and hematopoietic prostaglandin synthase (HPGDS). As TH2 cell counts positively correlated with blood eosinophilia, they are also termed pathogenic effector (peTH2) cells. [3] HPGDS catalyses the synthesis of prostaglandin D2 (PGD2), a powerful lipid mediator for allergic inflammation. PGD2 exerts its effect by promoting the recruitment of CRTH2 expressing cells to induce airway hyperreactivity.[4-6] Given that CRTH2 is found on most of the cell types involved in the eosinophilic response pathway including eosinophils, basophils, ILC2, type 2 CD8+ T cells (TC2), macrophages and non-classical monocytes [2, 3, 7-9] , we sought to determine the contribution of each cell type in mediating pathogenesis of AR.

In a prior study, we established a sensitisation rate of approximately 80% of the local population in Singapore to house dust mite (HDM) allergens. [10] This results in an incidence rate of about 40% for AR. In order to identify the key driver behind airway allergies, we analysed the CRTH2+ subset in the PBMCs of our Singapore Systems Immunology Cohort (SSIC) longitudinal cohort. [10, 11]

Here, we employed unsupervised PhenoGraph clustering to analyse whole blood and PBMCs from the SSIC cohort. Flow cytometry and ultra-sensitive cytokine arrays revealed positive associations between circulating eosinophil numbers and plasma IL-5, confirming the eosinophilic nature of AR. Furthermore, supervised cluster analysis of PBMCs revealed strong correlation between CD161+ TH2 and self-reported symptoms of AR as well as between eosinophil numbers and IgE titres. Notably, IL-5 was found to be exclusively produced by CD45RB low expressing (CD45RB<sup>lo</sup>) CD161+ TH2 subset, suggesting the role of this population in driving eosinophilia in AR. Finally, in-depth analysis in an active disease cohort by functional mass cytometry further established that a specific subset of CD45RB<sup>lo</sup>HPGDS+ CD161+ TH2 is directly associated with AR. [12, 13] In particular, this subset co-expressed CD27, KLRG1, CD38, ICOS, C45RO and TSLP-R. Upon stimulation, a complex set of cytokines comprising IL-5, IL-13, IL-4, IL-2, IL-3 and IL-9 is concomitantly released from our identified disease-driving subset, contributing towards inflammation in AR.

## METHODS

### Ethics Statement

This study was performed in accordance with the declaration of Helsinki and approved by the Institutional Review Board at the National University of Singapore (IRB numbers: NUS 10-445) and the SingHealth Centralised Institutional Review Board (CIRB Ref: 2017/2806). Written informed consent was obtained from donors prior to sample collection.

### Cohort Information

#### Singapore adult cohort

This study was carried using fresh blood samples from volunteer donors as well as frozen samples of PBMCs and blood plasma collected from the Singapore Systems Immunology Cohort (SSIC). [10, 11] Blood was also collected in Tempus[?] Blood RNA tubes (Life Technologies, Carlsbad, Calif) for RNA isolation. The cross-sectional cohort database was generated based on the recruitment of Singaporeans Chinese (n=216) from National University of Singapore as previously reported. [10, 11] Volunteers were classified as individuals with and without allergic rhinitis using demographics and medical history information collected from a survey questionnaire based on the Allergic Rhinitis Impact on Asthma (ARIA) [14, 15] and International Study of Asthma and Allergies in Childhood (ISAAC) guidelines. [16] Atopy status of donors were determined using a skin prick test (SPT) as previously described. [10] A positive SPT response is determined by wheal diameter of 3 mm or greater, when compared to positive (histamine) and negative (saline) controls.

#### Singapore children cohort

The active disease cohort is comprised of mixed-ethnicity Singaporean subjects below the age of 21 years old. Similarly, clinical phenotypic information and demographic parameters were obtained from volunteers

using a standardised survey questionnaire based on ARIA and ISSAC guidelines. All volunteers underwent a SPT using common HDM allergens *Dermatophagoides pteronyssinus* (DP) and *Blomia tropicalis* (BT) to ascertain atopy status. Fresh whole blood samples were collected from the volunteers into Tempus<sup>2</sup> Blood RNA tubes (Life Technologies, Carlsbad, Calif) for RNA isolation.

### Swedish cohort

BAMSE is a population-based Swedish cohort which recruited 4089 newborn children between 1994-1996. The participants have been followed repeatedly with questionnaires on lifestyle, exposures and symptoms of allergic disease, and have attended regular clinical follow-ups. At the 16-year follow-up, gene expression levels were measured (256 individuals of mean age 16.7 years) with the Affymetrix Human Transcriptome Array 2.0 (HTA).<sup>[17]</sup> Whole blood was collected from study subjects in PAXgene tubes, and RNA was extracted batch-wise (QIAGEN, Courtaboeuf, France). RNA yield and quality were assessed. RNA of highest quality was selected for amplification, labelling, and hybridization on HTA with WT PLUS kit (Affymetrix Inc). Gene expression levels were normalized with the Robust Multi-array Average (RMA) algorithm and transcript clusters were annotated as previously described.<sup>[17]</sup>

### Nucleic Acid Extraction and RNA Sequencing

Total RNA from whole blood was extracted using the MagMAX for Stabilized Blood Tubes RNA Isolation Kit (ThermoFisher), after which globin mRNA depletion was performed using Ambion GLOBINclear (ThermoFisher). Transcript abundance was measured using the Illumina Human HT-12-v4 Expression Bead Chip after preparing fluorescent-labelled PCR products according to the Illumina Human Whole-Genome Gene Expression DASL Assay Guide. Only Illumina probes free of any single nucleotide polymorphisms (SNPs) were used to determine the expression level of the genes to avoid allele-specific artifacts.

### RNA Sequencing Data Analysis

STAR aligner was used (splice junction annotations based on GENCODE version 27; [www.gencodegenes.org](http://www.gencodegenes.org)) to map paired-end raw reads to human genome build GRCh38. Based on GENCODE v27, mapped reads and gene annotations were enumerated using featureCounts. edgeR bioconductor package was used to compute log<sub>2</sub> transformed counts per million mapped reads (log<sub>2</sub>CPM) and log<sub>2</sub> transformed reads per kilobase per million mapped reads (log<sub>2</sub>RPKM). Removal of genes with the log<sub>2</sub>CPM inter-quartile range (IQR) of which across all samples was less than 0.5 preceded analysis of differentially expressed genes. Pairwise comparisons of differentially expressed genes between AR and non-AR was performed using edgeR. P-values were corrected for multiple testing using the Benjamini-Hochberg procedure (Benjamini, 2010) and false discovery rate (FDR). Corrected p-values were presented with a significance threshold of 0.05. Differentially expressed gene analysis was carried out using edgeR with the eosinophil percentage as a covariate. Default parameters were applied to run all software listed above.

### Immunophenotyping by flow cytometry

Fresh whole blood samples from SSIC cohort were lysed using RBC lysis buffer (containing 155 mM NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub> and 0.1mM EDTA). Cells were washed using PBS and stained using Anti-IgE (MB10-5C4, Miltenyi Biotec), FcERIA (AER-37, eBioscience), CCR3 (5E8, Biolegend), CD203c (97A6, Beckman Coulter), CD123 (6H6, eBioscience), CD14 (61D3, eBioscience), CD16 (3G8, Biolegend), HLA-DR (L243, BD Biosciences), CD1c (L161, BD Biosciences). The cells were acquired on LSRFortessa<sup>TM</sup> cell analyzer (BD) and analysed using FlowJo v10 (BD). Flow cytometry data on PBMCs were generated newly for this study by using frozen PBMC samples of the SSIC cohort. Cells were thawed using pre-warmed RPMI medium (Life Technologies). Dead cells were discriminated by staining PBMCs with LIVE/DEAD Fixable Blue Dead cell stain kit (Life Technologies). The cells were then washed using MACS buffer (0.5% BSA, 2 mM EDTA in PBS) and stained using the following antibodies: Anti-CD49d (clone 9F10, Biolegend), Anti-CD45RB (clone MEM-55, Immunotools), Anti-CD3 (clone UCHT1, BD Biosciences), Anti-CD45RO (clone UCHL1, Biolegend), Anti-CD161 (clone HP-3G10, Biolegend), Anti-CRTH2 (clone BM16, BD Bioscience), Anti-CD4 (clone RPA-T4, BD Biosciences), Anti-CD27 (clone O323, Biolegend) and Anti-CD38 (clone HB7,

BD Biosciences; O323). The cells were acquired on LSR II 5 lasers flow cytometer (BD) and analysis was performed using FlowJo v10 (BD) and unsupervised cluster analysis tools (see below).

### Plasma cytokine quantification

The plasma levels of IL-5, IL-13, IL-6, IL-17A and TNF[?] were quantified using Quanterix Single Molecule-Arrays (SiMoA) analyser using the Simoa[?] IL-5 Advantage Kit, Simoa[?] IL-13 Advantage Kit and Simoa[?] Cytokine 3-Plex B Advantage Kit respectively following the manufacturer's protocol.

### Detection of cytokine secretion by flow cytometry

Frozen cells were thawed using RPMI 1640 Medium (Gibco), supplemented with 10% FBS (BioWhittaker) and washed with homemade 1X MACS buffer (1X PBS + 0.5% BSA + 2mM EDTA). Cells were then pre-stained for 15 minutes at 37°C with 5% CO<sub>2</sub> using the following antibodies: anti-CD294 (clone BM16, BD Pharmingen) and anti-CD45RB (clone MEM-55, ImmunoTools). Cells were washed with 1X MACS buffer and then stimulated for 4 hours at 37°C with 5% CO<sub>2</sub> with 20ng/ml Phorbol myristate acetate (PMA) (Sigma-Aldrich), 1µM Ionomycin calcium salt (Sigma-Aldrich) and GolgiPlug (BD Biosciences).

After 4 hours of stimulation, cells were washed with 1X PBS (Gibco) and stained with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen) for 10 minutes at room temperature. Cells were then washed with 1X MACS buffer and stained for 15 minutes at 4°C using the following antibodies: anti-CD161 (clone HP-3G10, eBioscience). Intracellular staining was performed using the BD Cytotfix/Cytoperm Kit (BD Biosciences) according to the manufacturer's instructions. Cells were fixed with Fixation/Permeabilization solution (BD Biosciences) for 20 minutes at 4°C. Cells were washed and kept in 1X MACS buffer overnight at 4°C. On the next day, cells were permeabilized with 1X Perm/Wash buffer for 15 minutes at 4°C and stained with the following antibodies: anti-CD3 (clone UCHT1, BD Pharmingen), anti-CD4 (clone RPA-T4, BD Pharmingen) and anti-IL-5 (clone TRFK5, BioLegend). After 30 minutes, cells were washed with 1X Perm/Wash buffer and resuspended in 1X MACS buffer. Samples were acquired using BD LSR II flow cytometer and analysed using FlowJo v10 (BD).

### Functional analysis of stimulated PBMC by mass cytometry

All antibodies used in the study were labelled in house with metal tags. Purified antibodies for mass cytometry were obtained in carrier/protein-free buffer and coupled to lanthanide metals using the MaxPar DN3 antibody conjugation kit (Fluidigm Inc.) according to the manufacturer's recommendations. Upon conjugation, the yield of recovered antibody was determined by measurement of absorbance at 280 nm on a nanodrop before dilution of the antibodies in Candor PBS Antibody Stabilization solution (Candor Bioscience, Germany) for long-term storage at 4°C. Antibodies used are listed in **Supplementary Table 1**. In order to capture the phenotype of resting cells, most of the surface markers were stained prior to the stimulation. Frozen PBMCs were thawed using RPMI 1640 Medium (Gibco), supplemented with 10% FBS (BioWhittaker) and washed with homemade 1X MACS buffer. Cells were resuspended in pre-stimulation antibody cocktail and incubated for 30 minutes on ice. Cells were washed with CyFACS buffer (PBS with 4% FBS, 0.05% sodium azide) and transferred to a 6-well plate for stimulation. The cells were stimulated using the PMA/Ionomycin cocktail described earlier and incubated for 4 hours in a 37°C incubator. The cells were then transferred into a 96-well U bottom plate and washed with PBS, followed by post-stimulation surface antibody cocktail and incubation of 30 minutes at 37°C. Subsequently, cells were washed with CyFACS buffer and resuspended with the post-stimulation antibody cocktail and incubated for 30 minutes on ice. Cells were washed twice with CyFACS buffer and then treated with Cisplatin for 5 minutes on ice. Following double washes with CyFACS buffer and fixation overnight using 2% PFA, cells were permeabilized and stained with intracellular antibody cocktail for 30 minutes on ice. Washes were then carried out with permeabilization buffer followed by barcoding. Lastly, cells were stained with DNA intercalator (Cell-ID Intercalator-Ir, Fluidigm) in PBS for 20 mins at room temperature. After washing twice with CyFACS buffer, cells were washed with MiliQ water and filtered through a cell strainer snap cap tube in preparation of CyTOF acquisition. Cells were then diluted to  $0.5 \times 10^6$  cells/mL in MiliQ water containing 2% EQ Four Element Calibration Beads (Fluidigm). Cells were acquired using Helios mass cytometer at a rate of 280-350 cells/s on CyTOF

software version 7.0.5189 and analysed using FlowJo v10 (BD).

## Statistical Analysis

Flow cytometry, mass cytometry and plasma cytokine data analysis were done using non-parametric methods in R and GraphPad Prism (Dotmatics). Correlation of two continuous variables was performed using Spearman Rank correlation while comparison of continuous variables against binary categorical variables was done using Mann-Whitney U tests. PhenoGraph and UMAP analysis were done in R using the Cytokit2<sup>14</sup>[18]. P values less than 0.05 were deemed to be statistically significant.

PhenoGraph and cluster analysis was performed using Cytokit2 [18] followed by statistical analysis using Graphpad Prism (Dotmatics).

## RESULTS

### Transcriptomics highlight eosinophils and TH2 as disease-driving cells in AR

We assessed whole blood gene expression comparing AR and non-AR individuals from our previously described SSIC cohort using whole transcriptomic sequencing (**Supplementary Table 2A**). A total of 1160 unique probes associated to 1095 unique genes were differentially expressed at a nominal significance level of 0.05. A total of 23 probes representing 20 unique gene transcripts were robust after stringent correction for multiple testing at a false discovery rate (FDR) p-value of 0.05, with all transcripts upregulated in AR (**Table 1A**). Given that atopy is well known to be associated with eosinophil levels, sensitivity analysis with adjustment for eosinophil counts was performed, with all identified differentially expressed genes (DEGs) remaining significant post adjustment. To account for ethnicity and environmental influences on atopic gene expression, we validated our findings in BAMSE population-based cohort comprising of Swedish adolescents (**Table 1B**). Out of 20 DEGs identified in the SSIC cohort, 11 DEGs were replicated and reached significance in BAMSE, confirming the transferability of our findings irrespective of ethnicity and environmental differences (**Table 1B**).

For top DEGs that reached nominal significance in the SSIC cohort, we interrogated for biological function and performed Ingenuity Pathway Analysis (IPA). In the disease and disorders category, hypersensitivity and inflammatory responses were significantly associated with nominal AR-related DEGs (**Table 2A**). Top pathways enriched for hypersensitivity and inflammatory responses included cell-to-cell signalling and haematological system and cell-mediated immune response (**Table 2B, C**). Importantly, we observed strong functional enrichment for eosinophils, along with basophils and mast cells, in hypersensitivity responses associated with AR (**Table 2B**). Similarly, functional activation of TH2 and myeloid cells were highlighted to drive inflammatory responses in AR (**Table 2C**). Consistent with current understanding, transcriptomic findings emphasise eosinophils and TH2 cells as key drivers of AR.

### Blood eosinophilia and eosinophilic inflammation correlates strongly with AR

In order to identify the cellular drivers of airway inflammation in AR, we took a deeper look into the SSIC cohort. [10, 11] Using flow cytometry data from whole blood analysis, we performed unsupervised PhenoGraph clustering of whole blood subsets and correlated them with atopy status (**Figure 1A-C** and **Supplementary Table 3**). Of all cell subsets, eosinophils were identified as the only leukocyte cluster directly associated with atopy (**Figure 1B** and **C**). Atopic individuals were demonstrated to have higher frequencies of eosinophils than individuals without atopy (**Supplementary Figure 1A**). Following this, we did a correlation analysis of the symptoms of AR with eosinophil counts. While atopy status was defined objectively by SPT, assessment of AR manifestation and symptoms for SSIC cohort was based on a survey questionnaire. [11] Despite the subjective nature of these self-reported parameters, we observed strong associations between eosinophils and AR, where AR cases registered significantly higher eosinophil frequencies than non-AR controls (**Figure 1D**). The same trend was observed for total IgE levels (**Figure 1E**).

### CD161+ TH2 plays a role in driving eosinophilic inflammation in AR

In order to identify other atopy and AR-related subsets within lymphoid and myeloid populations, a flow cytometry panel was specifically designed for the detection of allergy-related PBMC subsets. [13] In contrast to whole blood analysis, PBMC clusters demonstrated non-significant associations with atopy and AR (**Supplementary Figure 2**). Given that TH2 was earlier identified as a disease-driving subset by transcriptomic sequencing, we gated for chemoattractant receptor homologue on TH2 (CRTH2+) positive cells to further enrich for key PBMC subsets with roles in disease progression of AR. CRTH2 is expressed by a small fraction (approximately 1.6%) of the PBMC population (**Figure 2A**) and is typically expressed on TH2-associated immune cells such as TH2 and ILC2. [19] In line with transcriptomic analysis, *PTGDR2* (gene encoding CRTH2) was also differentially expressed between non-AR and AR individuals (**Table 1**).

UMAP analysis segregated the entire CRTH2+ population into five distinct clusters representing basophils, TH2, ILC2, TC2 and non-classical monocytes (**Figure 2B**). PhenoGraph clustering subdivided these five main clusters into 28 distinct subpopulations (K1-K28; **Supplementary Figure 3**). While all CRTH2+ subsets are reportedly involved in allergy and allergic diseases, cluster ‘K9’ is highlighted here as the sole subset directly associated with atopy (**Figure 2C**). Cluster ‘K9’ (CD161+ TH2) is a TH2A-like subset [13] located within the TH2 cluster (**Figure 2D** and **Supplementary Figure 3**). Next, we interrogated CD161+ TH2 for associations with AR. Similar to eosinophils, frequency of CD161+ TH2 is significantly associated with both atopy and AR (**Supplementary Figure 1B** and **Figure 2E**). Furthermore, we observed a positive correlation between the frequencies of CD161+ TH2 and eosinophils (**Figure 2F**). Likewise, frequency of CD161+ TH2 is also positively correlated to titres of total IgE (**Figure 2G**). Taken together, CD161+ TH2 cells may represent a key cellular subset driving atopy and airway inflammation in AR.

### **CD45RB<sup>lo</sup> CD161+ TH2 promotes IL-5 production and disease progression**

As cellular phenotype is crucial to functional characteristics, we further refined the phenotype of the CD161+ TH2 subset driving AR. Of particular interest is the activation status of this cellular subset. Interestingly, surface expression of marker CD45RB was significantly downregulated on CD161+ TH2 cells of AR individuals compared to non-AR individuals, indicative of memory and differentiation characteristics (**Figure 3A**).

To assess the inflammatory profile of AR, we quantified IL-5, IL-13, IL-17A, IL-6 and TNF $\alpha$  plasma levels by ultra-sensitive SiMoA array. AR cases had significantly elevated plasma IL-5 levels as compared to non-AR cases, which confirmed Type 2 inflammation in AR (**Figure 3B** and **Supplementary Figure 4**). As the key survival factor of eosinophils, IL-5 plays a pivotal role in eosinophilic allergic diseases. [20-23] Given that TH2A and pe-TH2 populations were previously reported to be major producers of IL-5, [13, 24] we probed for IL-5 secretion in AR-relevant CD161+ TH2 subset. Interestingly, high circulatory plasma IL-5 levels were only observed in individuals with low CD45RB (CD45RB<sup>lo</sup>) expression on CD161+ TH2 cells (**Figure 3C**), suggesting that CD45RB<sup>lo</sup> CD161+ TH2 could be responsible for IL-5 secretion. To clarify the cell subsets responsible for IL-5 secretion, PBMCs were stimulated with PMA and Ionomycin, and respective cytokine secretion was analysed using flow cytometry. As stimulation results in an upregulation of CD45RB, PBMCs were pre-stained with surface markers (including CD45RB) prior to activation so that steady state phenotype could be preserved. IL-5 expression was then quantified by intracellular staining following stimulation. While we note a small population of IL-5 secreting conventional CD161- TH2 (cTH2), IL-5 secretion was significantly elevated in CD161+ TH2 cells (**Figure 3D** and **E**). Strikingly, IL-5 secretion was exclusively found within the CD45RB<sup>lo</sup> subset in both cTH2 and CD161+ TH2 (**Figure 3E**). These findings confirm CD45RB<sup>lo</sup>CD161+ TH2 as the main producers of IL-5.

Importantly, the proportion of IL-5 producing CD45RB<sup>lo</sup>CD161+ TH2 cells was significantly elevated in AR as compared to non-AR (**Figure 3F**). In contrast, there was no significant difference in the proportion of IL-5 producing CD45RB<sup>lo</sup> cTH2 between the non-AR and AR individuals. Taken together, these results suggest a role for IL-5 producing CD45RB<sup>lo</sup> CD161+ TH2 cells in the pathogenesis of AR.

### **CD45RB<sup>lo</sup> CD161+TH2 is the central pathogenic cell type in active disease**

As allergic manifestation in the aforementioned SSIC cohort were based on self-reported historical symptoms and is susceptible to recall bias, we therefore validated our findings in a second paediatric cohort with clinically diagnosed active AR manifestations (**Supplementary Table 2B**). As expected, there are significantly higher levels of eosinophils and CD161+ TH2 observed in AR cases, thus confirming eosinophilic inflammation in active AR (**Figure 4A, B**). In order to obtain a wide comprehensive phenotypic outlook of the disease-driving CD161+ TH2 subset, an extended 40plex mass cytometry allergy panel was designed to include antibodies specific for inflammatory cytokines and activation markers such as IL-2, IL-3, IL-4, IL-5, IL-9, IL-13, IL-17A, IL-22, IFN- $\gamma$ , TNF- $\alpha$  and HPGDS [12] (**Supplementary Table 1**). As the stimulation required to induce cytokine production triggers major shifts in the expression of surface markers, most of the lineage and surface markers (including CD45RB) were stained prior to stimulation, which enabled depiction of the actual *ex vivo* cellular phenotype.

Mass cytometry of PBMCs with the expanded 40-marker panel revealed all key populations as defined previously with the narrower FACS panel but at much higher granularity. Consistent with observations in the SSIC cohort (**Figure 3F**), a significantly higher proportion of IL-5 producing CD45RB<sup>lo</sup> CD161+ TH2 was found in individuals with active AR compared to non-AR controls (**Figure 4C**). This trend was not observed in CD161- cTH2.

To confirm our earlier observations in the SSIC cohort, we gated for CRTH2+ CD161+ cells and performed supervised phonograph clustering. A total of 9 sub-populations were segregated from the CD161+ TH2 subset (**Figure 4D and E**). Of all sub-populations, Cluster 3 was found to be significantly increased in individuals with active AR (**Figure 4F, G and Supplementary Table 5**).

In line with expectations, 'Cluster 3' is indeed an IL-5 secreting CD45RB<sup>lo</sup> population (**Figure 5A**). Moreover, this cluster appeared to be a highly differentiated population of mature CD161+ TH2 cells with an activated phenotype characterized by low expression of CD27, CD45RB, KLRG1 and high expression of CD45RO, HPGDS, ICOS, and CD38. The cytokine profile suggests that in addition to IL-5 production, this population also secretes a multitude of cytokines that includes IL-2, IL-3, IL-4, IL-9 and IL-13. Taking a deeper look, we further confirmed that cytokine secretion is highly correlative (**Figure 5B**). Thus, the severity of eosinophilic airway allergies such as AR seems to be driven by an activated terminally differentiated CD161+ TH2 subset that is able to concomitantly secrete a complex set of inflammatory cytokines.

## DISCUSSION

There has been growing evidence of heterogeneity within the human TH2 subsets discovered over the years. [12, 13, 25] Prussian and colleagues first reported high levels of IL-5+ producing TH2 subset in patients with allergic eosinophilic gastroenteritis. [12] This population was named 'peTH2' and was defined by their expression of HPGDS and CD161. [12] In parallel, the importance of IL-5 producing TH2 subset was further confirmed in mouse models of allergic airway inflammation and atopic dermatitis. [26, 27] Wambre et al defined a subpopulation of human allergen-specific TH2 subset which they coined as TH2A. [13] TH2A was identified as a memory subset that is over-represented in allergic individuals and secretes IL-5, IL-4 and IL-13 in response to stimulation. [13] As different cell markers were used across different groups to identify the subsets, this raises the question whether the identified TH2 populations pertinent for allergic diseases across these studies were homogenous.

In this study, we used unsupervised clustering tools (UMAP and PhenoGraph) on CRTH2+ PBMCs and identified a TH2A-like subset that correlated directly to the markers of atopy. A common feature between the different TH2 subsets reported in literature was the production of IL-5. [13, 19, 27] Similarly, we were also able to detect IL-5 in our identified CD161+ TH2 subset. Strikingly, IL-5 production was only limited to the CD45RB<sup>lo</sup> T cell population. As CD45RB<sup>lo</sup> T cells are typically terminally differentiated memory cells, this suggests a need for repetitive antigen stimulation before CD161+ TH2 subsets are capable of IL-5 secretion. This is supported the studies by Upadhyaya et al. and Islam et al, who found that the ability of TH2 cells to secrete IL-5 requires multiple rounds of *in vitro* stimulation. [25, 27] Similar requirements for repeated allergen exposure is needed to polarize IL-5 producing TH2 cells.

Intriguingly, CD45RB<sup>lo</sup>CD161<sup>+</sup> TH2 population was found to be directly correlated to AR in both cohorts with active and self-reported AR. Not only does this show the persistence of the CD45RB<sup>lo</sup>CD161<sup>+</sup> TH2 population, it also implies the pertinence of this population in the pathogenesis of AR and possibly other allergic diseases. Both SSIC and active AR cohorts described in this study were collected in Singapore, whereby majority of the individuals are sensitized against HDM. [10] HDM is a perennial allergen in tropical nations such as Singapore, thus T cells in atopic individuals undergo constant stimulation. [11] This could explain the strong association observed between CD45RB expression on CD161<sup>+</sup> TH2 cells and atopy markers despite the fact that not all subjects demonstrated active AR symptoms during the collection of SSIC cohort.

A high dimensional 40-marker panel was used to further characterize the CD161<sup>+</sup> TH2 population. As mentioned previously, pre-staining of cell surface markers was performed prior to stimulation in order to preserve the steady state cell identities. We identified a specific subset within the CD161<sup>+</sup> TH2 population that associated strongly with AR. Other than exhibiting a terminally differentiated phenotype (defined by low expression of CD27 and CD45RB with concomitant high expression of CD45RO and ICOS) with intracellular expression of HPGDS, this subset was also able to secrete multiple cytokines upon stimulation. Amongst several others, this included allergy related cytokines such as IL-5, IL-4 and IL-13. Taken together, our current study unifies the markers previously reported for allergic-specific TH2 subsets and provides clarity for the pathogenic TH2 subset previously reported in different allergic diseases. The persistence of CD45RB<sup>lo</sup> CD161<sup>+</sup> TH2 may allow for public health surveillance of allergic individuals. Moreover, these cells may also be leveraged as a biomarker for the effectiveness of immunotherapy as well as a potential disease target in the treatment of AR and allergic diseases.

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**Table 1. Differential gene expression (DEGs) comparison between non-AR and AR from whole blood samples in (A) SSIC and (B) BAMSE cohorts**

A) Differentially Expressed Genes (DEGs) for AR from SSIC	A) Differentially Expressed Genes (DEGs) for AR from BAMSE
Gene	logFC
<i>IL5RA</i>	0.475

<i>PYROXD2</i>	0.202
<i>SIGLEC8</i>	0.575
<i>THBS4</i>	0.390
<i>ALOX15</i>	0.526
	0.411
<i>OLIG2</i>	0.614
<i>ADGRE4P</i>	0.411
<i>CLC</i>	0.189
<i>LINC00323</i>	0.422
<i>CCL23</i>	0.678
	0.604
<i>HES1</i>	0.356
<i>SMPD3</i>	0.356
<i>HRASLS5</i>	0.367
<i>PRSS33</i>	0.595
<i>SLC29A1</i>	0.411
	0.275
<i>TFF3</i>	0.614
<i>PTGDR2</i>	0.401
<i>IL17RB</i>	0.345
<i>P2RY2</i>	0.310
<i>RASL11B</i>	-0.286
<b>B) DEGs for AR replicated in BAMSE</b>	<b>B) DEGs for AR replicated in BAMSE</b>
<b>Gene</b>	<b>logFC</b>
<i>SLC29A1</i>	0.125
<i>IL5RA</i>	0.366
<i>ALOX15</i>	0.313
<i>SMPD3</i>	0.137
<i>CLC</i>	0.415
<i>HRASLS5</i>	0.109
<i>SIGLEC8</i>	0.148
<i>OLIG2</i>	0.085
<i>P2RY2</i>	0.09
<i>PRSS33</i>	0.069
<i>HES1</i>	0.058

**logFC** - logarithm base 2 of fold change effect size for AR phenotype; **Avg Expr** - mean expression across all samples; **P-value** – False discovery rate (FDR) corrected p-value of gene expression to AR; **P.eosadj** - P-value for AR after correcting for eosinophil percentages. In the case of *ALOX15*, *CCL23* and *SLC29A1*, there were dual probes present that tagged to the same gene where expression values of both probes achieved FDR-significance.

**Table 2: Ingenuity Pathway Analysis for nominally significant AR-related DEGs from the SSIC cohort.**

<b>(A) Enrichment for AR candidates for diseases and disorders</b>	<b>(A) Enrichment for AR candidates for diseases and disorders</b>
<b>Diseases and Disorders</b>	<b>P-value</b>
Hypersensitivity response	1.98E-05 – 4.08E-02
Inflammatory response	1.98E-05 – 4.99E-02
Haematological disease	2.27E-04 – 4.26E-02
Immunological disease	2.27E-04 – 1.69E-02

**(B) Significant terms enriched in the hypersensitivity response**

Categories	Function	P-value	Gene Symbol
Cell-to-Cell Signalling	Activation of eosinophils	1.98E-05	IL1RL1, IL5RA, PTGDR2
Haematological system	Quantity of eosinophils	6.25E-05	IL1RL1, IL5RA, PTGDR2, SIGLEC8
Cellular movement	Infiltration by eosinophils	2.68E-04	IL1RL1, PTGDR2, SIGLEC8
Cell-to-Cell Signalling	Response of eosinophils	3.60E-04	IL1RL1, IL5RA
Cellular function and movement	Function of eosinophils	3.72E-03	IL1RL1, SIGLEC8
Cellular function & haematological system	Infiltration by basophils	3.78E-03	IL1RL1, PTGDR2
Hypersensitivity Response	Tolerization of mast cells	3.78E-03	PTGDR2
Cell-to-Cell Signalling	Inflammation of eosinophils	1.13E-02	IL1RL1

(C) Significant terms enriched in the inflammatory response	(C) Significant terms enriched in the inflammatory response	(C) Significant terms enriched in the inflammatory response	(C) Significant terms enriched in the inflammatory response
Categories	Function	P-value	Gene Symbol
Cell-to-Cell Signalling	Activation of Th2 cells	3.60E-04	IL1RL1, PTGDR2
Cell-mediated immune response	Chemotaxis of T lymphocytes	1.48E-02	CCL23, PTGDR2
Cellular movement	Leukocyte migration	1.93E-02	ALOX15, CCL23, IL1RL1, THBS4, SIGLEC8, PTGDR2
Cell-to-Cell Signalling	Activation of myeloid cells	9.52E-04	IL1RL1, IL34, IL5RA, PTGDR2, SIGLEC8

A total of 1160 unique probes with nominal P-value <0.05 associated with 1095 unique genes were evaluated with IPA analysis. P-value for each category is reported. (A) Diseases and disorders enriched in DEG candidates from Table 1A. Top pathways enriched in the (B) hypersensitivity and (C) inflammatory response from the enrichment candidates in (A).

*Figure Legends*

**Figure 1. Eosinophilic allergic rhinitis (AR) in SSIC cohort is associated with high levels of HDM-specific IgE** Whole blood leukocytes from SSIC cohort were stained and analysed with flow cytometry. (A) Representative scatterplot showing lymphocytes (green), monocytes (red) and granulocytes (blue) subsets in whole blood (n=216) and these subsets are analysed using unsupervised phenograph clustering and umap dimensionality reduction. (B) Umap clusters were annotated based on their lineage markers and (C) associated with atopy phenotype. P-value of the respective cluster was presented as blue/red shading based on statistical analysis. Red denotes direct correlation while blue denotes negative correlation. Scatter plots comparing (D) percentage of eosinophils in total leukocytes and (E) total plasma immunoglobulin E (IgE) between non-AR and AR individuals. Spearman correlation was performed in **Figure 1C** while pairwise two-tailed Mann-Whitney *U* test was performed in **Figure 1D-H**. A p-value of less than 0.05 is considered statistically significant. (\*\* $p < 0.01$ , \*\*\* $p < 0.001$ )

**Figure 2. CD161+ CD4+ TH2 cells are associated with AR.** PBMCs of SSIC individuals were stained and analysed using flow cytometry. (A) CRTH2+ subsets from PBMCs (n=216) were gated and used for analysis by phenograph clustering and umap dimensionality reduction. (B) Umap clusters were

annotated based on lineage markers and associated with (C) atopy phenotype. Statistical analysis was performed using Wilcoxon rank sum test. Umap clusters were shaded red according to statistical significance. (D) Representative scatterplot showing CD4 and CD161 expression on cluster K9 (derived from umap cluster in (C)) overlaid on total CRTH2+ PBMCs. Number in the plot refers to the representative percentage of cluster K9 of all CRTH2+ PBMCs. (E) Scatter plot comparing the numbers of peripheral CD161+ TH2 cells between non-AR and AR individuals. Two-tailed Mann-Whitney  $U$  test was performed. Association of peripheral CD161+ TH2 numbers with (F) whole blood eosinophil numbers and (G) total plasma IgE. Non-atopic individuals were shaded in grey while atopic individuals were shaded in black. Statistical analysis was performed using Spearman correlation. P-value of less than 0.05 is considered statistically significant. (\*\* $p < 0.01$ , \*\*\* $p < 0.001$ )

**Figure 3. CD45RB<sup>lo</sup>CD161+ TH2 subset produces high level of IL-5 and are associated with AR.** PBMCs of SSIC individuals were stained and analysed using flow cytometry. (A) Scatterplots of CD45RB log mean fluorescence intensity (MFI) on CD161+ TH2 comparing between non-AR and AR individuals. (B) Scatterplot comparing plasma IL-5 level between non-AR and AR in SSIC individuals. Two-tailed Mann-Whitney  $U$  test was performed. (C) Scatterplot showing association of plasma IL-5 levels against CD45RB MFI on CD161+ TH2. PBMCs were pre-stained with CD45RB and stimulated using PMA/ionomycin. Intracellular staining was performed to determine IL-5 production using flow cytometry. (D) Paired analysis of IL-5 MFI on CD161- or CD161+ TH2 cells from non-AR or AR individuals. Wilcoxon paired analysis was performed. (E) Representative scatterplots showing expression of IL-5 and CD45RB in CD161- TH2 (cTH2) and CD161+ TH2 subsets. Numbers in quadrant represents percentage of cells in each quadrant of the plot. (F) Scatterplot showing association of CD45RB<sup>lo</sup>IL-5+ cTH2 or CD161+ TH2 in non-AR or AR individuals. Statistical analysis was performed using one-way ANOVA followed by Tukey post test. P-values less than 0.05 are considered statistically significant. (\*\* $p < 0.01$ , \*\*\* $p < 0.001$ )

**Figure 4. CD45RB<sup>lo</sup>CD161+ TH2 subset is associated with active AR manifestation.** Whole blood leukocytes from the pediatric cohort were stained and analysed with flow cytometry. Scatterplots comparing the percentage of (A) eosinophils and (B) CD161+ TH2 between non-AR and AR individuals. Statistical analysis was performed using two-tailed Mann-Whitney  $U$  test. PBMCs were pre-stained with cell surface markers and stimulated using PMA/ionomycin. Intracellular staining was performed to determine cytokine production using CyTOF. (C) Scatterplot showing association of CD45RB<sup>lo</sup>IL-5+ cTH2 or CD161+ TH2 in non-AR or AR individuals. (D) Representative scatterplot showing gating of CD161+ TH2 from total CD4+ T cells to be used for umap cluster analysis in (E). CD161+ TH2 cells were gated, downsampled to 200 cells per sample and analysed using unsupervised phenograph clustering with 38 markers. (E) Umap dimensionality reduction was performed and cells were segregated into different clusters. Numbers in the plot represent cluster numbers. (F) Scatterplot comparing percentage of cluster 3 (derived from (E)) between non-AR and AR individuals. Statistical analysis was performed using two-tailed Mann-Whitney  $U$  test. P-value of less than 0.05 is considered statistically significant. ( $*p < 0.05$ ) (G) Umap scatterplot showing the distribution of cluster 3 (in red) in concatenated non-AR (n=6) and AR (n=14) samples respectively.

**Figure 5. AR-associated CD45RB<sup>lo</sup>CD161+ TH2 subset secreting diverse range allergy-related cytokines** (A) Heatmap showing marker and cytokine expression on the different clusters derived from Figure 4E. (B) Scatterplots showing expression of hematopoietic prostaglandin D synthase (HPGDS), CD45RB, IL-5, IL-4 and IL-13 on cluster 3 overlaid on total CD161+ TH2.

*Supplementary figure and tables legends*

**Supplementary Figure 1. Scatterplots comparing percentage (A) eosinophils and CD161+ TH2 on Non-atopic and atopic individuals in SSIC cohort**

**Supplementary Figure 2. Umap clustering of PBMCs from SSIC cohort and the association to atopy.** Representative scatterplot showing representative PBMCs population. PBMCs from SSIC cohort (n=216) were analysed using unsupervised phenograph clustering and umap dimensionality reduction. Umap clusters were annotated based on their lineage markers and associated with atopy phenotype.

Supplementary Figure 3. Heatmap showing phenograph cluster K1-28 and their associated markers

Supplementary Figure 4. Quantification of plasma (A) IL-13, (B) IL-17A, (C) IL-6 and (D) TNF $\alpha$  in SSIC cohort individuals

Supplementary Table 1. List of CyTOF antibodies used for surface and intracellular staining

Supplementary Table 2. Demographic table of SSIC and Pediatric cohort

Supplementary Table 3. Association of umap clusters from SSIC CRTH2+ PBMCs to atopy

Supplementary Table 4. Association of surface markers on CD161+ TH2 to AR

Supplementary Table 5. Association of umap cluster from pediatric cohort CD161+ TH2 to AR

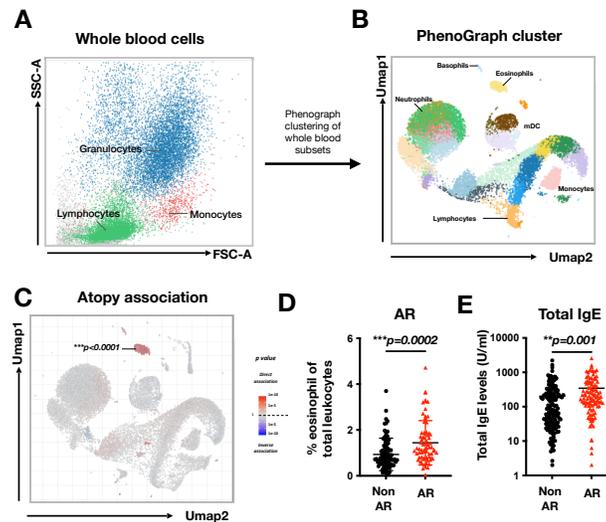


Figure 1

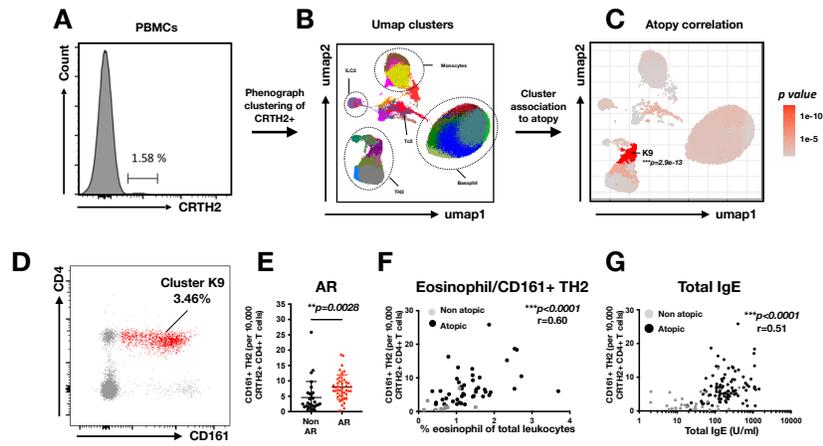


Figure 2

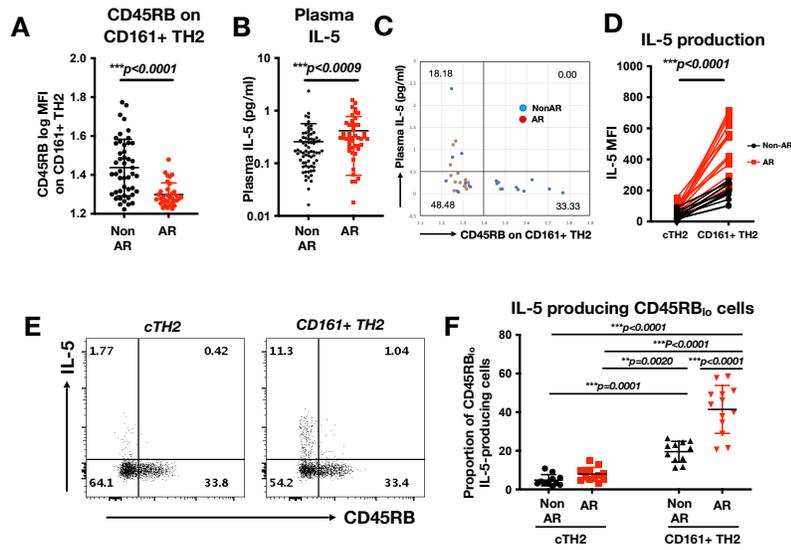


Figure 3

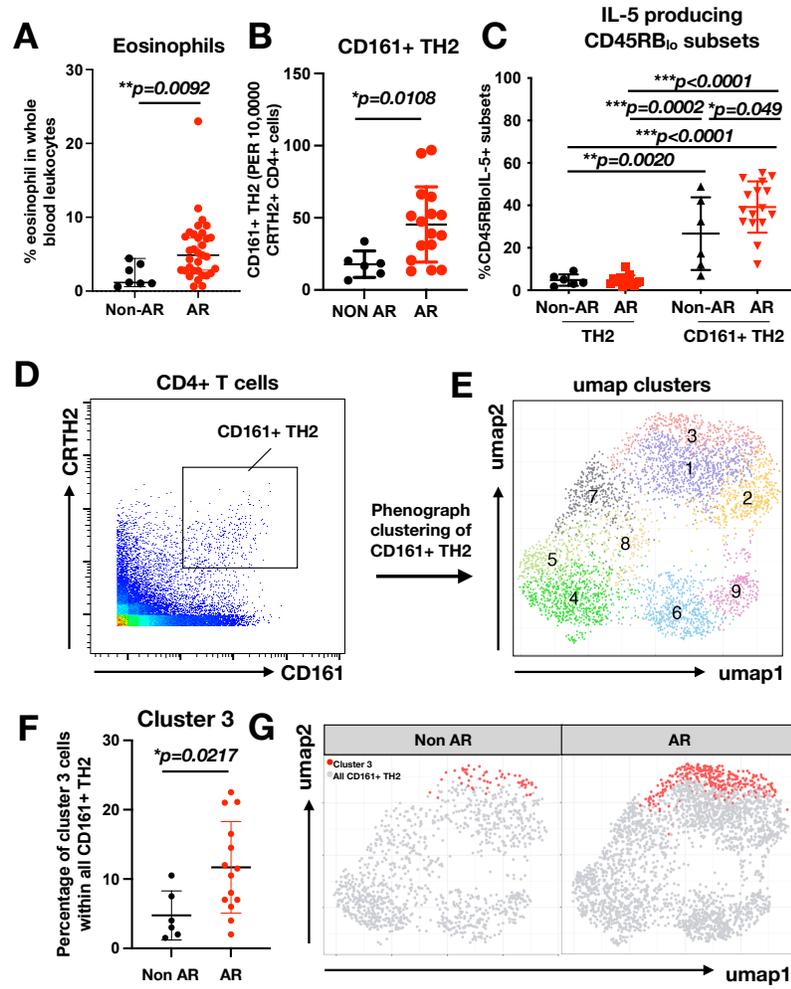
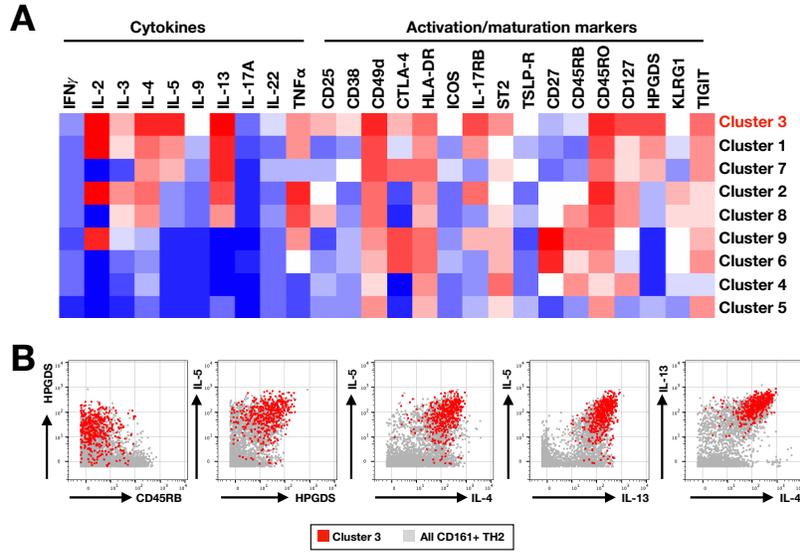


Figure 4



**Figure 5**