

Comprehensive Profiling of Peripheral Immune Cells and Subsets in Patients with Intermittent Allergic Rhinitis Compared to Healthy Controls and After Treatment with Glucocorticoids

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Abstract—Intermittent allergic rhinitis (IAR) is a common allergic disease, which is associated with local infiltration of T cells, eosinophils, and basophils. However, changes of circulating inflammatory cells may reflect local and systemic allergic inflammation and potentially, also the response to treatment with glucocorticoids (GCs). In this study, we comprehensively profiled peripheral blood immune cells and subsets from 12 patients with IAR during the birch pollen season before and after GC treatment and nine healthy controls by flow cytometry. Orthogonal partial least squares discriminant analysis (OPLS-DA) identified that peripheral immune cells and subsets markedly separated symptomatic patients and controls. Eosinophils, basophils, and Th2 cells contributed most to the separation. However, there was no good separation between patients before and after GC treatment. Local allergic inflammation in the nasal mucosa is associated with increased circulating Th2 cells, eosinophils, and basophils. Local GC treatment has limited effects on circulating immune cells.

KEY WORDS: glucocorticoids; orthogonal partial least squares discriminant analysis; peripheral blood; intermittent allergic rhinitis.

INTRODUCTION

Clinically, intermittent allergic rhinitis (IAR) is mainly characterized by local signs and symptoms, which

is associated with local infiltration of T cells, eosinophils, and basophils. However, several patients with IAR also have asthma and eczema as well as general symptoms such as fatigue. Extensive clinical and molecular studies have shown that the local nasal inflammation induces systemic inflammatory signals, which activate the bone marrow [1, 2]. Pioneering work by Denburg *et al.* demonstrated that local allergen challenge led to the release of progenitor cells for both basophils and eosinophils from the bone marrow [3]. This suggests that changes of circulating inflammatory cells may reflect local allergic inflammation and potentially, also the response to treatment with local glucocorticoids (GCs). Despite this, there is little data on such changes. Increased peripheral eosinophils have been shown in patients with IAR during the pollen season, compared to before season, while CD161+ T cells were identified to be increased in patients with IAR compared to healthy controls [4, 5]. However, surprisingly, a study of asthmatic patients showed that oral GC treatment had no effect on the expression of regulatory CD4+ T cells [6].

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It is also of note that IAR depends on altered interactions between multiple cells other than eosinophils and basophils [7]. According to the Th2 cell paradigm, antigen-presenting cells present allergen to CD4+ T cells. In allergic patients, this leads to Th2 cell differentiation and the release of Th2 cytokines, which activate eosinophils, B cells, and mast cells. This paradigm has been expanded to include several other T helper cell subsets [7–10]. CD8+ T cells may also play a role [11]. In this study, we first aimed to detect differences in peripheral immune cells and subsets between patients with IAR and controls using flow cytometric analysis followed by multivariate analysis, namely, orthogonal partial least squares discriminant analysis (OPLS-DA). Second, we aimed to determine if peripheral immune cells differed between patients with IAR before and after local treatment with GCs.

MATERIALS AND METHODS

Ethics Statement

The study was approved by the Ethics Committee of the Medical Faculty of the Linköping University. Written consents and questionnaire data sheets were obtained from all subjects and a written consent was also obtained from the parents of one patient who was a minor. The written consent was obtained on a special form with information about the study and the conditions of the study, according to instructions from the ethics committee (this form was also approved by the ethics committee, which considers this of great importance for their approval).

Subjects

Twelve patients with IAR and nine healthy controls were included in the study. All subjects were seen during the birch pollen season and all patients were symptomatic. The median (range) age of patients was 24.5 (17–54) and five were women, while the median (range) age of healthy controls was 25 (20–29) and four were women. IAR was defined based upon a positive history and a positive ImmunoCAP Rapid (Pharmacia Diagnostics AB, Uppsala, Sweden) to birch pollen, but no history of perennial rhinitis. Patients with any other diseases, including tumor, diabetes, infectious diseases, and pregnancy were excluded. All patients were investigated by an Ear–Nose–Throat (ENT) specialist to exclude severe deviation of nasal septum or nasal

polyps. The controls were all symptom-free, had no history of seasonal or perennial rhinitis or any other atopic disease, and had negative tests of IgE. None of the patients with IAR and the controls had received any local or systemic treatment with GCs or antihistamines within 2 months immediately before samples were taken [12].

Grass-, birch-, house dust mite-, horse-, cat-, and dog-IgE were measured by ImmunoCAP Rapid (Pharmacia Diagnostics AB, Uppsala, Sweden). Exhaled nitric oxide (ENO) was measured using NIOX MINO (Aerocrine AB, Solna, Sweden). ENO and peak expiratory flow (PEF) were used to search for signs of asthma [13, 14]. All patients were asked to mark their symptoms on a visual analog scale (VAS) of 0–10 [15] before and after 14 days of treatment with two doses of 50 µg per dose of fluticasone nasal spray in each nostril once daily. To check for compliance, the fluticasone bottles were weighed before and after the start of treatment. The median (range) of decreased bottle weights were 5.58 (3.78–7.59) g.

Flow Cytometry

Blood samples were collected from patients before GC treatment and controls. Red blood cells were excluded by a red blood lysis kit from Miltenyi Biotec (Bergisch Gladbach, Germany). Prior to staining, cells were blocked with human IgG. Different immune cells were analyzed according to established surface markers (Table 1). Matched isotype controls were used in all flow cytometry analyses. The flow cytometry was done using FACSaria flow cytometer (BD Biosciences, San Diego, CA, USA) and the data was analyzed by FlowJo 7.6 (TreeStar Inc., San Carlos, CA, USA).

Mouse anti-human CD4-FITC, rat anti-human CD294-PE (CRTH2), mouse anti-human CD45RO-PerCP/Cy7, mouse anti-CD123-APC, mouse anti-HLA-DR-PE, mouse anti-human CD8-Pacific Blue™, mouse anti-human CD3-FITC, and all matched isotype controls were purchased from BD Pharmingen (San Diego, CA, USA). Mouse anti-human CD56-PE, mouse anti-human CD11c-PerCP/Cy7, mouse anti-human CD3-FITC, mouse anti-human CD14-FITC, mouse anti-human CD16-FITC, mouse anti-human CD19-FITC, mouse anti-human CD56-FITC, mouse anti-human CD27-PE, mouse anti-human CD19-PerCP/Dy5, mouse anti-human CD20-APC, and all matched isotype controls were purchased from ImmunoTools GmbH (Friesoythe, Germany). Mouse anti-human CD16-APC, mouse anti-

Table 1. Phenotypes of Immune Cells from Human Peripheral Blood

Cell types	Phenotypes
Eosinophil	CD16+SSChigh
Neutrophil	CD16–SSChigh
Monocyte	FSChighSSClowCD14+
Lymphocyte	SSClowCD3+
Basophil	CD123+HLADR–
NK cell	CD3–CD56+
pDC	CD3–CD14–CD16–CD19–CD56–CD123+HLADR+CD11c–
mDC	CD3–CD14–CD16–CD19–CD56–CD123–HLADR+CD11c+
CD4+ T cell	CD3+CD4+SSClow
CD4+ TCM	CD4+CCR7+CD45RA–CD45RO+
CD4+ TEM	CD4+CCR7–CD45RA–CD45RO+
Th1	CD4+CXCR3+CCR6–CD161–
Th2	CD4+CXCR3–CRTH2+
Th17	CD4+CXCR3–CCR6+CD161+
Treg	CD4+CD127lowCD25+
CD8+ T cell	CD3+CD8+SSClow
CD8+ TCM	CD8+CCR7+CD45RA–CD45RO+
CD8+ TEM	CD8+CCR7–CD45RA–CD45RO+
B cell	CD19+CD20+
Naive B cell	CD19+CD20+CD27–
Memory B cell	CD19+CD20+CD27+

TCM T central memory cell, *TEM* T effector memory cell, *mDC* myeloid dendritic cells, *pDC* plasmacytoid dendritic cells

human CD14-PE/Cy7, mouse anti-human CD197-PE (CCR7), mouse anti-human CD4-Pacific Blue™, mouse anti-human CD45RA-APC, mouse anti-human CD161-FITC, mouse anti-human CD183-PerCP/Cy5.5 (CXCR3), mouse anti-human CD196-PE/Cy7 (CCR6), mouse anti-human CD127-APC (IL-7R α), mouse anti-human CD25-APC/Cy7, and all matched isotype controls were purchased from BioLegend (San Diego, CA, USA).

Data Analysis

OPLS-DA was performed in SIMCA-P+12.0.1 software (Umetrics, Umeå, Sweden) to analyze differences in peripheral blood immune cell expression between either symptomatic patients and healthy controls, or patients before and after GC treatment [16]. Prior to OPLS-DA modeling, flow cytometric data were preprocessed with log transformation and Pareto (par) scaling. OPLS-DA is a supervised multiple regression analysis for classification in which systematic variation in the *X* block (flow cytometric data) is divided into two model parts, plus the residual noise: the first part that models the *X* variation correlated to *Y* variable (subjects) and is referred to as the predictive component and the other

part that comprises the *X* variation that is uncorrelated to the discriminant *Y* variable and is referred to as the orthogonal component [17, 18], as judged by a leave out data cross validation (all data are left out once in a seven leave out series). The cross validated, i.e., jack knifed, loadings were used to select variables that best contributed to the discrimination between two groups [18].

For analysis of the differences of selected variables that best correlated to the discrimination, the Mann–Whitney *U* test and the Wilcoxon signed-rank test were used for unpaired and paired samples, respectively. *P* values were corrected for multiple hypotheses testing using Benjamini–Hochberg false discovery rate (FDR) correction. Data were expressed as mean $\pm$ SEM. A *P* value less than 0.05 was considered significant.

RESULTS

Characteristics of Subjects

In this study, we included 12 patients with IAR and nine healthy controls. Their characteristics are described in Table 2. All patients had a VAS of >5 before GC treatment, while all controls had normal VAS. In the patients, GCs led to a significant decrease of VAS from 8.22 ± 0.33 to 3.58 ± 0.70 ($P < 0.0001$). ENO was significantly higher in patients than in controls, 66.50 ± 15.72 compared to 22.44 ± 3.17 , and this value decreased to 48.75 ± 5.31 after treatment with GCs (both $P < 0.01$). No significant difference in PEF was found (Fig. 1).

Expression of Peripheral Blood Immune Subsets is Correlated to the Discrimination Between Patients with IAR and Controls

In order to examine if differences in peripheral blood immune cells and subsets correlated to the discrimination between patients with IAR and healthy controls or between patients with IAR before and after GC treatment, we analyzed a combination of immune cells and subsets in peripheral blood from patients with IAR before and after GC treatment and healthy controls. The flow cytometry-based profiling analysis included the major known peripheral immune cells consisting of eosinophils, neutrophils, monocytes, lymphocytes, NK cells, myeloid dendritic

Table 2. Characteristics of Subjects

ID	Age	Gender	VAS	IgE					
				Birch	Grass	Dog	Horse	Cat	Mite
HC1	25	M	0	–	–	–	–	–	–
HC2	27	M	0	–	–	–	–	–	–
HC3	29	M	0	–	–	–	–	–	–
HC4	21	F	0	–	–	–	–	–	–
HC5	25	M	0	–	–	–	–	–	–
HC6	21	M	0	–	–	–	–	–	–
HC7	20	F	0	–	–	–	–	–	–
HC8	23	F	0	–	–	–	–	–	–
HC9	27	F	0	–	–	–	–	–	–
P1	30	M	7.2	+	+	+	+	+	–
P2	26	F	8.2	+	–	+	+	+	–
P3	23	M	8.1	+	+	–	–	–	–
P4	24	M	9.4	+	+	+	+	+	+
P5	52	F	6.6	+	–	–	–	–	–
P6	20	F	8.3	+	–	+	+	+	+
P7	25	M	8.3	+	–	–	–	–	–
P8	24	M	9.3	+	–	+	–	–	–
P9	19	M	9.8	+	+	+	–	+	–
P10	54	M	9.6	+	–	–	–	–	–
P11	38	F	6.8	+	+	–	–	–	–
P12	17	F	7	+	+	+	–	–	–

HC healthy control, P patients with IAR, VAS visual analog scale

cells (mDC), plasmacytoid dendritic cells (pDC), and basophils (Table 1). We also analyzed CD4⁺ T cells, CD8⁺ T cells, and B cells and corresponding naive and memory subsets (Table 1). In addition, primary Th1, Th2, Th17, and Treg cells were also examined [19–22]. This led to the inclusion of 25 variables for multivariate modeling analysis.

Firstly, we searched for differences in peripheral blood immune subsets between symptomatic patients and healthy controls using OPLS-DA modeling. Such an approach identifies correlation patterns that discriminate groups and estimates the relative importance of each included immune subset expression value for the discrimination [17, 23, 24]. OPLS-DA modeling with flow cytometric data from symptomatic patients and healthy controls provided a model with one predictive component and one orthogonal component. The predictive variation between flow cytometric data and the discriminator variable used 55.3 % of the flow cytometric data (according to R^2X) with an explained discrimination value of 89.9 % (according to R^2Y). The total predicted discrimination variation was 53.7 % (according to Q^2). The model was indicated to be significant according to the SIMCA software's default significance test (cross-validation and eigen vector size).

This indicated that symptomatic patients with IAR and healthy controls were discriminated by the expression of peripheral blood immune cells and subsets (Fig. 2a). We extracted the top five variables (20 % of input variables) that best correlated to the discrimination between symptomatic patients and healthy controls using OPLS-DA predictive loadings plot with significant confidence intervals (Fig. 2b). This led to the identification of a list of immune subsets that best correlated to the discrimination between symptomatic patients and controls including eosinophils, basophils, Th2 cells, CD4⁺, and CD8⁺ T central memory cells (Fig. 2b). Mann–Whitney U test of these variables showed that eosinophils (adjusted $P < 0.05$), basophils (adjusted $P < 0.05$), and Th2 cells (adjusted $P < 0.05$) markedly increased in symptomatic patients, while CD4⁺ and CD8⁺ T central memory cells decreased (adjusted $P < 0.05$), compared to healthy controls (Fig. 3).

Expression of Peripheral Blood Immune Subsets Does Not Discriminate Patients with IAR Before and After GC Treatment

Next, we aimed to examine if differences in peripheral blood immune cells and subsets separated

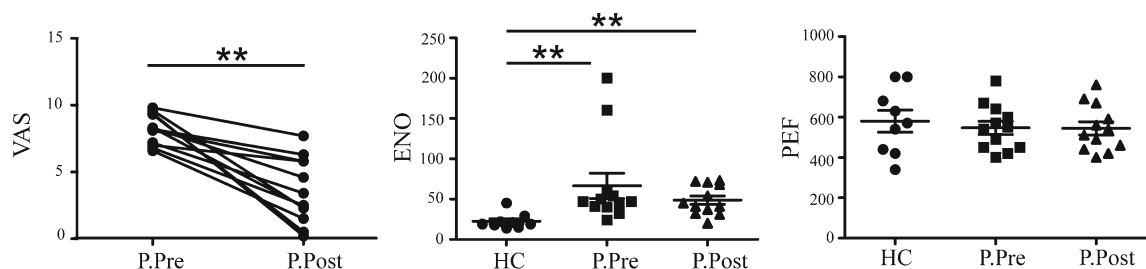


Fig. 1. Analysis of clinical symptoms and signs in patients with IAR and healthy controls. *Pre* patients before GC treatment, *post* patients after GC treatment, *HC* healthy controls, *VAS* visual analog scale, *ENO* exhaled nitric oxide, *PEF* peak expiratory flow. ** $P < 0.01$.

patients before and after GC treatment. We repeated the OPLS-DA modeling using the flow cytometric data from patients with IAR before and after GC treatment as *X* variables. This resulted in one model with one predictive component. Flow cytometric data of 17.2 % (according to R^2X) was used to explain 41.9 % discrimination variation (according to R^2Y) and the predicted discrimination variation was 1.4 % (according to Q^2). Although the model was indicated to be significant, it lacks reliability according to the predicted discrimination value ($Q^2 = 1.4$ %) (Fig. 4). This indicates that GC treatment has a limited effect on the expression of peripheral blood immune cells and subsets. This was supported by a Wilcoxon signed-rank test of the 25 variables from patients

with IAR before and after GC treatment, which only identified NK cells ($P < 0.05$), CD4+ TCM and CD8+ TEM cells ($P < 0.05$), Th1 cells ($P < 0.05$), and memory and naive B cells ($P < 0.05$) that changed following GC treatment without FDR control. However, with FDR control, none of them significantly changed following GC treatment (Fig. 5).

DISCUSSION

In the present study, we directly investigated potential differences of peripheral immune cells and subsets between patients with IAR and controls as well

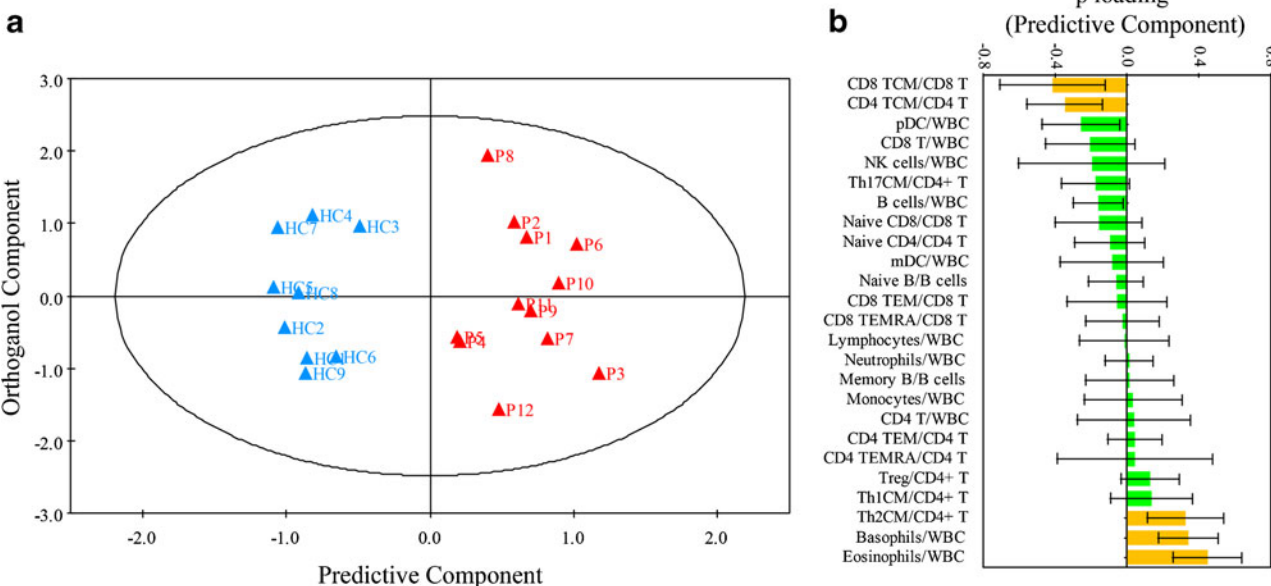


Fig. 2. OPLS-DA modeling of the peripheral immune subsets from patients with IAR and healthy controls. OPLS-DA modeling of the flow cytometric data of peripheral blood immune cells was performed to analyze the discrimination between symptomatic patients with IAR (*red*) and healthy controls (*blue*). **a** A score scatter plot of the OPLS-DA model showed the between (predictive component) and within-group (orthogonal component) separation. **b** A *p* loading of the predictive component was used to select top 5 variables (*yellow*) that contributed to the separation between symptomatic patients with IAR and healthy controls. *P* patients with IAR before GC treatment, *HC* healthy controls.

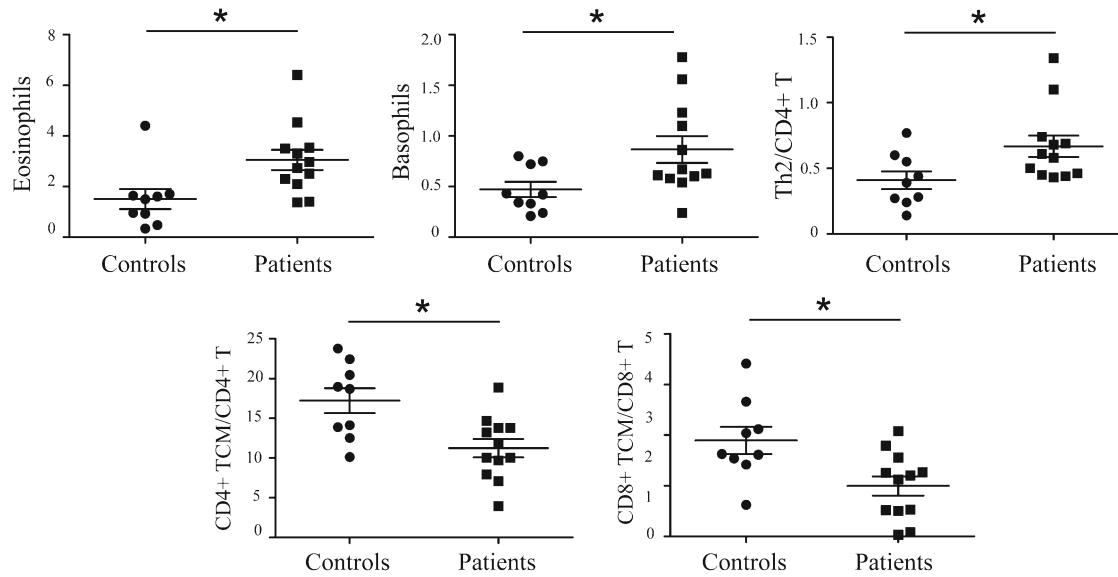


Fig. 3. Differences of the top immune subsets correlated to the discrimination between patients with IAR and healthy controls. *TCM* T central memory cells. * adjusted $P < 0.05$.

as between patients with IAR before and after GC treatment. We found that peripheral blood immune cells and subsets markedly separated symptomatic patients with IAR and healthy controls. Circulating eosinophils, basophils, and Th2 cells contributed most to the separation of patients and controls and were markedly

higher in patients. However, despite highly significant decreases of symptom scores, local GC treatment did not have significant effects on peripheral blood immune cells. Thus, while our findings agree with IAR being a systemic disease, we did not find that local GC treatment had systemic effects on immune cells.

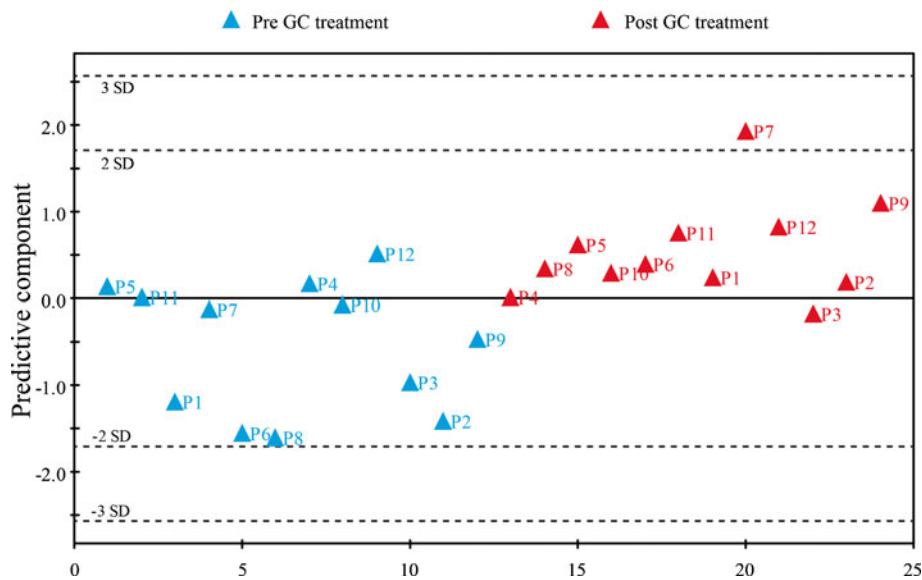


Fig. 4. OPLS-DA modeling of the peripheral immune subsets from patients with IAR before and after GC treatment. OPLS-DA modeling of the flow cytometric data of peripheral blood immune cells was performed to analyze the discrimination between symptomatic patients before and after GC treatment. A score scatter plot of the OPLS-DA model showed a mixed separation between groups (predictive component). Patients with IAR before and after GC treatment are shown in blue and red, respectively.

The eosinophil is one of the key effector cells, which accumulates in nasal mucosa in patients with IAR and contributes to enhanced membrane permeability and tissue damage [7, 25]. In agreement with a previous study, we found increased eosinophils in peripheral blood in patients compared to controls [4]. Increased eosinophils in patients with IAR may be explained by elevated expression of inflammatory cytokines such as IL-5, which is one of key regulatory cytokines of eosinophils [26–28]. This indicates that enhanced concentrations of eosinophils in peripheral blood from patients with IAR may contribute to the influx of eosinophils to the local nasal mucosa in patients with IAR during the pollen season. Basophils also accumulate in the nasal mucosa in patients with IAR and play a critical role in the pathogenesis of allergic disorders by producing inflammatory mediators as well as various cytokines and chemokines [29–31]. An early source of IL-4 derived from basophils is critical for the differentiation of Th2 cells in patients with IAR in response to inhaled allergen challenge [25, 32]. We also found increased Th2 cells in peripheral blood from patients with IAR. This finding is in agreement with the paradigm that Th2 cells attract and activate eosinophils as well as basophils [33, 34]. Similar to eosinophils and basophils, a local increase of IL-4-producing T cells have been demonstrated in the nasal mucosa from patients with IAR [35]. Taken together, these findings support that local nasal cellular reactions in IAR are

mirrored by similar changes in the blood. Interestingly, we found decreased CD4+ T and CD8+ T central memory cells in patients with IAR. The values for CD4+ TCM cells in healthy individuals in our study are similar to an independent study by Nausch *et al.* [36], which confirms the accuracy of our methodology. A possible explanation for reduced T memory cells in patients with IAR is that during active inflammation, these cells migrate from the blood to the nasal mucosa. Further studies of larger materials, including analyses of the corresponding subsets in the nasal mucosa are warranted to elucidate this finding. It is of interest that in a study of autoimmune diseases, with a Th1-like profile, increased circulating CD8+ T cells were observed [37]. By contrast, in asthmatic children, decreased number of CD8+ T cells were found [38]. The role of CD8+ T cells in allergy has not been clarified, but decreased levels could point to a counter-regulatory role.

It is well known that GCs are one of the most effective drugs in the treatment of allergy and other inflammatory diseases [39, 40]. This can be explained at the cellular level because GCs have wide-ranging inhibitory effects on a variety of inflammatory cells as well as because GCs induce Treg cells, which play a critical anti-inflammatory role [41–44]. For instance, topical GC treatment decreases the number of dendritic cells and nasal T cells in allergic rhinitis [45–47]. It has also been shown to inhibit class switching to IgE in

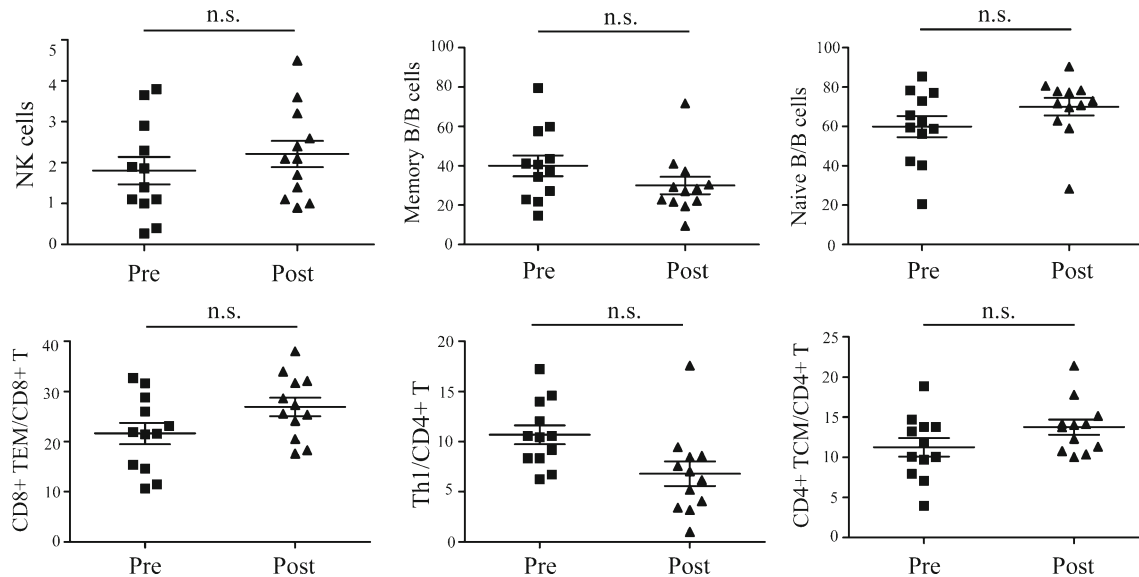


Fig. 5. Differences of selected immune cells between patients with IAR before and after GC treatment. *Pre* patients before GC treatment, *post* patients after GC treatment, *TCM* T central memory cells, *TEM* T effect memory cells, *n.s.* not significant.

nasal B cells but have no effect on the number of nasal B cells [48]. Topical GC treatment decreases not only the numbers of Th2 cells and the production of Th2 cytokines including IL-4, IL-5, and IL-13 but also Th1 cells and their key cytokines IFN- γ and IL-12 [49–51]. In this analysis, we found that GC treatment had no statistical significance on peripheral blood immune cells and subsets. This indicates that GC treatment has limited effects on circulating immune cells, compared to its wide-ranging effects on local immune cells.

In conclusion, we comprehensively profiled circulating immune cells and subsets in IAR. Our findings are compatible with the Th2 paradigm, in that we found increased number of circulating Th2 cells, eosinophils, and basophils, while local GC treatment had limited effects.

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