Urbanization in Indonesia and its impact on non-communicable diseases: a clinical, epidemiological, and immunological study

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Chapter 6

TH2A AND CD38+ TH2A CELLS IN PERIPHERAL BLOOD AND NASAL MUCOSA OF INDIVIDUALS WITH ALLERGIC RHINITIS IN URBAN AND RURAL INDONESIA

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ABSTRACT

Background: The prevalence and severity of AR are generally higher in urban than in rural areas. It is hypothesized that urbanization alters the immune system and thereby, AR manifestation.

Aims: To evaluate the peripheral blood and nasal mucosal immune cells of Indonesian young adults with AR originating from rural and urban areas.

Methods: AR subjects were skin prick test (SPT) positive to allergens and had rhinitis symptoms based on ISAAC questionnaire. The healthy control (HC) subjects were negative for SPT and had no rhinitis symptoms. Total IgE levels were determined by ELISA and eosinophil counts through microscopy. Whole blood and nasal mucosal samples of 18 urban (10 AR and 8 HC) and 12 rural (6 AR and 6 HC) subjects were analyzed using mass cytometry.

Results: In comparison to HC, both urban and rural AR subjects had higher total IgE levels, while eosinophil counts were only elevated in urban AR group. Major differences were seen when examining the nasal mucosa, where basophils, mast cells, CD4 Th2, Th2A, and $CD38^+$ Th2A cells were upregulated, but only in the urban AR. In addition, the expression of the upper respiratory tract homing marker CCR3 on $CD38^+$ Th2A cells was restricted to the urban AR group. In contrast, the differences in peripheral blood were modest, with only a significantly higher $CD163^+$ mDCs in rural AR.

Conclusion: Urban AR showed strong inflammatory immune responses in the nasal mucosa compared to rural AR, which might explain the higher disease activity in urban areas globally.

INTRODUCTION

Allergic rhinitis (AR) is a chronic inflammatory allergic disease involving the nasal mucosa, characterized by rhinorrhea, nasal congestion, sneezing, and itchy eyes.[1] The worldwide prevalence of AR in the adult population is approximately 18.1% (ranging from 1.0% to 54.5%).[2] Although AR is not a life-threatening condition, it causes a high economic burden, including indirect costs due to high rates of absenteeism and decreased work productivity.[3] It also has major effects on sleep, daily activity, emotional well-being, and quality of life (QOL).[3,4]

Indonesia has one of the lowest AR prevalence (5.2%) globally based on the result of The International Study of Asthma and Allergies in Childhood (ISAAC) in 1998.[5] However, the prevalence of AR has increased significantly, as reported by several recent studies, between 13.5% to 38.4%.[6-9] This rising prevalence could be associated with the rapid urbanization associated with rapid socio-economic growth in Indonesia.[10]

Urbanization causes significant alterations in the social, environmental, and lifestyle aspects of human lives, such as: dietary intake,[11] farming exposure,[12] parasitic infections,[13] hygiene and sanitation,[14] biodiversity,[15] and pollution.[16] These changes affect the immune system and thereby, disease pathogenesis and outcome.[17] As a systemic immune disease with nasal mucosa as its effector site, AR manifestation is likely to be modified by these potential effects of urbanization on the immune system. Many studies have shown a higher prevalence of AR in urban compared to rural populations,[18-20] although no data is available for Indonesian. Additionally, a less severe manifestation of allergic rhinitis in rural compared to urban population has been reported previously.[21,22]

Numerous studies have been performed to evaluate the role of the immune system in AR pathophysiology, either in peripheral blood or nasal site. The majority of these studies reported an upregulation of basophils,[23,24] eosinophils,[25,26] mast cells,[27] group 2 innate lymphoid cells (ILC2s),[28,29] and CD4 Th2 cells in AR patients both in systemic and nasal compartments.[30,31] Recent studies have identified a specific subset of Th2 cells, the pathogenic Th2A cells, that play an important role in

AR.[32,33] However, despite the differences in the prevalence and disease severity, no study has compared the immune system in AR individuals of urban and rural areas. In this study, we analyzed the immune profiles of whole blood and nasal mucosal compartments using mass cytometry in Indonesian young adults originating from urban and rural areas, with and without AR.

METHODS

Study design and population

This cross-sectional study included part of the subjects from a larger cohort study evaluating the effect of urbanization on metabolic health and allergy in an Indonesian young adult population. This study was conducted in the Depok campus of the University of Indonesia (UI), involving freshmen UI bachelor students. The study was approved by the Health Research Ethical Committee of Faculty of Medicine Universitas Indonesia (No. 1181/UN2.F1/ETIK/2017). All participants provided informed consent prior to the study.

The detailed procedures for recruitment of study participants can be found in our previous study.[11] The subjects were classified into the urban group if they were born and lived in urban areas, such as in Jakarta metropolitan areas or in one of the provincial capital cities. While, the rural group comprised subjects that were originally born and lived in rural areas, defined as the villages that are located at the district levels across Indonesia, and just recently (less than three months) migrated to an urban area. Subjects with pregnancy, any current infections, history of autoimmune diseases, and the usage of anti-inflammatory drugs, anti-histamines, or steroids were excluded from the study. All subjects' measurements and biological samples collections were performed in the first three months of the start of the academic year, between August to November 2019.

From the larger cohort, 18 urban (8 allergic rhinitis/AR and 10 healthy control/HC) and 12 rural (6 AR and 6 HC) subjects with availability of whole blood and nasal samples were selected for mass cytometry measurements. These subjects were referred as mass cytometry cohort. To confirm the findings observed in the whole blood mass

cytometry measurements, flow cytometry was performed in the remaining subjects from the larger cohort, and referred as the flow-cytometry cohort. This latter dataset consisted of 13 urban (5 AR and 8 HC) and 18 rural (11 AR and 7 HC) subjects (see **Fig. S1** for the flow chart for the inclusion of study participants). Both cohorts compared AR and HC subjects from each urban and rural group.

Skin prick test, ISAAC questionnaire, and allergic rhinitis definition

All subjects underwent a skin prick test (SPT) using extracts of five common aeroallergens: two species of house dust mites (*Dermatophagoides farinae/Der F and Dermatophagoides pteronyssinus/Der P*), cockroach/*Blatella germanica*, dog epithelial, and cat epithelial (ALK-Abello BV, Almere, The Netherlands). Saline was used as a negative control and histamine chloride (10 mg/mL) as the positive control. The SPT was performed on the volar side of the subject's lower arm using skin prick lancets. After fifteen minutes of application, the wheal sizes were measured. Skin test reactivity was considered positive if the longest diameter plus the diameter perpendicular of wheal size divided by two was 3 mm or larger than the negative control.[34]

For evaluating rhinitis symptoms, all subjects were asked to filled in the core questionnaire for rhinitis from the ISAAC (International Study of Asthma and Allergies in Childhood) questionnaire, which has been validated previously for the Indonesian population.[35,36] Subjects were defined as having rhinitis symptoms if they answered 'Yes' to the questions *"Have you ever had a problem with sneezing, or a runny, or a blocked nose when you DID NOT have a cold or the flu?"* and *"In the past 12 months, have you had a problem with sneezing, or a runny, or a blocked nose when you DID NOT have a cold or the flu?"*.

Allergic rhinitis (AR) subjects were defined as having reactive SPT results for any of the aeroallergens and positive rhinitis symptoms based on the ISAAC questionnaire (SPT+RHI+). Additionally, the question *"In the past 12 months, how much did this nose problem interfere with your daily activities?"* in the ISAAC questionnaire was used to evaluate the severity of rhinitis symptoms in these AR subjects. This was categorized as no, mild, moderate, or severe activity disturbance. The subjects who had negative SPT results and without rhinitis symptoms (SPT-RHI-) were included as healthy control (HC) subjects.

Biological samples collection

Blood samples collection were performed after an overnight fasting. EDTA blood samples were used to prepare Giemsa-stained peripheral blood smear for evaluation of eosinophil counts. While, SST containing blood samples were centrifuged to obtain the serum samples and stored in a −80°C freezer until measurement. Approximately 200 µL sodium heparin whole blood sample was mixed together with 1 mL cryopreservation solution medium (CryoStor® CS10, STEMCELL Technologies, Cologne, Germany) in cryovials and was transferred to a −80°C freezer for a minimum of 4 hours. Subsequently, cryovials were stored in liquid nitrogen until analysis for immune cells profiling. Cellular frequencies of such whole blood cryopreserved samples were previously shown to correlate with immune frequencies measured on fresh blood samples.[37]

To obtain immune cells from the nasal mucosal layer, nasal curettages were collected using a small probe with a previously described protocol.[38] Briefly, the nasal inferior turbinate was visualised using a headlamp with the subject in a seated position and head slightly tilted posteriorly and then, curettage was performed using small probe/curette (ASL Rhino-Pro©, Arlington Scientific, Utah, USA) to collect the cells from the mucosal layer. A total of four scrapes, two from each nostril, were obtained from each subject. After each curettage, the nasal probe was flicked into 1 mL cryopreservation solution medium (CryoStor® CS10, STEMCELL Technologies, Cologne, Germany) inside a cryovial until all biological material was dislodged. Subsequently, these cryovials were transferred to a freezing unit in a −80°C freezer for a minimum of 4 hours and then stored in liquid nitrogen for further analysis.

Total IgE and eosinophil counts measurements

The levels of total IgE were measured in the serum samples by ELISA as described previously.[39] The results were expressed in International Units (IU/mL). As some parts of Indonesia are helminth endemic areas and previous study showed that helminth infections could increase the total IgE levels and upregulated the pathogenic Th2A immune cells population,[40] healthy subjects (SPT-RHI-) with total IgE >500 IU/mL were excluded for immune cells profiling. The cut-off of 500 IU/mL was based on the

median total IgE levels of all subjects without helminth infection in urban and rural population, calculated from our previous study.[41] The Giemsa-stained peripheral thin blood smears were assessed to obtain the differential white blood cell counts, resulting in a relative percentage of eosinophils (eosinophil counts).

Mass cytometry

Mass cytometry measurement was performed on the cryopreserved whole blood (WB) and nasal curettage samples of 18 urban and 12 rural mass cytometry cohort subjects. Two antibody panels were designed for this experiment, one for phenotyping the immune cells ex vivo (**Table S1**), while the other was for sample's barcoding (**Table S2**). Samples were measured with a HeliosTM mass cytometer (Fluidigm, USA) in several batches and the obtained .FCS files were exported and pre-processed using 'CyTOFclean' (v1.0.3.)[42] and 'CATALYST' (v1.22.0)[43] packages. For WB samples, granulocytes were defined as $EpCAM^{-}CD66b^{+}$ and other immune cells as $EpCAM^{-}CD45^{+}CD66b^{-}$. For nasal mucosal samples, epithelial cells were defined as additional population expressing $EpCAM^{+}CD45^{-}$, leading to two blood and three nasal populations exported (**Fig. S2**). The resulting .FCS files were then exported to the OMIQ software (Dotmatics, Boston, USA) separately for WB and nasal mucosal immune cells. Batch correction of the phenotypic markers was performed with CytoNorm (k=5 for WB, k=3 for nasal mucosal samples) using concatenated samples as reference control, and results were assessed visually.[44] We performed Uniform Manifold Approximation and Projection (UMAP) [45] algorithm to visualize the high dimensional data, and FlowSOM [46] consensus metaclustering (k=50 for WB, k=25 for nasal mucosal) algorithm was applied to generate immune cell clusters. A total of 49 immune cells clusters were identified from the WB samples (**Table S3**), while 23 clusters from nasal mucosa (**Table S4**), which were exported as .CSV files for further statistical analysis. For the identification of pathogenic Th2A in the WB and nasal mucosal mass cytometry dataset, manual gating was performed in the OMIQ software. The pathogenic Th2A was defined as $CD4^{+}CD27^{-}CCR7^{-}CRTH2^{+}CD161^{+}$, as described previously.[33] An additional gating for CD38 was applied in this pathogenic Th2A population to obtain the $CD38^{+}$ Th2A (see **Fig. S3A & S3B** for gating strategy).

Flow-cytometry

To confirm the findings regarding the pathogenic Th2A immune cells population from the mass cytometry analysis, we performed flow cytometry using cryopreserved WB samples from 13 urban and 18 rural flow cytometry cohort subjects. After preparation and staining with extracellular antibody cocktail (**Table S5**), the samples were acquired on a Cytek Aurora 5L spectral flow cytometer and unmixed using SpectroFlo software (Cytek Biosciences, USA). The .FCS files were then exported to the OMIQ software and a similar gating strategy for mass cytometry data was applied for the identification of pathogenic Th2A immune cells population. An additional gating for CCR3, an important marker for T-cell homing in the human upper airway mucosa,[47] was performed to obtain the CD4 Th2 $CCR3^{+,}$ Th2A $CCR3^{+,}$ and $CD38^{+}$ Th2A $CCR3^{+}$ immune cell populations (**Fig. S3C**).

Statistical analysis

For clinical variables, data were presented as means and its standard deviations (SD) if normally distributed and as median (25^{th}, 75^{th} percentile) if not normally distributed. To evaluate the differences of total IgE levels and eosinophil counts between AR and HC subjects for each urban and rural group, Mann-Whitney test was performed.

All the CSV files generated from OMIQ were imported and analysed in R software (x64 version 4.1.2) within RStudio version 1.4 and analyzed using a generalized linear mixed model (lme4 and lmerTest package v3.1-3) [48,49] to evaluate the differences between AR and HC subjects for each urban and rural group. We adjusted the P-values with Benjamini Hochberg procedure to correct for multiple testing hypothesis.[50] Lastly, for the differences of CD4 Th2, Th2A, and $CD38^{+}$ Th2A between AR vs. HC subjects for each urban and rural group, separate generalized linear mixed model test was applied. For all tests, statistical significance was considered at the two-sided 5% level.

More detailed information on mass cytometry and flow cytometry procedures, as well as statistical analysis, are available at the **Supplementary Methods.**

RESULTS

Characteristic of study population

As indicated in **Table 1**, the age and BMI were comparable between AR and HC subjects in both urban and rural groups, but the proportion of male participants was higher in the AR group. Additionally, higher total IgE levels were observed in both urban and rural AR compared to HC subjects. Eosinophil counts were higher only in the urban AR than HC subjects (**Fig. S4A**). Similar patterns for these clinical characteristics, total IgE levels, and eosinophil counts were also observed for the flow cytometry cohort (**Table 1**, **Fig. S4B**) as well as the larger cohort that the study subjects were selected from (**Table S6**), indicating a representative group were analyzed.

Systemic immune profiles of allergic rhinitis vs. healthy control subjects from urban and rural areas

A total of 2.19 x 10^6 WB immune cells from thirty subjects were included for unsupervised clustering using FlowSom algorithm in the OMIQ software to identify cell subsets, as indicated and visualized with UMAP. The expression of immune cell markers used for clustering can be seen in **Fig. 1A and S5**. Furthermore, 49 immune cell clusters were captured within B cells, CD4 and CD8 T cells, unconventional T cells, γδ T cells, NK and innate lymphoid cells, as well as monocytes and dendritic cells (**Fig. 1B and C, Table S3**).

A higher frequency of cluster 28 ($CD163^+$ myeloid dendritic cells/mDCs) was seen in rural but not in urban AR compared to HC counterparts (**Fig. 1D**). Similar trends were observed for clusters 29 ($CD4^+CD25^{hi}CD127^-$/Tregs) and 35 (non-classical monocytes), which were not statistically significant after fdr-correction. When considering the type-2 immune responses, which are known to be associated with allergic disorders, we found no differences in the frequency of cluster 19 (CD4 Th2), cluster 27 (basophils), and cluster 26 (ILC2s) between AR and HC subjects in both groups (**Fig. S6, Table S7**). Altogether, despite the clear differences in the systemic total IgE levels between AR and HC subjects in both urban and rural groups, there were less prominent differences in peripheral blood immune cells known to have an important role in the pathogenesis of AR. The only statistically significant difference was in the $CD163^+$ mDCs, which was upregulated in the rural AR individuals compared to HCs.

Table 1. Comparison between allergic rhinitis (AR) and healthy control (HC) subjects for urban and rural group in the subsets for mass cytometry and flow cytometry (confirmation study) measurement.

Variables	Mass cytometry subjects				Confirmation study (flow cytometry) subjects			
	Urban (N=18)		Rural (N=12)		Urban (N=13)		Rural (N=18)	
	SPT+RHI+ (AR) (n=8)	SPT+RHI+ (AR) (n=8)	SPT+RHI+ (AR) (n=6)	SPT-RHI- (HC) (n=6)	SPT+RHI+ (AR) (n=5)	SPT-RHI- (HC) (n=8)	SPT+RHI+ (AR) (n=11)	SPT-RHI- (HC) (n=7)
Age (years old)#, median (25^{th}; 75^{th} percentile)	18.1 (17.4; 18.6)	18.5 (18.1; 19.1)	18.7 (18.0; 19.1)	18.9 (18.6; 19.1)	18.5 (17.9; 19.6)	18.5 (18.1; 18.8)	18.9 (17.9; 19.3)	18.7 (17.9; 18.9)
Sex, n male (%)	4 (50.0)	5 (50.0)	5 (83.3)	3 (50.0)	2 (40.0)	3 (37.5)	9 (81.8)	2 (28.6)
BMI (kg/m2)#, median (25^{th}; 75^{th} percentile)	19.9 (18.3; 23.0)	22.6 (19.2; 24.1)	21.1 (19.4; 26.9)	19.6 (17.4; 22.2)	20.5 (18.1; 24.5)	18.9 (17.5; 23.0)	21.0 (18.8; 23.6)	19.0 (18.0; 23.6)
Activity disturbances, n positive (%)								
- No	2 (25.0)	NA	3 (50.0)	NA	2 (40.0)	NA	4 (36.4)	NA
- Mild	3 (37.5)		2 (33.3)		2 (40.0)		6 (54.5)	
- Moderate	3 (37.5)		1 (16.7)		1 (20.0)		1 (9.1)	

#non-normally distributed continuous variables, presented as median (25^{th}; 75^{th} percentile).

SD: standard deviation; BMI: body mass index; SPT+RHI+: reactive SPT result combined with the presence of rhinitis symptoms; SPT-RHI-: negative SPT results without any rhinitis symptoms; AR: allergic rhinitis; HC: healthy control; NA: not applicable.

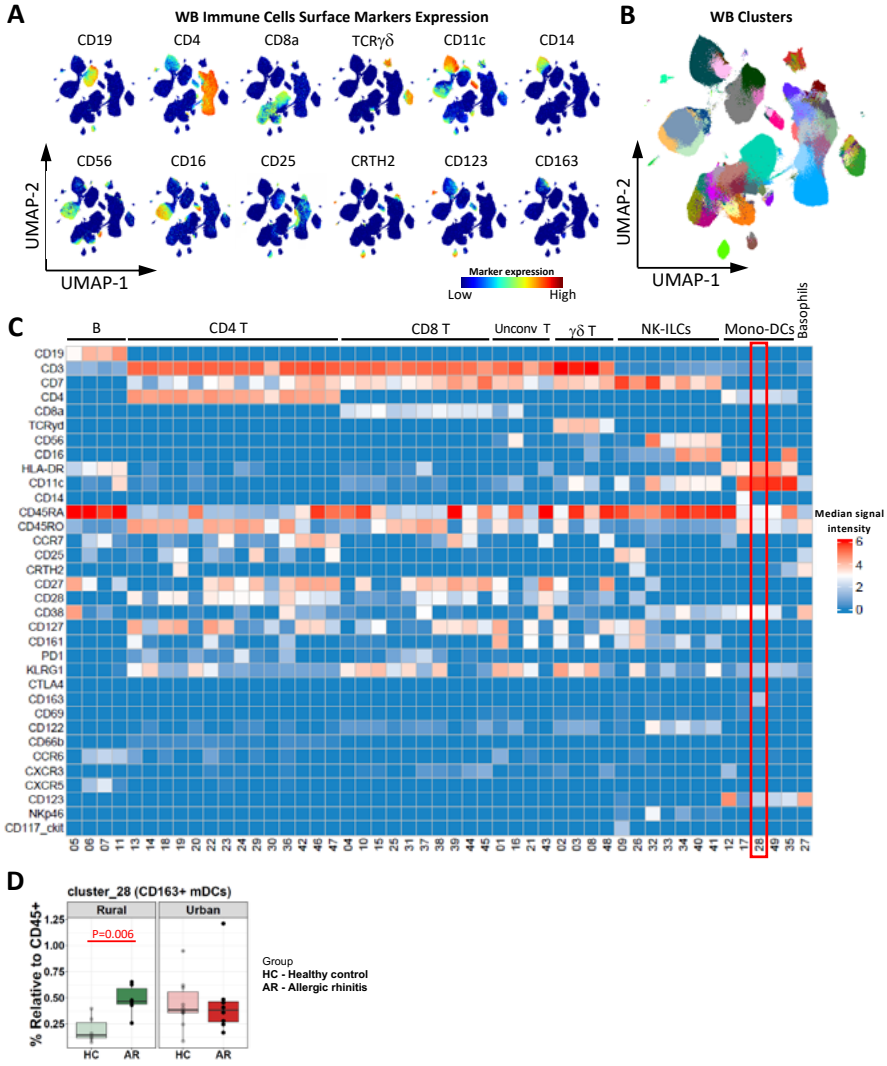

Figure 1. Higher frequency of systemic CD163+ mDCs was observed in rural allergic rhinitis compared to healthy control subjects but not in the urban group.

A. The expression of several immune cell surface markers obtained from mass-cytometry measurement, used for the clustering of whole-blood immune cell populations. **B.** Uniform Manifold Approximation and Projection (UMAP) of whole-blood immune cells clustering in mass-cytometry data based on the cell markers expression. **C**. A heatmap summary of median expression values of cell markers expressed by whole-blood immune cell clusters identified in hierarchical clustering. Red line box in the heatmap indicated the cluster with statistically significant differences (adjusted P-value <0.05) between AR vs. HC subjects. **D**. Box plots depicting the percentage of immune cell cluster (relative to CD45+ cells) significantly different between AR vs. HC subjects. To evaluate the differences between AR vs. HC subjects for each rural and urban group, generalized linear mixed model test was performed for all identified clusters with Benjamini-Hochberg correction for multiple testing to obtained the adjusted P-values.

AR: allergic rhinitis subjects; HC: healthy control subjects.

Altogether, despite the clear differences in the systemic total IgE levels between AR and HC subjects in both urban and rural groups, there were less prominent differences in peripheral blood immune cells known to have important role in the pathogenesis of AR. The only statistically significant difference was in the $CD163^+$ mDCs, which was upregulated in the rural AR individuals compared to HCs.

Nasal mucosal immune profiles of allergic rhinitis vs. healthy control subjects in urban and rural group

From 1.01 X 10^5 nasal immune cells included for unsupervised clustering and based on the expression of immune cell markers (**Fig. 2A and S7**), a total of 23 immune cell clusters were included for further statistical analysis (**Fig. 2B and 2C, Table S4**). Higher frequencies of cluster 06 (basophils), cluster 07 ($CD4^+CD25^{hi}CD127^-$), and cluster 10 (mast cells) were found in urban AR compared to HC, but not in rural subjects. A similar trend was also observed for an activated memory T cell cluster 08 ($CD4^+CD45RO^+CD38^+PD\text{-}1^{+)}$ (**Fig. 2D, Table S8**). Moreover, trends of decreased frequencies of cluster 05 ($HLA\text{-}DR^+CD11c^+CD16^+$) and the NK cluster 19 ($CD7^+CD56^+CD11c^+NKp46^+$) were seen in rural AR versus HC, but not in the urban group (**Fig. S8, Table S8**). Thus, more differences in the nasal mucosal immune cell clusters were observed in the urban than rural AR subjects when compared to the HCs.

Systemic and nasal mucosal pathogenic Th2A and $CD38^+$ Th2A in urban and rural allergic rhinitis subjects

Although CD4 Th2 cells have long been established as a major player in allergic rhinitis,[31,51] recent studies revealed a distinct subpopulation of Th2 cells, which was identified as pathogenic Th2A ($CD4^+CRTH2^+CD27^-CD161^+$), that might have a crucial role in the pathogenesis of allergic diseases [32,33]. Therefore, we manually gated this cell population within the WB mass cytometry dataset (**Fig. S3A**). We did not observe differences in the percentages of systemic CD4 Th2 cells in either urban or rural AR, in comparison to the HC subjects. However, a higher frequency of pathogenic Th2A was observed in rural AR subjects but not in the urban AR when compared with corresponding HC. In addition, the activation marker CD38 was similarly upregulated

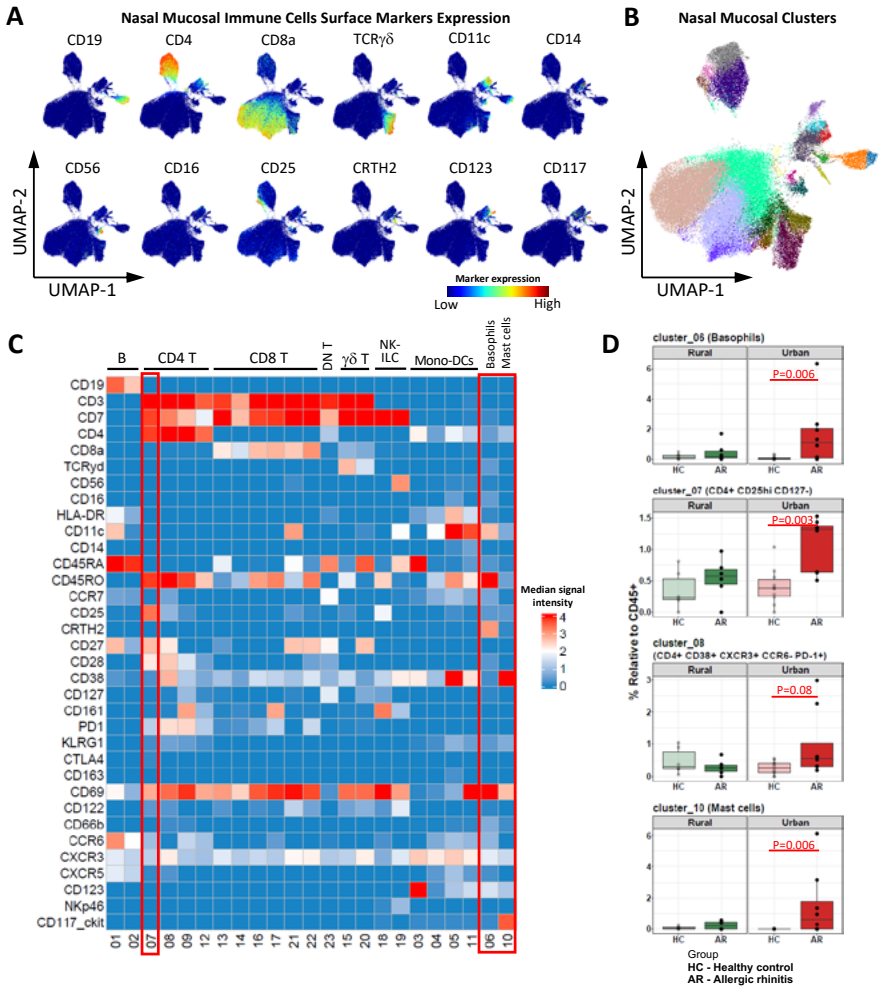

Figure 2. Higher frequency of nasal mucosal basophils, mast cells, and CD4+CD25hiCD127- were observed in urban allergic rhinitis compared to healthy control subjects but not in the rural group.

A. The expression of several immune cell surface markers obtained from mass-cytometry measurement, used for the clustering of nasal mucosal immune cell populations. **B**. Uniform Manifold Approximation and Projection (UMAP) of nasal mucosal immune cells clustering in mass-cytometry data based on the cell markers expression. **C**. A heatmap summary of median expression values of cell markers expressed by nasal mucosal immune cell clusters identified in hierarchical clustering. Red line box in the heatmap indicated the cluster with statistically significant differences (adjusted P-value <0.05) between AR vs. HC subjects. **D**. Box plots depicting the percentages of several systemic immune cell clusters (relative to $CD45^+$ cells) significantly different between AR vs. HC subjects. To evaluate the differences between AR vs. HC subjects for each rural and urban group, generalized linear mixed model test was performed for all identified clusters with Benjamini-Hochberg correction for multiple testing to obtained the adjusted P-values.
AR: allergic rhinitis subjects; HC: healthy control subjects.

on these Th2A cells in both urban and rural AR compared to their HC counterparts (**Fig. 3A**). These findings in the WB mass cytometry dataset were confirmed in a second cohort using flow cytometry (**Fig. S9A**). Furthermore, these Th2A and CD38$^+$ Th2A cells were positively correlated with the levels of activity disturbances, especially in the urban AR subjects (**Fig. S9B**).

Previous unsupervised clustering in the nasal mucosal immune cells did not identify the CD4 Th2 cells as a separate cluster. By performing manual gating, we could identify this population, as well as the pathogenic Th2A cells (**Fig. S3B**). We found elevated percentages of nasal mucosal CD4 Th2 and Th2A cells, as well as the upregulation of CD38 in the Th2A immune cells, only in the urban AR versus HC subjects, but not for rural group (**Fig. 3B**). Subsequently, positive correlations were observed for these frequencies of nasal mucosal CD4 Th2, Th2A, and CD38$^+$ Th2A cells with the levels of activity disturbances, also only in urban but not rural group (**Fig. S9C**).

Taken together, our study confirmed the role of peripheral blood pathogenic Th2A and/or CD38$^+$ Th2A in AR, but the presence of these cells in the nasal mucosa was only seen in urban AR, which might be related with the severity of AR clinical manifestation.

CCR3 expression on Th2 cells in urban and rural allergic rhinitis subjects

To explain the discrepancies in the findings between systemic and nasal mucosal compartments regarding the Th2A and CD38$^+$ Th2A, we evaluated the expression of CCR3 on these cells (**Fig. S3C**), a marker that characterizes immune cells from peripheral blood which migrate to the upper respiratory tract mucosa.[47] A higher frequency of Th2A CCR3$^+$ cells was observed in urban AR compared to HC. Moreover, upregulation of CD38$^+$ Th2A CCR3$^+$ cells was only found in the urban but not rural AR, in comparison to the HC subjects (**Fig. 4**). Thus, increased frequency of activated Th2A cells expressing homing marker (CCR3) in peripheral blood in the urban AR group, is in agreement with the increased presence of these cells in the nasal mucosa of urban individuals with AR.

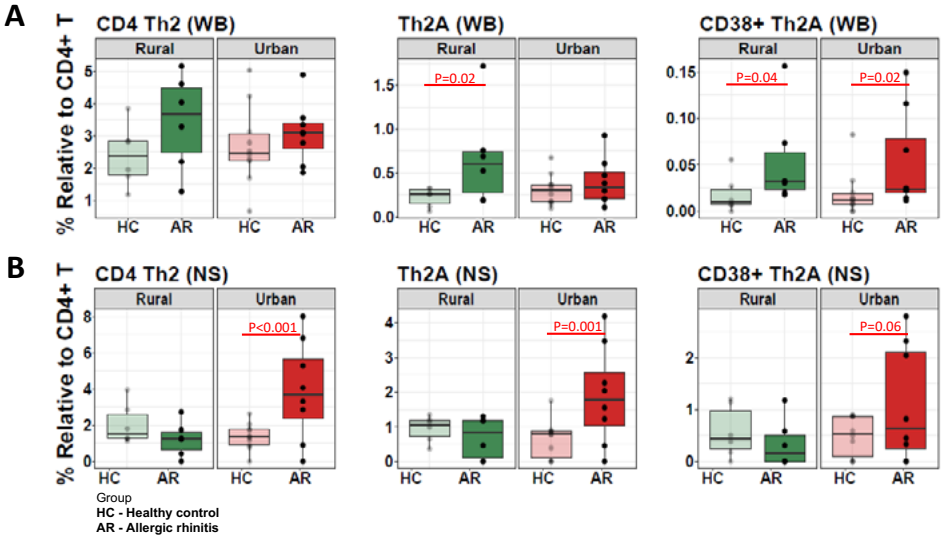

Figure 3. Discrepancies in the findings of CD4 Th2, Th2A, and CD38⁺ Th2A cell percentages between systemic and nasal mucosal compartments in urban and rural allergic rhinitis subjects compared to healthy controls.

A. Box plots depicting the differences of the percentages of systemic CD4 Th2, Th2A, and CD38⁺ Th2A cells (relative to CD4 T cells) between allergic rhinitis vs. healthy control subjects for each rural and urban group, in the whole-blood mass cytometry dataset. **B**. Similar box plots as (**A**) for the nasal mucosal mass cytometry dataset. To evaluate the differences of the percentages of the immune cell populations between allergic rhinitis vs. healthy control subjects for each rural and urban group, generalized linear mixed model test was performed.
AR: allergic rhinitis subjects; HC: healthy control subjects, WB: whole-blood, NS: nasal mucosa.

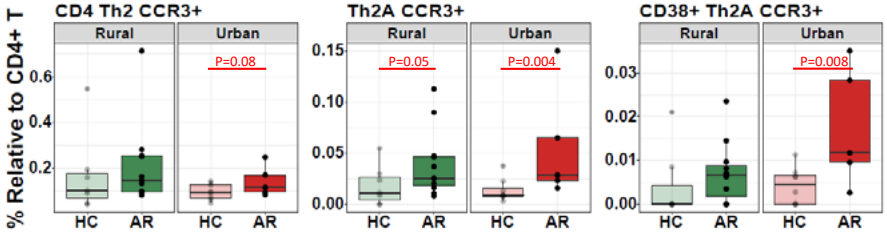

Figure 4. Higher frequency of systemic Th2A CCR3⁺ and CD38⁺ Th2A CCR3⁺ were observed in urban allergic rhinitis compared to healthy control subjects but not in the rural group.

Box plots depicting the differences of the percentages of systemic CD4 Th2 CCR3⁺, Th2A CCR3⁺ , and CD38⁺ Th2A CCR3⁺ between allergic rhinitis (AR) vs. healthy control (HC) subjects for each rural and urban group, in the confirmation study (flow-cytometry) dataset. To evaluate the differences between AR vs. HC subjects for each rural and urban group, generalized linear mixed model test was performed.
AR: allergic rhinitis subjects; HC: healthy control subjects.

DISCUSSION

Here, we performed high-dimensional immune profiling using mass-cytometry in urban and rural AR subjects evaluating both peripheral blood and nasal mucosal immune compartment. Systemic immune profiling revealed an increase of $CD163^+$ mDCs and Th2A in rural but not in urban AR subjects, while $CD38^+$ Th2A were upregulated in AR subjects from both rural and urban groups. At the same time, striking differences were observed for the nasal mucosal immune cells in the urban AR compared to HC subjects, but not for the rural group.

Although we did not observe higher numbers of CD4 Th2, ILC2, and basophils in peripheral blood of either urban or rural AR subjects when compared to their HC counterparts, the findings of elevated percentages of $CD38^+$ Th2A in both rural and urban AR confirmed a role for these cells in AR individuals.[33] Interestingly, these differences did not manifest in the nasal mucosal site for rural AR subjects. Indeed, more pronounced differences were seen in the nasal mucosal immune cells of urban AR subjects, including the upregulation of CD4 Th2, Th2A, and $CD38^+$ Th2A compared to the urban HCs, which was not observed in the rural group. Moreover, the positive correlations between the percentages of these effector cells in the nasal mucosa with the level of activity disturbances, suggest a higher disease activity in the urban AR.[52] Thus, our study findings support the importance of evaluating the immune system in AR not only in the peripheral blood, but importantly also in the nasal mucosal site as the effector organ.

The elevated $CD163^+$ mDCs in the systemic compartment of rural AR subjects might explain the lack of cellular perturbations, in particular, in the nasal mucosa of these subjects compared to the healthy controls. The $CD163^+$ subset of dendritic cells has been reported to have more anti-inflammatory and tolerogenic properties.[53-55] This systemic skewing of immune cells towards more regulatory condition in these rural AR subjects is also supported by the trends of higher numbers of Tregs and non-classical monocytes compared to HCs. Tregs have been shown to suppress Th2 cells and their activation and migration to the inflammatory sites, and induce IgG4 instead of IgE production by B cells.[56,57] In addition, non-classical monocytes could alter Th2 cells activation [58] and were upregulated after allergen immunotherapy.[59,60]

C-C chemokine receptor 3 (CCR3) has an essential role in the clinical manifestation of AR and is expressed by many effector cells associated with the pathogenesis of AR, such as eosinophils,[61] basophils,[62] mast cells,[63] and CD4 Th2.[61] Our study extended the role of CCR3 in AR, further to Th2A and CD38^{+} Th2A. The elevated numbers of the nasal mucosal CD4 Th2, Th2A, and CD38^{+} Th2A immune cell populations in the urban AR compared to HC subjects was supported by the similar findings of the CD4 Th2 CCR3$^{+,}$ Th2A CCR3$^{+,}$ and CD38^{+} Th2A CCR3^{+} percentages in the WB, suggesting more migration of these immune cells from the systemic to the effector site.

To our knowledge, this is the first study comprehensively comparing the immune profiles of both peripheral blood and nasal mucosal compartments in AR subjects from urban and rural sites, that evaluated the pathogenic Th2A cells and the migratory marker CCR3. Nevertheless, there are some limitations to this study. First, we did not include CCR3 in our mass cytometry antibody panel and the CCR3 expression was only measured in the flow cytometry dataset. However, as the clinical characteristics and the findings related to CD4 Th2, Th2A, and CD38+ Th2A were similar for both datasets, we could infer the results regarding the CCR3^{+} populations for mass cytometry dataset. The lack of functional study to confirm more regulatory states in the rural AR subjects and the antigen specificity of Th2A cells are another limitation of this study. Lastly, quantifications of cytokines both in WB and nasal mucosa would add valuable information to the differences of the immune profiles between urban and rural AR.

To summarize, we observed distinct immune profiles between Indonesian young adults with AR originating from rural and urban areas. Urban AR showed more differences in the nasal mucosal immune cells when compared to their HC counterparts, but this was not seen in the rural group. In addition, these alterations of immune cells in urban AR might be associated with more severe clinical manifestation, as reported previously [21,22]. Our study also confirmed the important role of Th2A and CD38^{+} Th2A cells in AR. Altogether, this study improves our understanding on the pathogenesis of AR and might be useful when considering clinical treatment, although further study are needed.

Conflict of Interest

All authors declare that they have no relevant conflicts of interest.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author (F.K. and M.Y.) upon reasonable request.

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SUPPLEMENTARY MATERIALS

Table S1. Antibody panel mass-cytometry for nasal-mucosal and whole-blood.

Label	Specificity	Clone	Vendor	Catalogue number	Dilution
^{89}Y	CD45	HI30	Fluidigm[a]	3089003B	1/200
^{141}Pr	CD196 (CCR6)	G034E3	Fluidigm	3141003A	1/100
^{142}Nd	CD19	HIB19	Fluidigm	3142001B	1/200
^{143}Nd	CD117 (c-Kit)	104D2	BioLegend[b]	313223	1/100
^{144}Nd	CD66b	REA306	Miltenyi[c]	130-108-019	1/50
^{145}Nd	CD4	RPA-T4	Fluidigm	3145001B	1/100
^{146}Nd	CD8a	RPA-T8	Fluidigm	3146001B	1/200
^{147}Sm	CD183 (CXCR3)	G025H7	BioLegend	353733	1/100
^{148}Nd	CD14	M5E2	BioLegend	301843	1/100
^{149}Sm	CD25 (IL-2Ra)	2A3	Fluidigm	3149010B	1/100
^{150}Nd	CD185 (CXCR5)	J252D4	BioLegend	356902	1/100
^{151}Eu	CD123	6H6	Fluidigm	3151001B	1/100
^{152}Sm	TCRγδ	11F2	Fluidigm	3152008B	1/50
^{153}Eu	CD7	CD7-6B7	Fluidigm	3153014B	1/100
^{154}Sm	CD163	GHI/61	Fluidigm	3154007B	1/100
^{155}Gd	CD69	FN50	BioLegend	313502	1/200
^{156}Gd	CD294 (CRTH2)	BM16	BioLegend	350102	1/50
^{158}Gd	CD122 (IL-2Rb)	TU27	BioLegend	339015	1/100
^{159}Tb	CD197 (CCR7)	G043H7	Fluidigm	3159003A	1/100
^{160}Gd	Epcam	51.1	BioLegend	324229	1/50
^{161}Dy	KLRG1 (MAFA)	REA261	Miltenyi	Special order	1/100
^{162}Dy	CD11c	Bu15	Fluidigm	3162005B	1/200
^{163}Dy	CD152 (CTLA-4)	BNI3	BioLegend	369602	1/100
^{164}Dy	CD161	HP-3G10	Fluidigm	3164009B	1/100
^{165}Ho	CD127 (IL-7Ra)	AO19D5	Fluidigm	3165008B	1/200
^{167}Er	CD27	O323	Fluidigm	3167002B	1/200
^{168}Er	HLA-DR	L243	BioLegend	307651	1/200
^{169}Tm	CD45RA	HI100	Fluidigm	3169008B	1/100
^{170}Er	CD3	UCHT1	Fluidigm	3170001B	1/100
^{171}Yb	CD28	CD28.2	BioLegend	302937	1/100
^{172}Yb	CD38	HIT2	Fluidigm	3172007B	1/200
^{173}Yb	CD45RO	UCHL1	BioLegend	304239	1/100
^{174}Yb	CD335 (NKp46)	92E	BioLegend	331902	1/100
^{175}Lu	CD279 (PD-1)	EH 12.2H7	Fluidigm	3175008B	1/100
^{176}Yb	CD56	NCAM16.2	Fluidigm	3176008B	1/100
^{209}BI	CD16	3G8	Fluidigm	3209002B	1/400

[a]Fluidigm, South San Francisco, CA, USA. [b]Biolegend, San Diego, CA, USA. [c]Miltenyi Biotech, Bergisch Gladbach, Germany. CCR, C-C chemokine receptor. CD, cluster of differentiation. CRTH2, prostaglandin D2 receptor 2. CXCR, CXC chemokine receptor. EpCAM, epithelial cell adhesion molecule. HLA-DR, human leukocyte antigen-D-related. IL-2R, interleukin-2 receptor. IL-7Rα, interleukin-7 receptor α. KLRG1, killer cell lectin-like receptor subfamily G member 1. MAFA, mast cell function-associated antigen. PD-1, programmed cell death protein-1. TCR, T-cell receptor. All markers were stained on the cell surface.

Table S2. Barcoding panel for mass cytometry.

Label	Specificity	Clone	Vendor	Catalogue number	Dilution
^{106}Cd	B2M	2M2	BioLegend[a]	316302	1/50
^{110}Cd	B2M	2M2	BioLegend	316302	1/50
^{111}Cd	B2M	2M2	BioLegend	316302	1/50
^{112}Cd	B2M	2M2	BioLegend	316302	1/50
^{114}Cd	B2M	2M2	BioLegend	316302	1/50
^{116}Cd	B2M	2M2	BioLegend	316302	1/50
^{198}Pt	B2M	2M2	BioLegend	316302	1/50

[a]Biolegend, San Diego, CA, USA. B2M, Beta-2 microglobulin.

Table S3. Whole-blood clusters' identification.

Clusters' number	Clusters' identification
cluster_01	DN T cells (EM) CD27+ CD28+ CD38-
cluster_02	yd T cells (RO+ CD27+)
cluster_03	yd T cells (RA+ CD27- CD161-)
cluster_04	CD8 T cells (EMRA) CD27- CD127- KLRG1+
cluster_05	Plasma cells
cluster_06	B cells memory
cluster_07	B cells naïve
cluster_08	yd T cells (RA+ CD27- CD161+)
cluster_09	ILC3
cluster_10	CD8 T cells (EMRA) CD27+ KLRG1+ CXCR3-
cluster_11	B cells CD11c+
cluster_12	pDCs
cluster_13	CD4 T cells (EM) CD27- CD28+ CD38- CD127+ CD161+ KLRG1+
cluster_14	CD4 T cells (EM) CD27- CD28- CD38- CD127+ PD1+ KLRG1+
cluster_15	CD8 T cells (EM) CD27- CD28- CD127+ KLRG1+
cluster_16	CD8 NKT cells
cluster_17	Classical monocytes
cluster_18	CD4 T cells (EM) CD25+ CD27- CD28+ CD127+
cluster_19	CD4 Th2
cluster_20	CD4 T cells (EMRA)
cluster_21	DN T cells (EM) CD27lo CD28lo CD38-
cluster_22	CD4 T cells (EM) CD27+ CD28+ CD38- CD127+ CD161+ KLRG1+
cluster_23	CD4 T cells (EM) CD28+ CD127+
cluster_24	CD4 T cells (EM) CD27+ CD28+ CD38- CD161- PD1+
cluster_25	CD8 T cells (EM) CD27- CD28- CD127- KLRG1+
cluster_26	ILC2
cluster_27	Basophils
cluster_28	CD163+ mDCs
cluster_29	CD4 T cells (EM) CD25+ CD127- (Tregs)
cluster_30	CD4 T cells (EM) CD28+ CD127+
cluster_31	CD8 T cells (EM) CD27+ PD1+ KLRG1+
cluster_32	NK cells (NKp46+)
cluster_33	NK cells (CD16-)
cluster_34	NK cells (CD16+) CD38+ CD161+ KLRG-
cluster_35	Non-classical monocytes
cluster_36	CD4 T cells (CM) CD27+ CD28+ CD38+ CD161+ PD1+
cluster_37	CD8 T cells (EM) CD27+ CD28+ CD38+ PD1+
cluster_38	CD8 T cells (EM) CD27+ CD28+ CD127+ KLRG1+
cluster_39	CD8 T cells (naïve)
cluster_40	NK cells (CD16+) CD38+ CD161- KLRG+-
cluster_41	NK cells (CD16+) CD38+ CD161+ KLRG+
cluster_42	CD4 T cells (naïve) CD25- CD27+ CD38+ CD127+
cluster_43	DN T cells (naïve) CD38+
cluster_44	CD8 T cells (CM) CD27+ CD28+ CD38- CD127+
cluster_45	CD8 T cells (EMRA) CD27+ KLRG1- CXCR3+
cluster_46	CD4 T cells (naïve) CD25- CD27+ CD127+
cluster_47	CD4 T cells (naïve) CD25+ CD27+ CD38+ CD127+
cluster_48	yd T cells (RA+ CD27+ CD161-)
cluster_49	CD163- mDCs

Table S4. Nasal mucosal clusters' identification.

Clusters' number	Clusters' identification
cluster_01	B cells memory
cluster_02	B cells naïve
cluster_03	pDCs
cluster_04	HLA-DR+ CD11c+ (mDCs)
cluster_05	HLA-DR+ CD11c+ CD16+
cluster_06	Basophils
cluster_07	CD4+ CD25+ CD127- CD69+
cluster_08	CD4 T cells RO+ CD25lo CD69+ CD38+ CXCR3+ PD1+
cluster_09	CD4 T cells RO+ CD27- CD127+ CD69hi CD161+ PD1+
cluster_10	Mast cells
cluster_11	HLA-DR+ CD11c+ CD14+ (CD14+ monocytes)
cluster_12	CD4 T cells CD69+ CD27- CD127- CD161- PD1+
cluster_13	CD8 T cells RA+ CCR7- CD69+ CD27lo CD122lo
cluster_14	CD8 T cells CD27- CD69+ CD38- CD161- PD1-
cluster_15	yd T cells (RO+ CD27-)
cluster_16	CD8 T cells RO+ CD69+ CD27- CD38+ CD161- PD1+
cluster_17	CD8 T cells RO+ CD69+ CD27- CD38+ CD161+ PD1+
cluster_18	ILCs
cluster_19	CD7+ CD56+ CD11c+ NKp46+ (NK cells)
cluster_20	yd T cells (RA+ CD27+)
cluster_21	CD8 T cells RO+ CCR7- CD69+ CD27+ CD122+
cluster_22	CD8 T cells RO+ CD69+ CD27+ CD38+ CD161- PD1+
cluster_23	DN T cells

Table S5. Antibody panel flow-cytometry (confirmation study) for Th2A analysis in whole-blood.

Marker	Fluorochrome	Vendor	Catalogue number	Dilution
Live/dead				
CD3	BV510	Biolegend[a]	317332	1/200
CD4	SB550	Biolegend	344656	1/100
CD8	PE	In Vitro[b]	12-0088-42	1/200
CD161	pe-cy5	BD Biosciences[c]	551138	1/100
CD294 (CRTH2)	BV711	Biolegend	350124	1/50
CD27	FITC	BD Biosciences	555440	1/100
CD197 (CCR7)	pe-cy7	BD Biosciences	557648	1/200
CD38	apc-fire810	Biolegend	303550	1/100
CCR3	AF647	Biolegend	310709	1/100

[a]Biolegend, San Diego, CA, USA. [b]In Vitro Technologies, Mt Wellington, Auckland, New Zealand. [c]BD Biosciences Franklin Lakes, NJ, USA. CCR, C-C chemokine receptor. CD, cluster of differentiation. CRTH2, prostaglandin D2 receptor 2.

Table S6. Comparison between allergic rhinitis (AR) and healthy control (HC) subjects in whole study population urban and rural group.

Variables	Urban (N=88)		Rural (N=79)	
	SPT+RHI+ (AR) (n=36)	SPT-RHI- (HC) (n=52)	SPT+RHI+ (AR) (n=32)	SPT-RHI- (HC) (n=47)
Age (years old), mean (SD)	18.50 (0.70)	18.54 (0.70)	18.96 (0.95)	18.79 (0.60)
Sex, n male (%)	21 (58.3)	18 (34,6)*	21 (65.6)	16 (34.0)*
BMI (kg/m2), mean (SD)	22.08 (4.78)	21.71 (4.16)	21.98 (4.23)	20.47 (3.09)
Total IgE (IU/mL)#, median (25^{th}; 75^{th} percentile)	450 (283; 914)	54 (20; 119)*	364 (180; 715)	67 (33; 212)*
Eosinophil counts# (%), median (25^{th}; 75^{th} percentile)	3.0 (2.0; 5.0)	1.0 (0.5; 3.0)*	3.0 (1.8; 6.0)	2.5 (1.0; 4.0)
Activity disturbances, n positive (%)				
- No	8 (22.2)	NA	10 (31.3)	NA
- Mild	16 (44.4)		16 (50.0)	
- Moderate	12 (33.3)		5 (15.6)	
- Severe	0 (0)		1 (3.1)	

#non-normally distributed continuous variables, presented as median (25^{th}; 75^{th} percentile).

*statistically significant differences (P<0.05) between allergic rhinitis vs healthy control subjects for each urban and rural group.

SD: standard deviation; BMI: body mass index; SPT: skin prick test; SPT+RHI+: reactive SPT result combined with the presence of rhinitis symptoms; SPT-RHI-: negative SPT results without any rhinitis symptoms; AR: allergic rhinitis; HC: healthy control;

NA: not applicable.

Table S7. Statistical analysis for the comparison of whole-blood immune cell clusters between allergic rhinitis vs. healthy control subjects for each rural and urban group.

	Rural				Urban			
Clusters	**Intercept**	**Group_AR**	**p_group_AR**	**fdr_group_AR**	**Intercept**	**Group_AR**	**p_group_AR**	**fdr_group_AR**
cluster_01	-3,55767	-0,54261	0,148998	0,892306	-3,43947	-0,12626	0,586195	0,921133
cluster_02	-3,80563	0,061606	0,860665	0,983213	-3,44024	0,029917	0,902846	0,991261
cluster_03	-6,13988	0,947396	0,207174	0,892306	-4,57296	-0,60109	0,225303	0,710842
cluster_04	-3,34224	0,090367	0,821248	0,983213	-3,80633	0,482496	0,163887	0,710842
cluster_05	-6,00724	0,38784	0,386994	0,892306	-5,66692	-0,48564	0,346505	0,77176
cluster_06	-3,56128	-0,04103	0,86454	0,983213	-3,5361	-0,16292	0,471889	0,856392
cluster_07	-2,3516	-0,09744	0,70642	0,983213	-2,53146	-0,37527	0,025432	0,587003
cluster_08	-6,24144	0,289572	0,747326	0,983213	-5,74545	0,042371	0,943747	0,991261
cluster_09	-8,2815	0,179265	0,636861	0,983213	-7,801	-0,04422	0,871145	0,991261
cluster_10	-4,44398	-0,05421	0,882885	0,983213	-4,88498	0,259467	0,332654	0,77176
cluster_11	-4,83964	0,121549	0,508626	0,892306	-4,5138	-0,36429	0,090472	0,710842
cluster_12	-6,2091	0,194338	0,407058	0,892306	-5,9509	-0,00411	0,985367	0,991261
cluster_13	-4,49637	0,261459	0,473786	0,892306	-4,57402	-0,11206	0,541563	0,915054
cluster_14	-5,71435	0,529654	0,254048	0,892306	-5,43439	0,128427	0,820664	0,991261
cluster_15	-5,52616	0,06298	0,846384	0,983213	-5,5878	-0,16041	0,613914	0,921133
cluster_16	-5,5162	-0,69191	0,474596	0,892306	-6,47317	0,972854	0,169029	0,710842
cluster_17	-2,16845	0,054704	0,881916	0,983213	-2,01932	-0,16676	0,470223	0,856392
cluster_18	-4,52343	0,293236	0,156177	0,892306	-4,3603	0,054272	0,723326	0,957918
cluster_19	-5,42008	0,286091	0,359188	0,892306	-5,5394	0,460345	0,038857	0,587003
cluster_20	-7,22466	0,385508	0,715607	0,983213	-7,10484	0,331644	0,579428	0,921133
cluster_21	-4,78606	-0,42164	0,180375	0,892306	-4,76118	0,050428	0,785978	0,991261
cluster_22	-4,07875	0,299905	0,410331	0,892306	-4,10484	0,236415	0,146589	0,710842
cluster_23	-2,85693	0,121889	0,448565	0,892306	-2,81173	0,147479	0,232112	0,710842
cluster_24	-5,69943	0,239748	0,40576	0,892306	-5,64789	0,162749	0,331791	0,77176
cluster_25	-4,02079	0,00181	0,99597	0,99597	-4,5851	0,333127	0,375414	0,799796
cluster_26	-7,33508	0,566889	0,072137	0,88368	-7,13338	0,110324	0,630962	0,921133
cluster_27	-5,52127	0,474089	0,507684	0,892306	-5,21812	0,467054	0,188352	0,710842
cluster_28	-6,3888	1,020754	0,000136	0,006673	-5,56576	0,003016	0,991261	0,991261
cluster_29	-4,93722	0,289195	0,040764	0,665805	-4,68805	-0,0079	0,960711	0,991261
cluster_30	-5,11902	0,004029	0,985162	0,99597	-5,37382	0,307747	0,18961	0,710842
cluster_31	-4,36921	0,100603	0,714037	0,983213	-4,78184	0,606083	0,012563	0,587003
cluster_32	-5,62688	-0,22977	0,464158	0,892306	-5,67664	0,205092	0,20368	0,710842
cluster_33	-4,49609	0,092423	0,859997	0,983213	-4,74745	0,753349	0,047919	0,587003
cluster_34	-3,37854	-0,0306	0,909154	0,989968	-3,25156	-0,24557	0,328906	0,77176
cluster_35	-5,25582	0,647368	0,011728	0,287339	-4,72946	-0,16021	0,639154	0,921133
cluster_36	-5,73263	0,265968	0,311791	0,892306	-5,61862	0,07161	0,715287	0,957918
cluster_37	-5,37863	-0,3204	0,408213	0,892306	-5,73337	-0,15682	0,284609	0,77176
cluster_38	-5,25219	-0,0324	0,942546	0,99597	-5,18497	0,179423	0,218342	0,710842
cluster_39	-1,92976	-0,33882	0,17775	0,892306	-2,02111	-0,03786	0,838414	0,991261
cluster_40	-4,485	0,681045	0,367426	0,892306	-3,73559	-0,75799	0,162517	0,710842
cluster_41	-3,91048	0,095051	0,860189	0,983213	-3,35189	-0,07444	0,860331	0,991261
cluster_42	-4,20841	-0,09524	0,672113	0,983213	-4,16447	0,152812	0,409384	0,835826
cluster_43	-6,265	-0,03651	0,872472	0,983213	-6,2363	-0,01983	0,927223	0,991261
cluster_44	-4,7826	-0,0511	0,831218	0,983213	-4,43467	-0,11875	0,513301	0,898276
cluster_45	-5,99488	0,308045	0,221806	0,892306	-5,77229	0,056988	0,860456	0,991261
cluster_46	-2,04208	-0,34386	0,189401	0,892306	-2,4052	0,291022	0,063359	0,620914
cluster_47	-4,92859	0,008166	0,980784	0,99597	-4,83024	0,144953	0,341395	0,77176
cluster_48	-5,8641	0,241216	0,509889	0,892306	-5,60893	-0,19927	0,438414	0,856392
cluster_49	-5,51903	0,509481	0,171193	0,892306	-5,31237	0,100064	0,67469	0,944566

Generalized linear mixed model analyses were performed to compare the differences between AR vs HC subjects for each rural and urban group with adjusted p-values were obtained using fdr-correction based on Benjamini-Hochberg method to correct for multiple testing hypothesis. Cells highlighted in yellow: p-values <0.05 and cells highlighted in red: adjusted p-values <0,05. AR: allergic rhinitis; HC: healthy control; fdr: false discovery rate.

Table S8. Statistical analysis for the comparison of nasal mucosal immune cell clusters between allergic rhinitis vs. healthy control subjects for each rural and urban group.

	Rural				Urban			
Clusters	**Intercept**	**Group_AR**	**p_group_AR**	**fdr_group_AR**	**Intercept**	**Group_AR**	**p_group_AR**	**fdr_group_AR**
cluster_01	-5,3928	-0,50912	0,460365	0,663235	-5,11841	-0,30311	0,512115	0,867185
cluster_02	-3,59012	-0,45238	0,212557	0,618828	-4,01548	-0,28037	0,557409	0,867185
cluster_03	-6,25172	0,189693	0,576007	0,779303	-6,3758	0,520506	0,464525	0,867185
cluster_04	-3,33407	-0,52538	0,109615	0,618828	-3,39083	0,169373	0,713547	0,867185
cluster_05	-4,51813	-0,94199	0,009783	0,225005	-5,76668	0,117491	0,82238	0,873545
cluster_06	-7,34547	1,302471	0,136119	0,618828	-8,03599	2,892854	0,000882	0,006762
cluster_07	-5,66	0,424663	0,252465	0,618828	-5,73141	1,062497	0,000125	0,002884
cluster_08	-5,62415	-0,24126	0,628838	0,78375	-6,00514	0,983091	0,014363	0,082586
cluster_09	-2,94355	0,049845	0,81989	0,852554	-3,1563	0,142224	0,650318	0,867185
cluster_10	-7,74556	1,001738	0,319463	0,618828	-10,4792	4,706901	0,000851	0,006762
cluster_11	-5,58745	-0,69142	0,348952	0,618828	-6,38334	-0,42852	0,644717	0,867185
cluster_12	-2,90447	-0,34099	0,358128	0,618828	-2,93333	0,059714	0,754074	0,867185
cluster_13	-3,40142	0,266795	0,333751	0,618828	-3,11594	-0,2234	0,501584	0,867185
cluster_14	-1,41181	0,211995	0,10161	0,618828	-1,32219	0,059984	0,740542	0,867185
cluster_15	-3,3992	0,240711	0,681522	0,78375	-3,70424	-0,1355	0,748552	0,867185
cluster_16	-1,78845	0,321794	0,269493	0,618828	-1,87336	0,213849	0,40279	0,867185
cluster_17	-1,01411	-0,24341	0,461381	0,663235	-1,16387	-0,02899	0,929617	0,929617
cluster_18	-6,31481	0,1601	0,760529	0,83296	-6,47394	0,810459	0,046696	0,2148
cluster_19	-4,72208	-0,62348	0,047777	0,549439	-5,23858	0,044894	0,835565	0,873545
cluster_20	-3,74794	-0,20034	0,376678	0,618828	-4,25026	-0,18253	0,752166	0,867185
cluster_21	-5,86452	0,091562	0,852554	0,852554	-6,1574	0,208773	0,711679	0,867185
cluster_22	-4,32784	0,143384	0,675566	0,78375	-4,44161	0,454245	0,124943	0,47895
cluster_23	-6,07245	-1,24415	0,302388	0,618828	-7,65866	0,714969	0,156411	0,513921

Generalized linear mixed model analyses were performed to compare the differences between AR vs HC subjects for each rural and urban group with adjusted p-values were obtained using fdr-correction based on Benjamini-Hochberg method to correct for multiple testing hypothesis. Cells highlighted in yellow: p-values <0.05 and cells highlighted in red: adjusted p-values <0,05. AR: allergic rhinitis; HC: healthy control; fdr: false discovery rate.

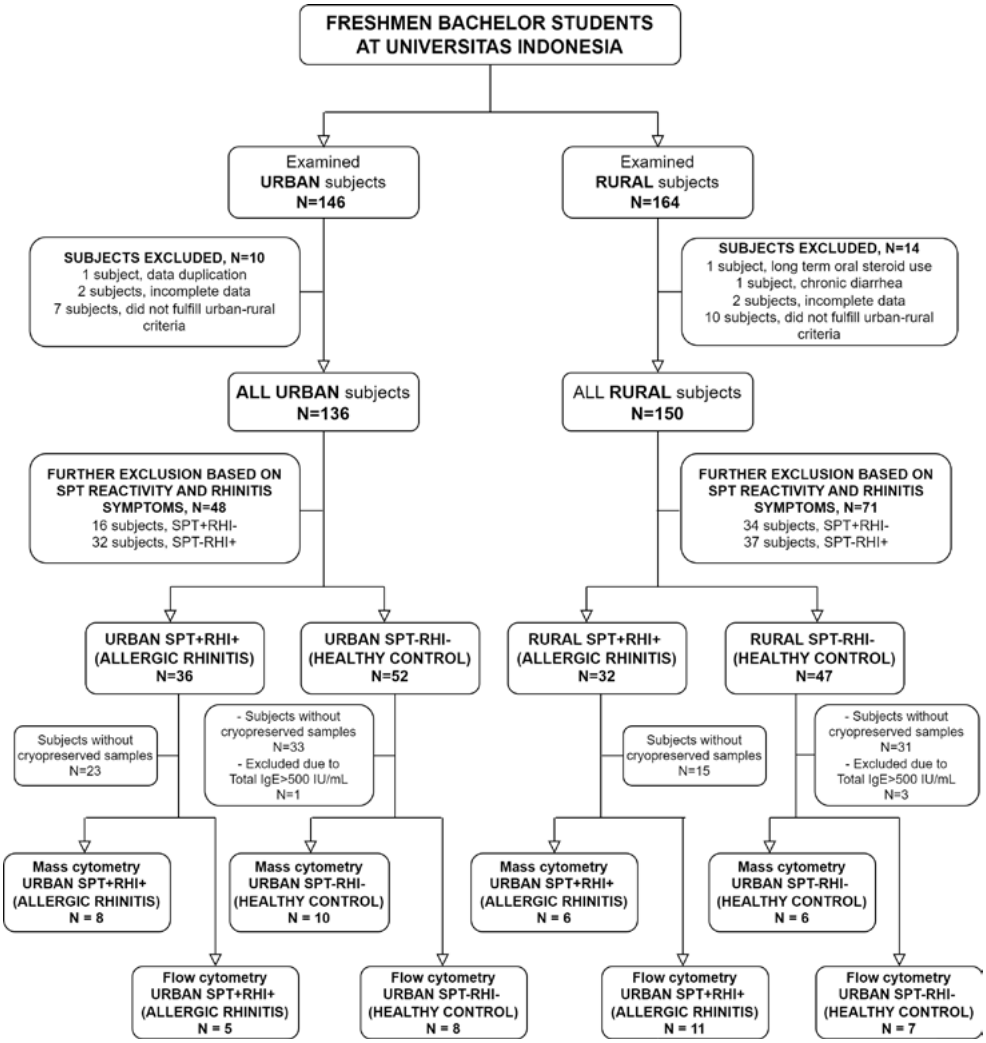

Figure S1. Flow chart for the inclusion of study participants.

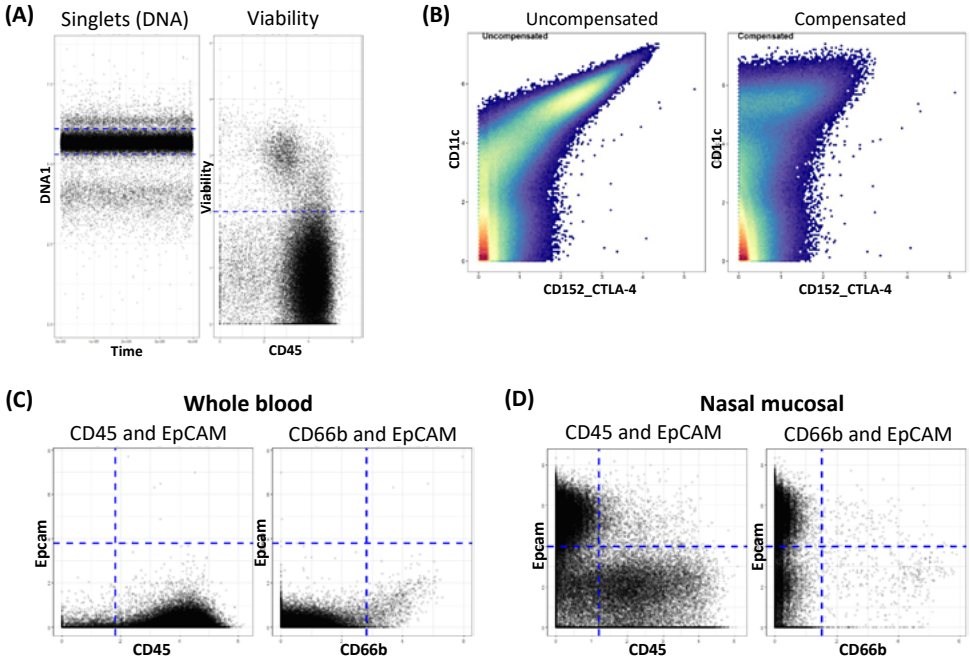

Figure S2. Pre-processing of mass cytometry data.
(A) Manual gating of the single and viable cells based on DNA dye (left) and viability dye (right). (B) Compensation of markers. Here, we showed the example of compensation between CD11c and CTLA4 markers before (left) and after (right) compensation. (C) Manual gating to obtain the two cell populations from whole blood samples, granulocytes ($EpCAM^-CD66b^+$) and other immune cells ($EpCAM^-CD45^+CD66b^-$). (D) Manual gating to obtain the three cell populations from nasal mucosal samples, epithelial cells ($EpCAM^+CD45^-$), granulocytes ($EpCAM^-CD66b^+$) and other immune cells ($EpCAM^-CD45^+CD66b^-$).

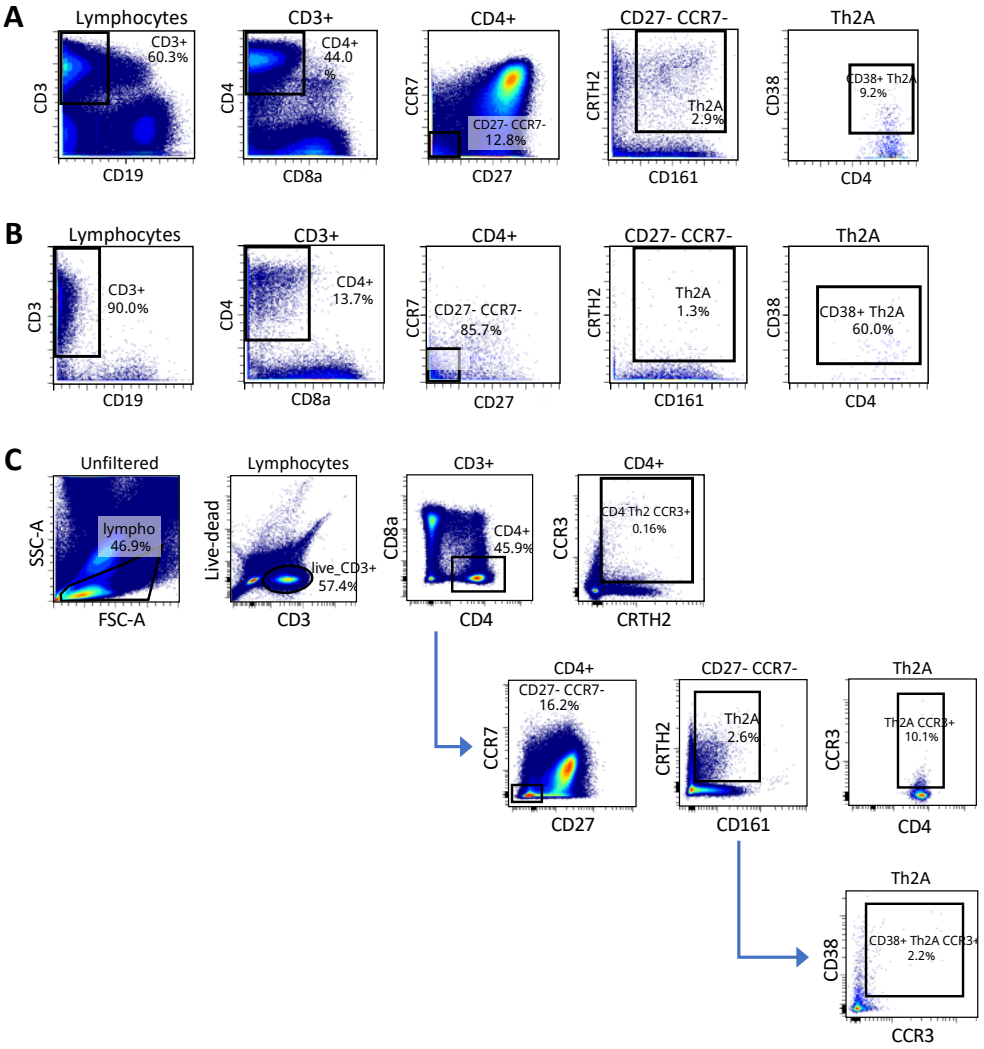

Figure S3. Gating strategy.

A. For the identification of Th2A and CD38^{+} Th2A immune cell populations from whole blood mass cytometry data. **B**. For the identification of Th2A and CD38^{+} Th2A immune cell populations from nasal mucosal mass cytometry data. **C**. For the identification of CD4 Th2 CCR3^{+}, Th2A, Th2A CCR3^{+}, CD38^{+} Th2A, CD38^{+} Th2A CCR3^{+} from whole blood flow cytometry data.

Pathogenic Th2A immune cells population was identified as CD4^{+}CD27^{-}CCR7^{-}CRTH2^{+}CD161^{+}.

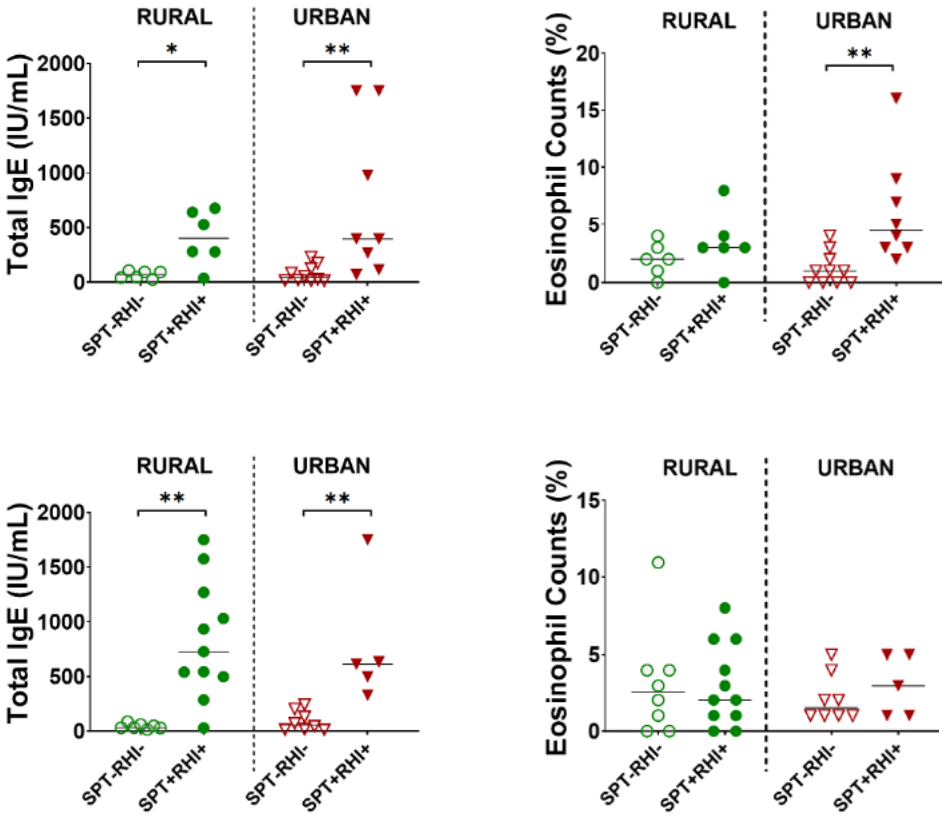

Figure S4. Comparison of total IgE levels and eosinophil counts between allergic rhinitis (SPT+RHI+) and healthy control (SPT-RHI-) subjects for urban and rural group in mass cytometry (A) and flow-cytometry (B) cohorts.

All parameters were presented as its median values and individual values for each subject. For the comparison between allergic rhinitis vs healthy control subjects, Mann-Whitney test was performed. SPT+RHI+: reactive SPT result combined with the presence of rhinitis symptoms, allergic rhinitis subjects; SPT-RHI-: negative SPT result without any rhinitis symptoms, healthy control subjects;
*P<0.05; **P<0.01

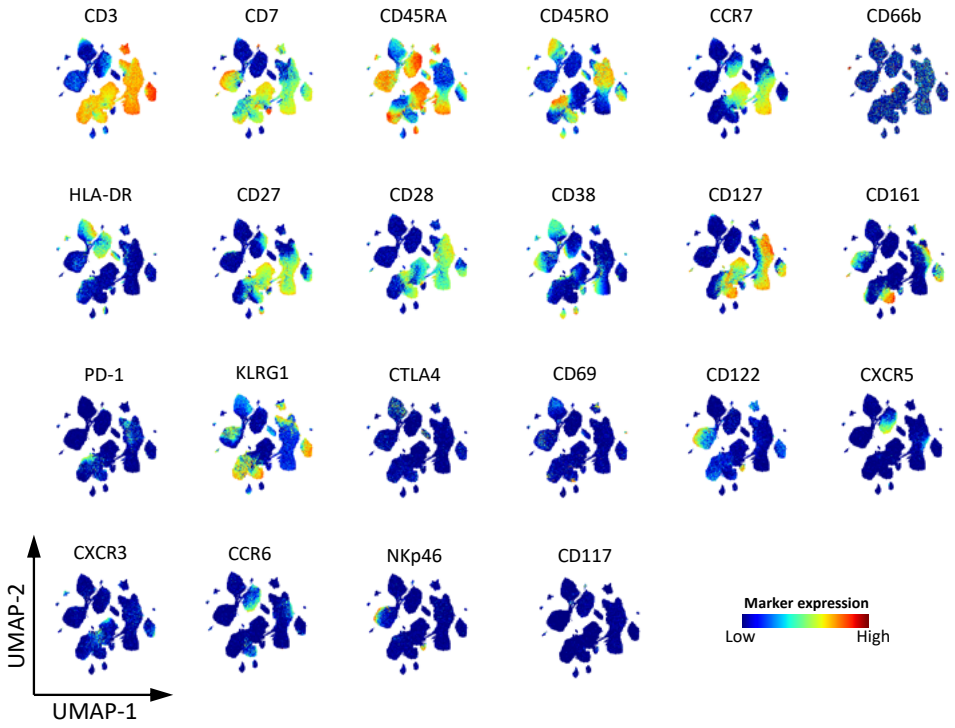

Figure S5. The expression of several immune cell surface markers obtained from mass-cytometry measurement, used for the clustering of whole-blood immune cell populations.

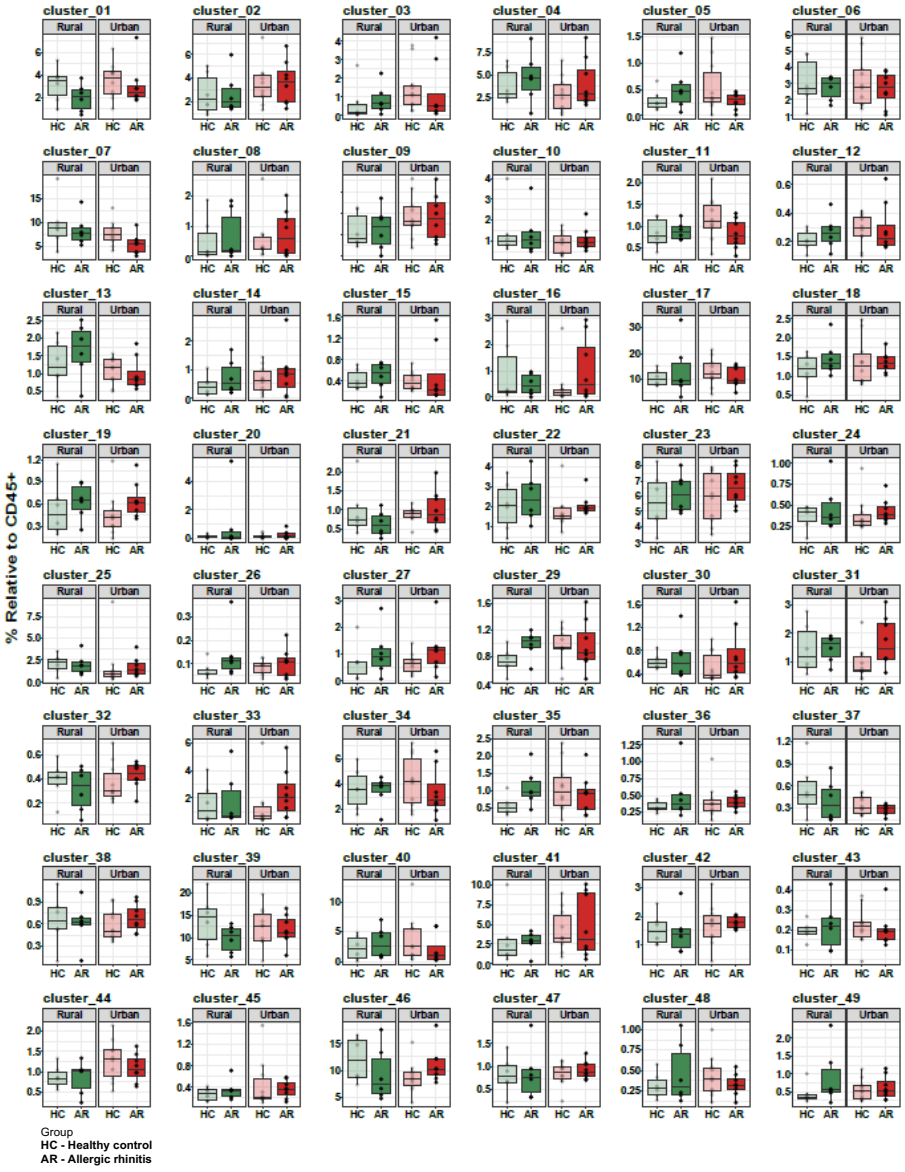

Figure S6. Comparison of the percentages (relative to CD45⁺ cells) of the remaining non-statistically significant whole-blood immune cell clusters between allergic rhinitis (AR) vs. healthy control (HC) subjects for each rural and urban group.

Individuals and boxplots with median percentage are depicted. Generalized linear mixed model test was performed with Benjamini-Hochberg correction for multiple testing hypothesis to obtain the adjusted P-values.

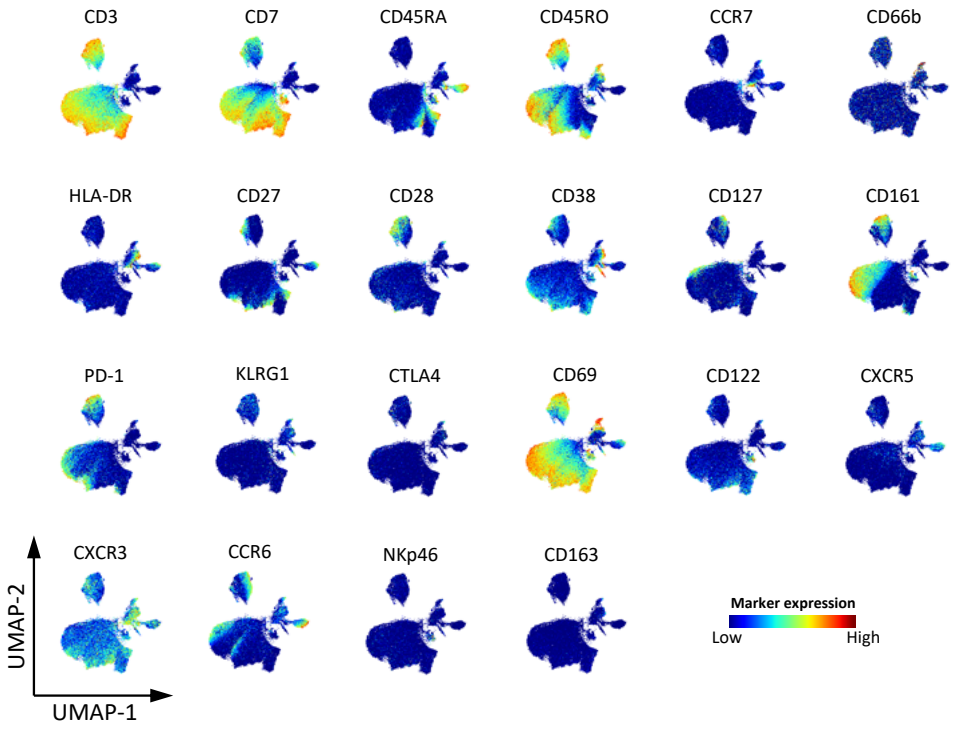

Figure S7. The expression of several immune cell surface markers obtained from mass-cytometry measurement, used for the clustering of nasal mucosal immune cell populations.

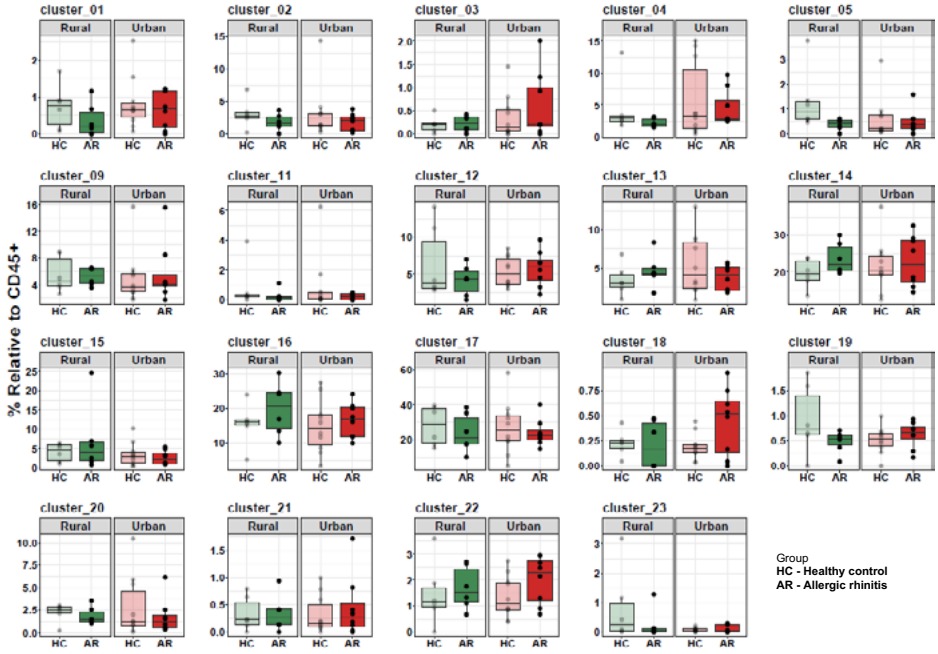

Figure S8. Comparison of the percentages (relative to CD45⁺ cells) of the remaining non-statistically significant nasal mucosal immune cell clusters between allergic rhinitis (AR) vs. healthy control (HC) subjects for each rural and urban group.

Individuals and boxplots with median percentage are depicted. Generalized linear mixed model test was performed with Benjamini-Hochberg correction for multiple testing hypothesis to obtain the adjusted P-values.

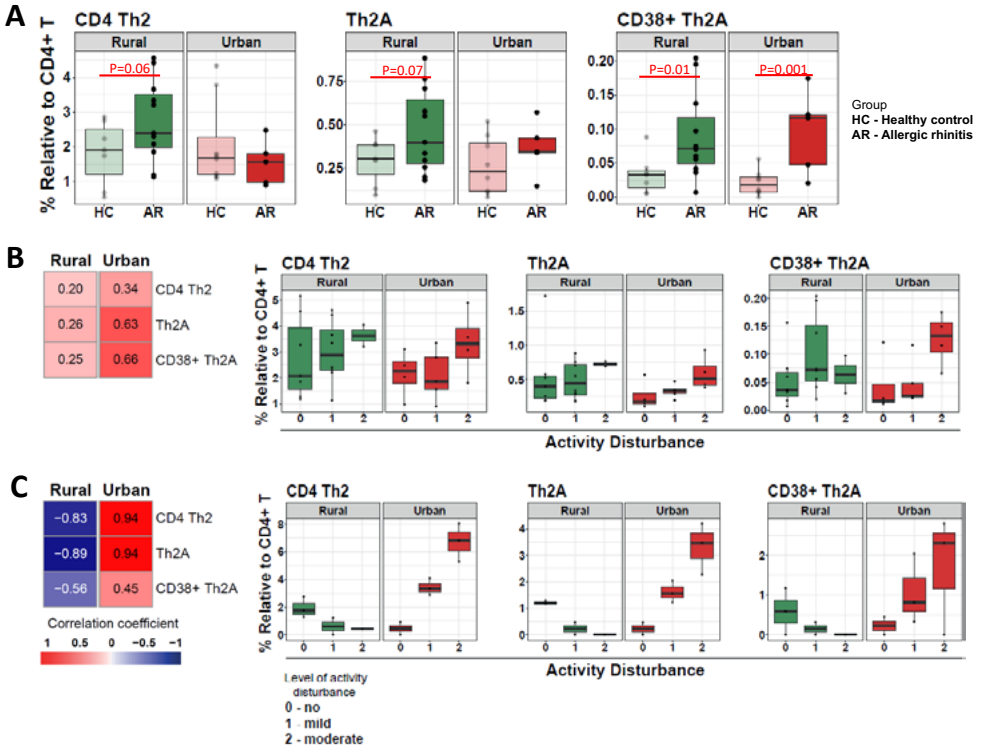

Figure S9. A. The percentages of systemic CD4 Th2, Th2A, and CD38⁺ Th2A cells (as relative to CD4⁺T cells) between allergic rhinitis (AR) vs. healthy control (HC) subjects for each rural and urban group, in the flow cytometry cohort. B. Correlation between the levels of activity disturbances with the percentages (relative to CD4⁺ T cells) of CD4 Th2, Th2A, and CD38+ Th2A in allergic rhinitis (AR) subjects for each rural and urban group in the combined whole blood mass and flow cytometry cohort, shown as correlation heatmap and individuals boxplots with median percentage. C. Correlation between the levels of activity disturbances with the percentages (relative to CD4⁺ T cells) of CD4 Th2, Th2A, and CD38⁺ Th2A in allergic rhinitis (AR) subjects for each rural and urban group in the nasal mucosal mass cytometry cohort, shown as correlation heatmap and individuals boxplots with median percentage.

Generalized linear mixed model test was performed for evaluating the differences between AR vs HC subjects in each urban and rural group for figure A. Spearman rank correlation test was performed to obtain the correlation coefficients in the heatmaps for figure B and C