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Investigating Immune Gene Signatures in Peripheral Blood from Subjects with Allergic Rhinitis Undergoing Nasal Allergen Challenge

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Nasal allergen challenge (NAC) is a human model of allergic rhinitis (AR) that delivers standardized allergens locally to the nasal mucosa allowing clinical symptoms and biospecimens such as peripheral blood to be collected. Although many studies have focused on local inflammatory sites, peripheral blood, an important mediator and a component of the systemic immune response, has not been well studied in the setting of AR. We sought to investigate immune gene signatures in peripheral blood collected after NAC under the setting of AR. Clinical symptoms and peripheral blood samples from AR subjects were collected during NAC. Fuzzy c-means clustering method was used to identify immune gene expression patterns in blood over time points (before NAC and 1, 2, and 6 h after NAC). We identified and validated seven clusters of differentially expressed immune genes after NAC onset. Clusters 2, 3, and 4 were associated with neutrophil and lymphocyte frequencies and neutrophil/lymphocyte ratio after the allergen challenge. The patterns of the clusters and immune cell frequencies were associated with the clinical symptoms of the AR subjects and were significantly different from healthy nonallergic subjects who had also undergone NAC. Our approach identified dynamic signatures of immune gene expression in blood as a systemic immune response associated with clinical symptoms after NAC. The immune gene signatures may allow cross-sectional investigation of the pathophysiology of AR and may also be useful as a potential objective measurement for diagnosis and treatment of AR combined with the NAC model. *The Journal of Immunology*, 2017, 199: 000–000.

Allergic rhinitis (AR) is the most prevalent allergic disease worldwide, affecting up to 40% of the global population. Prevalence has increased progressively over the last three decades in industrialized societies, with 30% of the population in Europe and the United States affected (1–4). AR is a symptomatic disorder of the nose induced after allergen exposure by an IgE-mediated inflammation of the nasal mucosa, driven by Th2 cells (1, 4). AR is characterized by early phase response (EPR) and, in some (3–75%) patients (5), a subsequent late phase response (LPR). LPR, although including sneezing, congestion, and rhinorrhea symptoms similar to EPR, is predominantly characterized by nasal congestion (6, 7).

Many studies have focused on the local inflammatory site. However, investigations into the pathophysiological systemic

immune responses of AR have been scant and not well elucidated (8). Preliminary data evaluating changes in epigenetic signatures after acute allergen challenge suggest that systemic mechanisms are indeed engaged (9). AR is associated with allergic dermatitis (eczema) and allergic asthma (10, 11), meaning that not only is this an epithelial organ-specific allergic response of the skin, lung, and nose, but it is also a systemic response. For example, complications of bone marrow transplantation (BMT), which are mainly graft-versus-host disease and immunodeficiency, provide an example of the role of the immune system in allergic conditions. After BMT from an allergic donor, nonallergic recipients have been reported to develop allergies. By contrast, a recipient's allergic dermatitis was resolved after a BMT from a nonallergic donor (11–13).

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The online version of this article contains supplemental material.

Abbreviations used in this article: AR, allergic rhinitis; BAU, bioequivalent allergen unit; BMT, bone marrow transplantation; CBC, complete blood count; EPR, early phase response; FCM, Fuzzy c-means; FDR, Benjamini–Hochberg false discovery rate; LME, linear mixed effect model; LPR, late phase response; M cohort, McMaster University study subjects; NAC, nasal allergen challenge; NLR, neutrophil/lymphocyte ratio; PNIF, peak nasal inspiratory flow; Q cohort, Queen's University study subjects; TNSS, total nasal symptom score.

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A systemic immune response investigational approach may provide a new cross-sectional view in understanding the pathophysiology of AR. As such, we designed an evaluation of the peripheral blood of AR patients undergoing a nasal allergen challenge (NAC; also known as nasal allergen provocation) in the Allergic Rhinitis–Clinical Investigator Collaborative project, part of the Allergy, Genes and the Environment Networks for Centres of Excellence, which has developed a standard NAC protocol (14). An NAC model is a human model of AR, in some ways similar to an environmental exposure unit/environmental challenge chamber. The NAC model, which delivers standardized allergens directly and locally to the nasal mucosa allowing for individual titration of the allergen, is more suitable for collection of clinical symptom scores, such as peak nasal inspiratory flow (PNIF) and total nasal symptom score (TNSS), and of biospecimens, such as peripheral blood, to investigate dynamic immune responses after allergen challenge (14–19).

If systemic immune response patterns in peripheral blood in an AR model such as NAC can be identified, they may be useful to help understand the pathophysiology of AR and to measure the effects of AR treatment. In this study, we examined the hypothesis that peripheral blood, collected after NAC, may provide distinct patterns of immune gene expression associated with the pathophysiology of AR.

Materials and Methods

Study approval

The Queen's University study (NCT01383590) and McMaster University study (NCT01383603) were registered on ClinicalTrials.gov. The Queen's University study (study code: DMED-1423-11) and healthy control study (DMED-1343-10) were granted ethical clearance by the Queen's University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board at Queen's University; the McMaster University study (Research Project 11-3551) was cleared by the Hamilton Integrated Research Ethics Board at McMaster University.

Subjects

This study was performed in 18 AR subjects (9 Queen's University study subjects [Q cohort] and 9 McMaster University study subjects [M cohort]) and 5 healthy nonallergic subjects who underwent NAC.

In AR subjects, inclusion criteria included a minimum 1-y documented history of AR on exposure to cats and positive skin prick test to cat allergen with a wheal diameter at least 3 mm larger than that produced by the negative control.

In healthy nonallergic subjects, inclusion criteria included a history of nonallergy to any aeroallergens and negative skin prick test to all of the common aeroallergens tested.

Participants were excluded if they had a diagnosis of asthma; a history of anaphylaxis to cat allergen; an $FEV_1 < 80\%$ of predicted; an FEV_1/FVC ratio < 0.7 ; vital signs (blood pressure, pulse rate, respiratory rate, and body temperature) that were outside normal limits; significant history of alcohol or drug abuse; any history of vasovagal reaction in response to needles or blood donation; a history of any significant disease or disorder; or subject was a smoker or quit smoking < 3 mo before screening date.

Nasal allergen challenge

The Q and the M cohorts used the similar NAC protocol except for the decision process of allergen dose administered at NAC visit. The NAC method is described in our previous article (14). NAC was implemented at screening visit and NAC visit. NAC visit followed screening visit by at least 6 d to allow a sufficient washout period. The allergen administered at both study sites was a standardized cat allergen extract (10,000 bioequivalent allergen unit/ml; ALK-Abello Pharmaceuticals, Hørsholm, Denmark) with the same DIN (02235299) and lot number (ID0142). At the screening visit an initial nasal wash with 5 ml of 0.9% saline was used to identify and exclude participants with nonspecific nasal hyper-responsiveness before allergen challenge. Participants were asked to record their baseline PNIF using the In-Check inspiratory flow measurement device (Clement Clarke International, Essex, U.K.) and TNSS. After the nasal wash, 100 μ l of serially diluted allergen (from 4.9 to 5000

BAU/ml, the interval was a 4-fold dilution factor between dilutions; time interval, 15 min) was sprayed into each nostril using the Aptar Bidose device (Aptar Pharma, Congers, NY) starting with the lowest concentration. The qualifying concentration was the concentration that met the criteria first (Table I). At NAC visit, the allergen challenge was once in the morning (before 10 AM). The allergen dose administered was differently decided between cohorts: a cumulative dose (from the lowest to the qualifying concentration dose) in the Q cohort, and the qualifying concentration dose (100 μ l of the qualifying concentration) in the M cohort (Table I).

Healthy nonallergic subjects underwent a similar NAC protocol except for the specific allergen: two times diluted stock birch allergen (39,000 protein nitrogen units/ml; ALK-Abello Pharmaceuticals), which was the maximum allergen dose used in the NAC model.

Symptom diary cards for TNSS

Participants recorded their nasal symptoms on diary cards (14): four symptoms categories (runny nose, nasal congestion, sneezing, and nasal itching). Each symptom was scored from zero to three (0: absence, 1: mild, 2: moderate and bothersome, 3: severe and intolerable). The card was designed for automatic scanning and reading using Optical Mark Recognition to allow automated data entry into the system.

Complete blood cells measurement with four-part differential blood cells counting

Complete blood count (CBC) with differential at NAC visit was generated using automated hematology analyzers (Q cohort and healthy nonallergic subjects: Sysmex XE-2100TM; M cohort: Sysmex XN-3000TM; Sysmex, Kobe, Japan).

NanoString assay

PAXgene blood RNA tubes collected at NAC visit were stored in -80°C freezers. The lysates were extracted from 5 ml of each sample by PAXgene Blood miRNA kit (PreanalytiX, Hombrechtikon, Switzerland). The lysates were profiled using NanoString methodology based on the manufacturer's protocol. In brief, 4 μ l of each lysate was applied to the NanoString nCounter PanCancer Immune Profiling Panel (NanoString, Seattle, WA) to measure differential mRNA expression of 770 genes (730 immune genes and 40 housekeeping genes). The lysate was mixed with 50 nt-sized Reporter probes and Capture probes of the target genes for hybridization at 65°C for 18 h. The hybridized samples were processed using the nCounter Prep Station using the High Sensitivity protocol and followed by quantitative detection by nCounter Digital Analyzer using the maximum resolution (MAX FOV). Raw data of NanoString assay was normalized by nSolver Analysis software (v2.6) using positive control and housekeeping genes.

Missing data

At NAC visit, clinical symptom scores from all subjects were collected before NAC (baseline, 0 h), and at 15 min, 30 min, and every hour from 1 to 12 h after NAC. Whole peripheral blood from AR subjects was collected in EDTA blood tubes and PAXgene blood RNA tubes at four time points: before NAC (baseline, 0 h), and 1, 2, and 6 h after NAC (Fig. 1B). Equivalent blood specimens were collected from healthy nonallergic subjects at two time points (before NAC and 1 h after NAC); we were unable to obtain the additional blood samples at 2 and 6 h after NAC due to these healthy subjects being part of a different study. However, 1 h after NAC blood sample represented a convenient comparator for some of our gene signature analyses.

Clinical symptom and blood data from AR subjects were partially missing because of either failed collecting or no procedure for collecting: in clinical symptom scores, the M cohort (one subject 0–12 h after NAC in PNIF, one subject 0–6 h after NAC in PNIF, one subject 7–12 h after NAC in PNIF, two subjects 7–12 h after NAC in TNSS); in CBC data, the Q cohort (one subject 2 and 6 h after NAC) and the M cohort (one subject 0, 1, 2, and 6 h after NAC); and in gene expression data, the Q cohort (one subject 2 and 6 h after NAC) and the M cohort (one subject 2 h after NAC).

Statistical analysis

Statistical analyses were performed using the R statistical computing program (Packages: nlme [version 3.1-128], GeneOverlap [version 1.6.0], compareGroups [version 3.2.4] and mixOmics [version 6.0.0]). Linear mixed effects models were used for the comparison between baseline and each time point after NAC in clinical symptoms, CBC, and gene expression

data. The similarities of clusters between cohorts were tested using the Fisher exact test (testGeneOverlap function of GeneOverlap package [version 1.6.0], <https://www.github.com/shenlab-sinai/geneoverlap>). A p value <0.05 was considered statistically significant. Benjamini–Hochberg false discovery rate (FDR) method was also used.

Fuzzy c-means clustering

Fuzzy c-means (FCM) clustering was performed using the R statistical computing program with packages nlme (version 3.1-128; J. Pinheiro, D. Bates, S. DebRoy, D. Sarkar, and R. Core Team. 2016. nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-128, <http://CRAN.R-project.org/package=nlme>) and Mfuzz (version 2.30.0) (20). The condition of FCM clustering (mfuzz function of Mfuzz package) was 1.8 fuzzification parameter ($m = 1.8$) and seven cluster centroids ($c = 7$). The function mfuzz used the FCM algorithm based on minimization of a weighted square error function.

Canonical correlation

We calculated the canonical correlation of immune cell frequencies in CBC and the cluster centers in gene expression data. The cluster centers are arithmetic means of the standardized expression of genes of each cluster at each subject's repeated measurements. To calculate the canonical correlation, we performed partial least squares regression using mixOmics package (version 6.0.0) (21).

Results

In our previous observations, nonallergic healthy subjects did not have any significant changes in clinical symptom score measurements after NAC (14). After we identified signatures of systemic immune responses in blood using the Q cohort, we validated the results of the analyses using the M cohort. In this article, we demonstrate the results of the study in both Q and M cohorts (Fig. 1).

NAC-derived AR response in the clinical symptoms measurement

Both Q and M cohorts had moderate negative correlations (Pearson correlation, $r < -0.5$) between PNIF and TNSS, and experienced peak TNSS or minimum PNIF score at 15 min after NAC (Fig. 2). PNIF of the Q cohort decreased significantly from 15 min to 5 and 9–10 h after NAC from baseline. This cohort experienced a significant increase of TNSS at 15 min to 4 and 10 h after NAC. PNIF of the M cohort significantly decreased at 15 and 30 min after NAC, and participants experienced a significant increase of TNSS at 15 min to 1 h after NAC.

Both cohorts used a different decision process of cat allergen doses administered at NAC visit

The fewer significant changes in the M cohort may be primarily related to the allergen doses, which were significantly ($p < 0.05$) different between the cohorts: six times lower than the Q cohort (Table I). Previous studies have demonstrated a relationship between the incidence of the stronger AR response such as LPR and higher allergen doses (22, 23).

NAC-derived changes of blood cells and neutrophil/lymphocyte ratio

In CBC data of the Q cohort, leukocytes and monocytes significantly ($p < 0.05$) increased at 2 and 6 h after NAC, whereas platelets significantly increased 6 h after NAC (Fig. 3A). In the M cohort, leukocytes significantly increased at 1 and 2 h after NAC, whereas platelets significantly increased at 1 and 6 h after NAC. Monocytes showed no significant change at any time point after NAC compared with baseline (Fig. 3B). Both cohorts displayed similar patterns in three leukocyte subtypes and neutrophil/lymphocyte ratio (NLR): neutrophils, increased at 1 and 2 h after NAC; eosinophils, decreased at 1 and 2 h after NAC; lymphocytes, increased at 6 h after NAC; and NLR, increased at 1 and 2 h after NAC (Fig. 3).

Seven clusters of immune gene expression patterns of the peripheral blood were identified

We sought signatures of immune gene transcripts of blood associated with immune cell frequencies to identify distinct pathophysiological systemic immune responses of AR. To investigate the signatures using a clustering method, we profiled 730 canonical immune genes using a NanoString nCounter assay on blood samples that were sequentially collected. We used two filtering procedures, a linear mixed effect model (LME) and a range threshold, to select significantly expressed immune genes after NAC. Using LME and considering missing data and subject differences as a nested random effect, we identified 224 genes as significantly ($FDR < 0.1$) different when compared between baseline and other time points after NAC. Then, 120 of these 224 genes were retained after applying a cutoff filter ($> \pm 1.2$ fold change) over time points in mean gene expression of all the subjects in the Q cohort before clustering. FCM clustering identified seven clusters based on the standardized (mean:

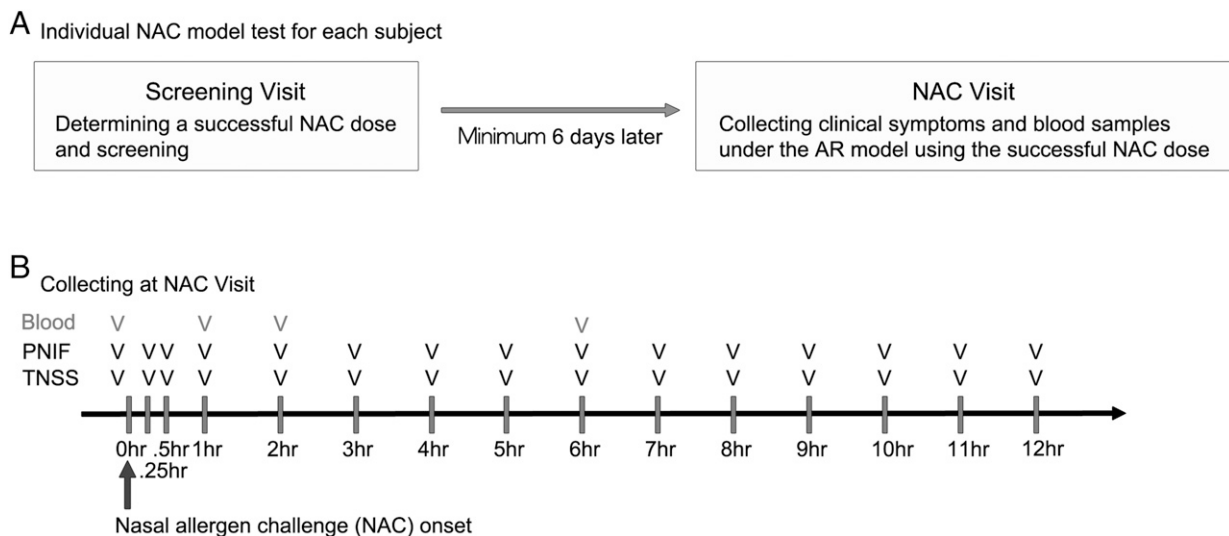


FIGURE 1. Diagram of an NAC model. **(A)** Individual NAC model test procedure from screening visit to NAC visit. **(B)** A schedule of collecting peripheral blood samples and clinical symptoms before and during NAC onset in both Q and M cohorts; in healthy nonallergic subjects, blood samples were collected at two time points (before NAC and 1 h after NAC).

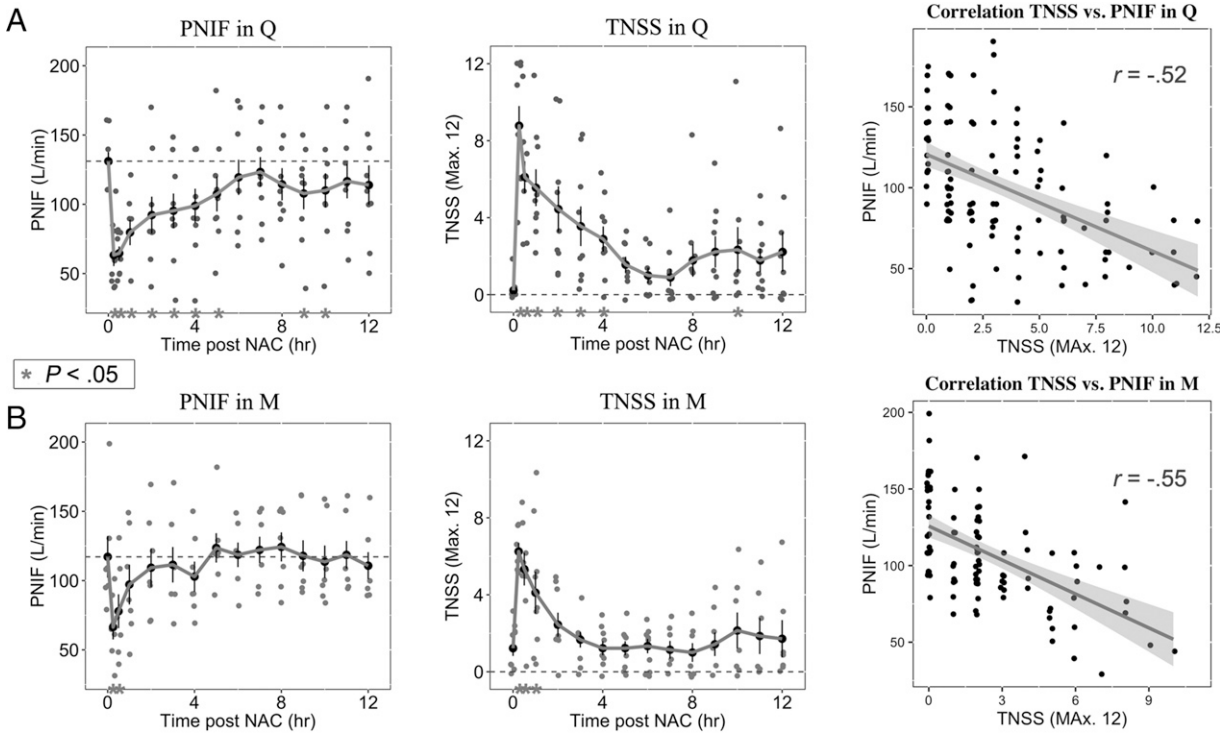


FIGURE 2. Clinical symptom scores after NAC. **(A)** Clinical symptom scores and Pearson correlation (r) of PNIF and TNSS in the Q cohort ($n = 9$). **(B)** Clinical symptom scores and Pearson correlation of PNIF and TNSS in the M cohort ($n = 9$, partial missing data). Error bars: mean $\pm$ SEM. * $p < 0.05$, LME.

0, SD: 1) mean gene expression pattern over the examined time points (Fig. 4A).

To validate the FCM clustering, we independently applied the analysis to the M cohort. The seven clusters were reproducible in the M cohort (Fig. 4B). A total of 207 genes from the profiled 730 immune genes were filtered and grouped into the seven clusters in the M cohort. For both cohorts, clusters 1, 2, and 3 displayed an increasing expression at 1 and 2 h after NAC; clusters 4 and 5 showed a maximum change at 6 h after NAC; and clusters 6 and 7 showed a decreasing expression at 1 and 2 h after NAC (Fig. 4A, 4B; see Supplemental Table I). The seven clusters in each cohort were tested for similarities using the Fisher exact test on 85 shared genes between 120 genes of the Q cohort and 207 genes of the M cohort (Table II). Five clusters

had significant ($FDR < 0.01$) similarities: Q2/M2, Q3/M3, Q4/M4, Q6/M6, and Q7/M7.

Relatedness of clusters and immune cells were investigated

To test the association between clusters and immune cell types, we performed cell enrichment analyses using Enrichr, a web-based, mobile software application (24), and Simple Enrichment Analysis in R (<https://www.github.com/cashoes/sear>), which has detailed cell subsets. Both analyses demonstrated similar results (Fig. 4C). Cluster Q2/M2 was primarily associated with myeloid cells such as neutrophils. Cluster Q3/M3 was also associated with myeloid cells such as neutrophils and monocytes. Cluster Q4/M4 was associated with B cells and T cells. Cluster Q6/M6 was associated with T cells.

Table I. Characteristics of the Q and M cohorts (Fisher exact test)

	Q Cohort	M Cohort	p Value
Sex (n)			<0.05
Men	0	5	
Women	9	4	
Age (y), mean $\pm$ SD	35 $\pm$ 8	40 $\pm$ 12	>0.05
Body mass index (kg/m^2), mean $\pm$ SD	26.8 $\pm$ 4.8	26.6 $\pm$ 3.4	>0.05
Race			>0.05
White	8	7	
Asian	1	2	
Study site (NAC model experiment)	Queen's University	McMaster University	
The criteria of qualifying concentration for successful NAC at screening visit:	AND	AND/OR	
1) a PNIF reduction of $\geq 50\%$ from baseline, and 2) a TNSS $\geq 8/12$.			
Allergen dose administered at NAC visit	Cumulative dose	Qualifying concentration dose	
Allergen dose administered at NAC visit (BAU), mean $\pm$ SD	408.5 $\pm$ 185.7	67.7 $\pm$ 55.1	<0.05

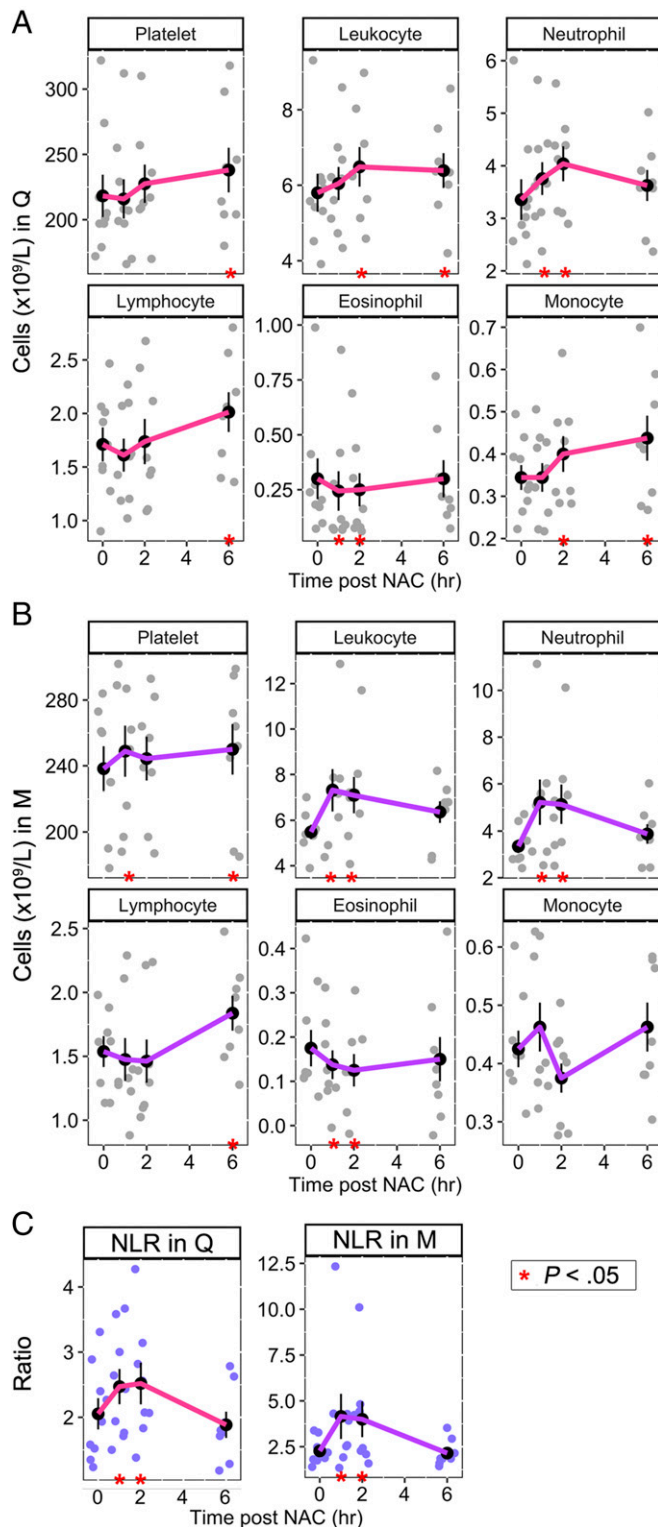


FIGURE 3. CBC after NAC. (A) CBC in the Q cohort ($n = 9$, partial missing data). (B) CBC in the M cohort ($n = 8$). (C) NLR in the Q ($n = 9$, partial missing data) and M ($n = 8$) cohorts. Error bars: mean $\pm$ SEM. * $p < 0.05$, LME.

We asked whether the clusters were correlated with the frequencies of the corresponding immune cells, which were demonstrated on the cell enrichment analyses, after NAC. To identify the correlations, we calculated the canonical correlations of immune cell frequencies with the cluster centers using partial least squares regression (Fig. 5). Clusters Q2/M2 and Q3/M3 were

strongly positively correlated to neutrophil count and NLR (canonical correlation > 0.75 , Q2 and NLR = 0.69) (Fig. 5). Cluster Q4/M4 was positively correlated (canonical correlation, Q4 = 0.86, M4 = 0.54) with lymphocyte count (Fig. 5). Interestingly, the M cohort, whose cluster M4 had weaker correlation with lymphocyte frequency than cluster Q4, had no significant LPR in the clinical symptom scores analysis (Fig. 2).

Correlation of clinical symptoms and clusters or immune cell frequencies were investigated in 13 AR subjects

After removing subjects who had missing data, we used 13 AR subjects (Q cohort: 8, M cohort: 5; sex: 2 male, 11 female; see Supplemental Table II) to investigate the association of clinical symptoms and the identified systemic immune response patterns (frequencies of neutrophils and lymphocytes, NLR, and clusters 2, 3, and 4): cluster 2 was associated with TRAIL and PDGFR- β signaling pathway, and clusters 3 and 4 were associated with IL-4-mediated signaling events in pathway enrichment analysis (FDR < 0.1 ; Enrichr). When the Pearson correlations (r) were calculated, the original values and the ratio values (a relative number at each time point after NAC compared with the baseline value) of the variables were used (Fig. 6; see Supplemental Fig. 1). A sum of clinical symptom scores in EPR (baseline to 6 h after NAC), LPR (7–12 h after NAC), or over all examined time points was used because of different time intervals between clinical symptoms and systemic immune response patterns: we separated EPR and LPR periods based on the change of the mean values of clinical symptoms.

The Pearson correlations between the sum of TNSS and the sum of PNIF or the sum of the ratio of PNIF were negligible ($r < +0.3$ or $r > -0.3$; see Supplemental Fig. 1). This may reflect that PNIF measures just one of four symptom categories of TNSS; although PNIF is an objective measurement, it is unlikely to represent overall symptoms after NAC.

The sum of TNSS in EPR was moderately associated with the ratio of lymphocyte (values at 2 h after NAC: $r = +0.51$, the sum over all time points: $r = +0.55$) and the ratio of NLR at 6 h after NAC ($r = -0.61$). The sum of TNSS over all time points was moderately associated with the ratio of NLR at 6 h after NAC ($r = -0.51$). The sum of PNIF in LPR was moderately associated with neutrophil at 2 h after NAC ($r = -0.51$). The sum of the ratio of PNIF in LPR was moderately associated with NLR at 6 h after NAC ($r = +0.67$; Fig. 6).

The immune genes shared in the same clusters of both cohorts were used for the correlation calculation. The geometric mean of the ratio of cluster 3 (19 genes in cluster Q3/M3; Table II) at 6 h after NAC was moderately ($r = -0.55$) associated with the sum of TNSS in EPR (Fig. 6).

Although neutrophil and lymphocyte frequencies were positively associated with the intensities of EPR or LPR, NLR and its ratio at 6 h after NAC were negatively associated with the severity of clinical symptoms (Fig. 6). In other words, considering NLR, higher lymphocytes at 2 and 6 h after NAC may be associated with the severity of AR; neutrophil count at 2 h after NAC was also positively related with the severity of AR, but inversely related at 6 h after NAC. NLR at baseline has been suggested as an indicator of inflammation and severity of AR (25, 26).

Comparison of immune gene signature patterns in 13 AR subjects with those in 5 healthy nonallergic control subjects

Five healthy nonallergic subjects whose blood collections were at two time points (baseline and 1 h after NAC) were used to compare the immune gene signature patterns with AR subjects. The healthy nonallergic subjects had no significant change in TNSS after NAC

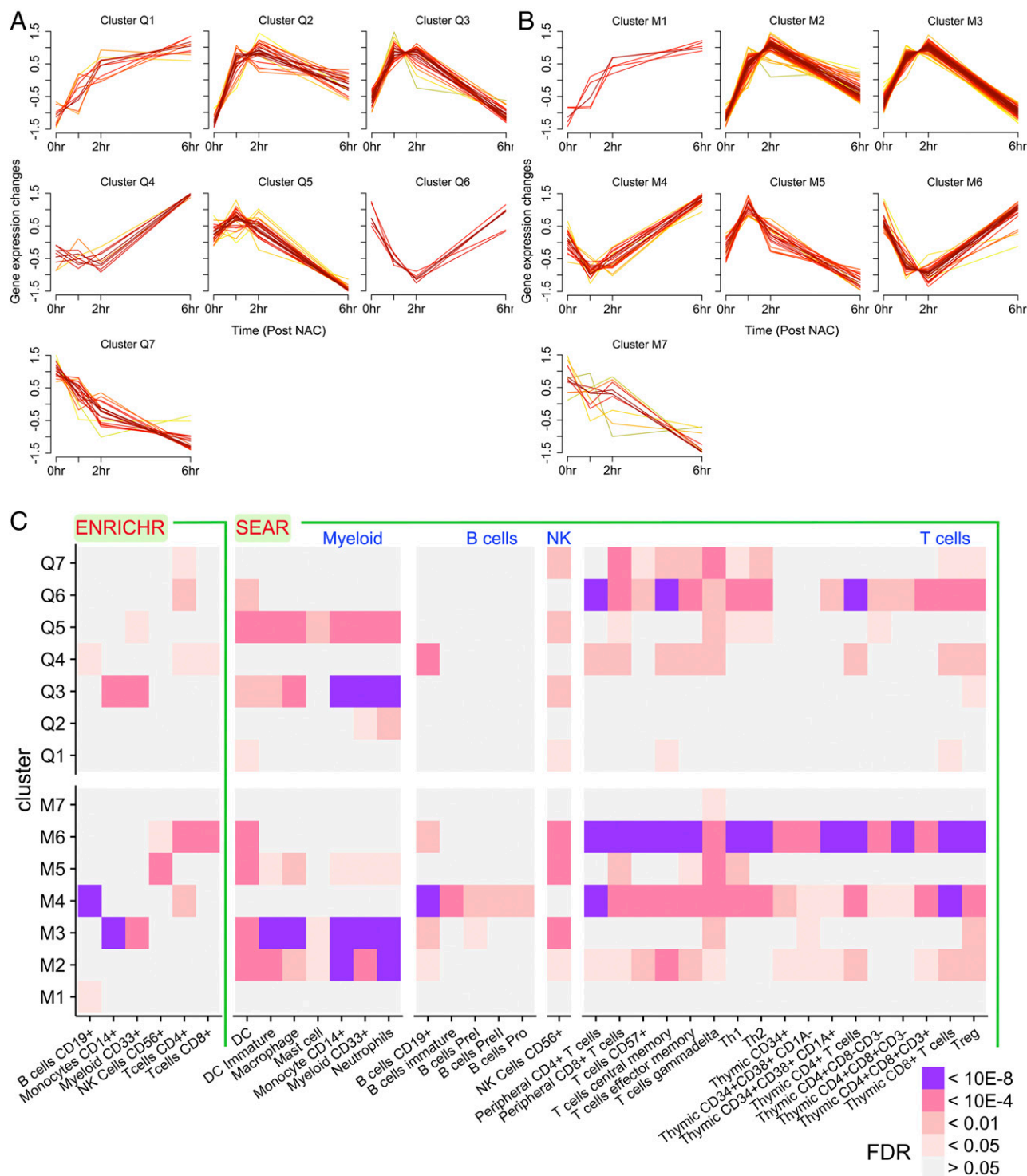


FIGURE 4. Seven clusters of differentially expressed immune genes in blood after NAC. **(A)** Seven clusters in the Q cohort ($n = 9$, partial missing data). **(B)** Seven clusters in the M cohort ($n = 9$, partial missing data). **(C)** Cell enrichment analyses of the seven clusters.

(baseline: 0). There was no significant difference ($p > 0.05$, Fisher exact test; see Supplemental Table II) in demographics (age, sex, race, and body mass index) between 13 AR subjects and 5 healthy nonallergic subjects.

Although immune cell frequencies (neutrophils, lymphocytes, and NLR) and their ratios in 13 AR subjects had significant changes after NAC, those in 5 healthy nonallergic subjects had no significant change (Fig. 7A), at least within the first hour after NAC. AR subjects and the healthy control subjects were significantly different in the variables except for neutrophils (Fig. 7A).

Ratios of clusters 2, 3, and 4 at 1 h after NAC were significantly different between the AR subjects and the healthy subjects. In comparison with baseline, ratios of clusters 2 and 3 at 1 h after NAC significantly increased in only the AR subjects, but the ratio of cluster 4 at 1 h after NAC significantly increased in only the healthy subjects (Fig. 7B).

Discussion

In this study, we demonstrated a systemic immune response signature, which is a clustered immune gene signature associated with

Table II. Similarities between clusters from the Q and M cohorts

FDR (Overlapped Genes)		Shared 85 of 120 Genes (Q Cohort)						
		3 Genes	19 Genes	23 Genes	8 Genes	16 Genes	6 Genes	10 Genes
		Cluster Q1	Cluster Q2	Cluster Q3	Cluster Q4	Cluster Q5	Cluster Q6	Cluster Q7
0 genes	Cluster M1	1	1	1	1	1	1	1
19 genes	Cluster M2	0.68 (2)	1.23×10^{-6} (14)	1 (3)	1	1	1	1
36 genes	Cluster M3	1	1 (4)	9.26×10^{-5} (19)	1	0.03 (12)	1	1 (1)
7 genes	Cluster M4	1	1	1	1.07×10^{-5} (6)	1	1	1
9 genes	Cluster M5	1	1 (1)	1 (1)	1	0.42 (4)	1	0.42 (3)
9 genes	Cluster M6	1 (1)	1	1	0.98 (2)	1	2.16×10^{-4} (5)	1 (1)
5 genes	Cluster M7	1	1	1	1	1	1	9.42×10^{-5} (5)

Numbers in parentheses indicate total number of shared genes (Fisher exact test).

corresponding immune cell frequencies in whole peripheral blood collected after the allergen challenge. The immune gene signatures, which were reproducible in cat allergy cohorts, associated with significantly changed immune cell frequencies. Cluster 3 was moderately associated with clinical symptoms at 6 h after NAC in the AR subjects. Clusters 2, 3, and 4 were significantly different between the AR subjects and the healthy nonallergic subjects, at least within the first hour after NAC, which was the only comparison we could perform given the more limited blood sampling available for the healthy nonallergic subjects.

The NAC model provides a well-established human experimental model of AR to collect clinical symptom scores and blood samples (14–16, 27–29). A sensitive and highly reproducible NanoString nCounter Gene Expression assay (30, 31) was used to profile 730 well-characterized immune genes in peripheral blood.

Using statistical and bioinformatics tools, we identified reproducible combinations of immune cells and gene expression transcripts associated with clinical symptom scores. FCM clustering, a widely used method to discover significant patterns in a given data set, was used to partition the gene expression data into clusters. We chose FCM over other clustering methods, such as K-means and hierarchical clustering, because FCM allows a more exploratory approach to find natural boundaries in data (32, 33). To solve the challenge of FCM, sensitivity to noise and outliers of a given data set (32), we applied two filtering procedures to our data before clustering: 1) a statistical test using an LME, and 2) a fold-change threshold.

We determined associations between seven immune gene clusters and immune cell frequencies (leukocytes, platelets, neutrophils, lymphocytes, monocytes, eosinophils, and NLR) using canonical correlation analysis, which is a multivariate form of the general linear model, adjusting for intersubject variability and time

points (21, 34). Finally, we investigated the Pearson correlation between clinical symptom scores and the identified systemic immune response pattern. Although TNSS is an ordinal variable, we used Pearson correlation because TNSS has been considered as an interval variable (14, 35) and we were interested in linear relationships between the intensity of clinical symptoms and other variables for a potential objective measurement.

The Q and the M cohorts experienced peak TNSS or minimum PNIF score at 15 min after NAC (Fig. 2), and significantly increased clinical symptoms were shown by several hours, even at 9 or 10 h in the Q cohort after NAC. This is consistent with previous studies, which demonstrated the peak of clinical symptoms at very early time point (2–20 min) after allergen challenges in AR and allergic conjunctivitis (5, 14, 36, 37).

We investigated immune cell frequencies in CBC data, which is a straightforward, rapid, and relatively inexpensive method that provides reliable counts of subtypes of leukocytes as a standard diagnostic tool, for example, neutrophils, lymphocytes, monocytes, and eosinophils (38–40). Basophil counts have been shown to be unreliable between hematology analyzers (38, 40). Considering the minimum detectable cell count (0.1×10^9 cells/l) of the analyzers, the basophil count was negligible in our CBC data (range $0.0\text{--}0.1 \times 10^9$ cells/l; median, 0.0). Thus, we excluded basophil count in this study, although basophils are an important effector cell type that is functionally and developmentally similar to mast cells associated with type 2 immune responses by type 2 Th cells (41).

Changes in immune cell frequencies of CBC are the net result of loss and gain of leukocytes in the blood. Many leukocyte subtypes are known to migrate to local inflammatory sites after NAC (2, 42–45). Leukocyte frequency was mainly affected by neutrophils and lymphocytes: Interestingly, the Q cohort had higher leukocyte

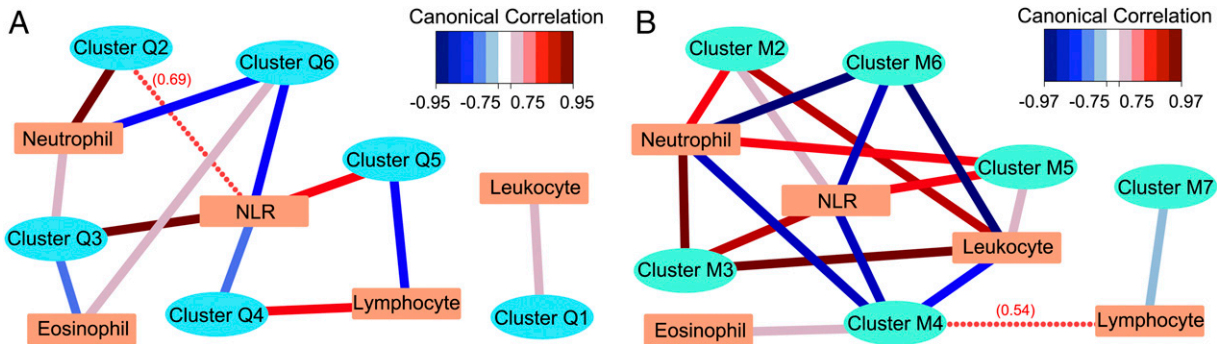


FIGURE 5. The relationship between clusters and immune cell frequencies. (A) Canonical correlation between cell frequencies and clusters in the Q cohort ($n = 9$, partial missing data). (B) Canonical correlation between cell frequencies and clusters in the M cohort ($n = 8$). Partial least squares regression was used.

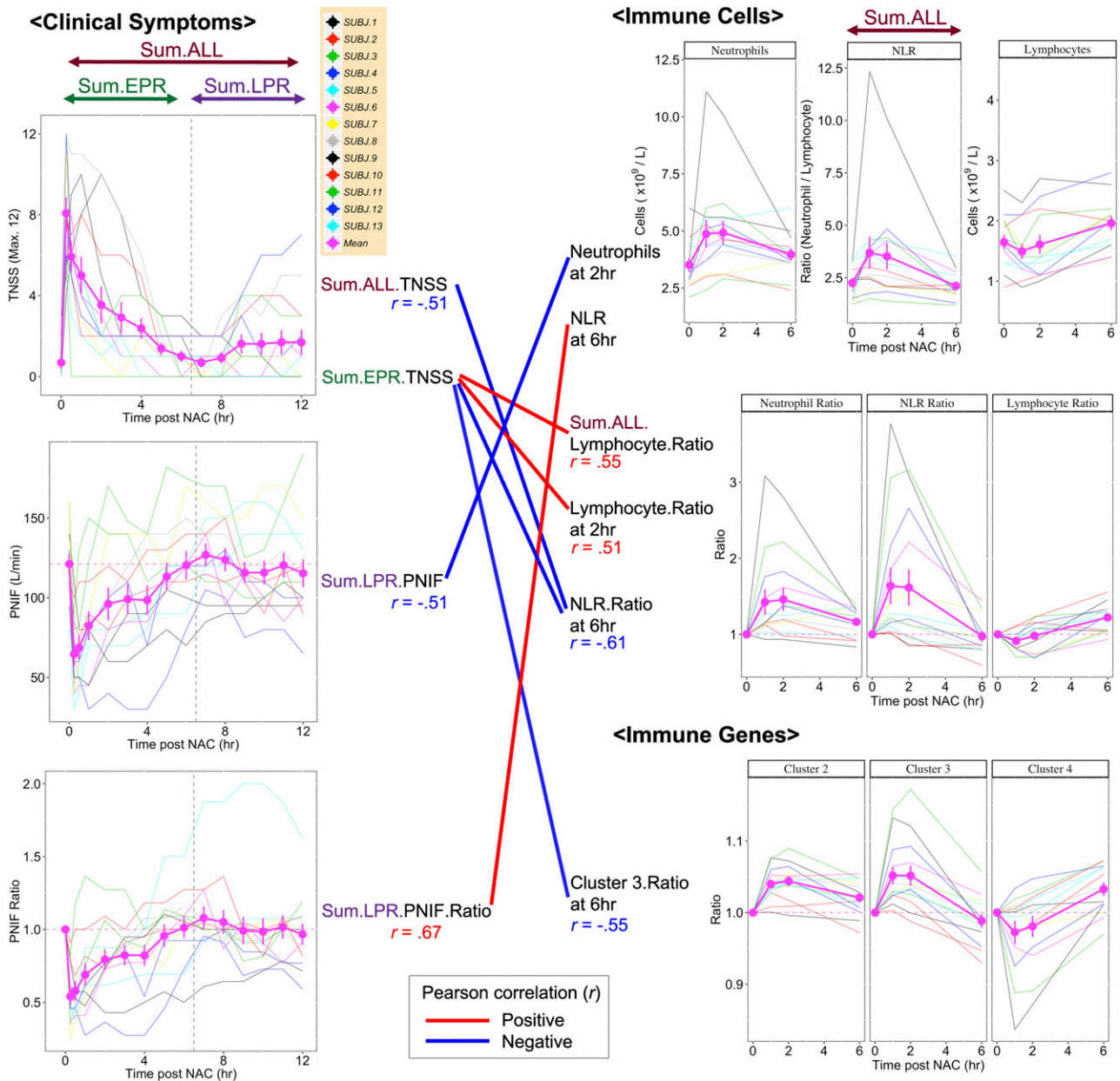


FIGURE 6. Investigation of correlations between clinical symptoms and systemic immune response in 13 subjects who had no missing data. The correlations ($r > +0.5$ or $r < -0.5$) between sum of clinical symptom scores (TNSS, PNIF, and PNIF.Ratio) and immune cell frequencies (lymphocytes, neutrophils, and NLR; lymphocytes.Ratio, and NLR.Ratio) or immune gene clusters (clusters 2, 3, and 4) were demonstrated ($n = 13$). Sum.ALL: a sum of scores over all measured time points (baseline to 12 h after NAC in clinical symptoms; baseline to 6 h after NAC in immune cell frequencies); Sum.EPR: a sum of scores over time points in EPR (baseline to 6 h after NAC); Sum.LPR: a sum of scores over time points in LPR (7–12 h after NAC). Error bars: mean $\pm$ SEM.

count at 6 h after NAC with higher monocytes in contrast with the M cohort. In a previous study, monocytes were significantly recruited at the site of allergen challenge in AR patients at 12 h after NAC (46).

The Q and the M cohorts displayed similar changes after NAC in NLR and frequencies of three leukocyte subtypes (neutrophils, eosinophils, and lymphocytes). However, the Q cohort, which had worse clinical symptoms at 2 h after NAC compared with the M cohort, showed significantly higher cell counts for neutrophils and lymphocytes at this time point compared with 1 h after NAC ($p < 0.05$, paired t test).

We observed lower eosinophil counts in the blood at 1 and 2 h after NAC (Fig. 3A, 3B). This may be associated with eosinophil influx into nasal lavage fluids, and is consistent with a study that

used intranasal heparin to reduce symptom scores at 1 and 6 h after NAC (43), while demonstrating lower influx of eosinophils. Another study reported eosinophils to be significantly increased in nasal and bronchial biopsies and blood from AR subjects at 24 h after NAC compared with baseline (47), although earlier time point samples were not collected.

In the correlation analysis using 13 subjects who had no missing data, counts of neutrophils and lymphocytes at 2 and 6 h after NAC or over all time points may be associated with the change of immune response phases, innate and adaptive immune response, cell-mediated and humoral immune response, and EPR and LPR. These may be directly associated with the severity of AR, with neutrophils representing the innate immune system (48) and lymphocytes representing the adaptive immune system. High

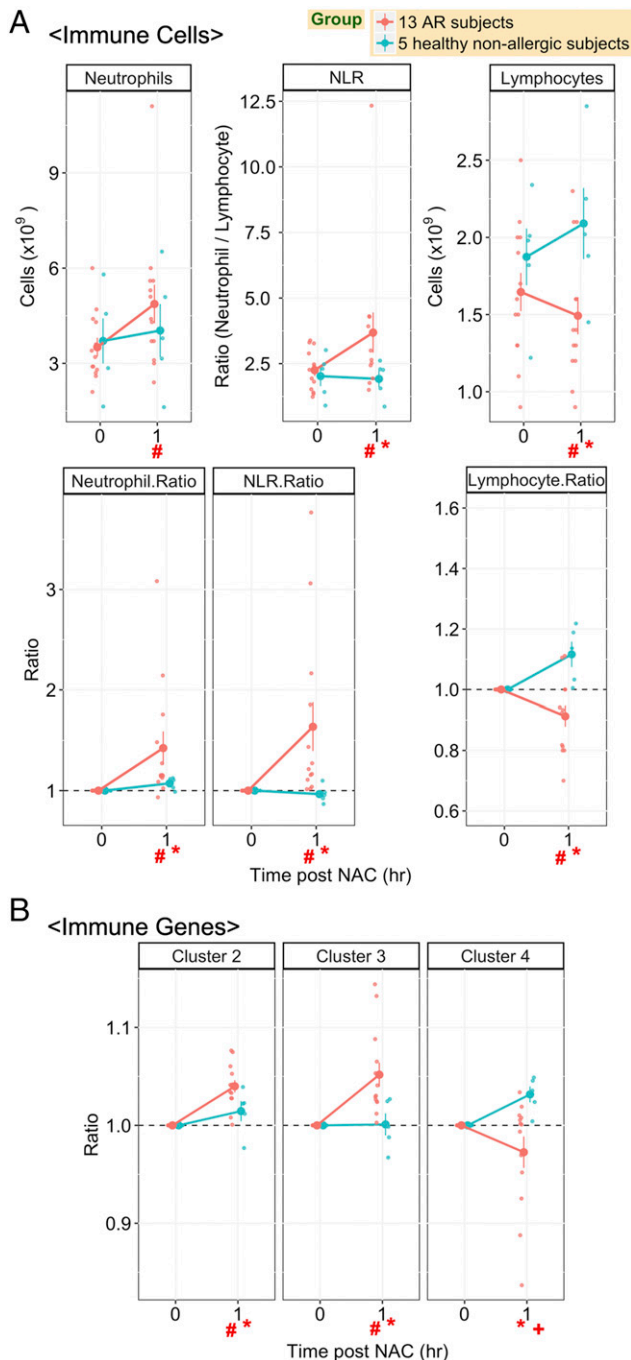


FIGURE 7. Comparison of immune gene signature patterns in 13 AR subjects with those in five healthy nonallergic subjects. **(A)** Frequencies and their ratios of neutrophils, lymphocytes, and NLR. **(B)** Ratios of immune gene clusters (clusters 2, 3, and 4). Error bars: mean $\pm$ SEM. * $p < 0.05$, Wilcoxon rank sum test (13 AR subjects versus five healthy subjects), # $p < 0.05$, Wilcoxon signed rank test/paired t test (baseline versus 1 h after NAC in the AR subjects), + $p < 0.05$, Wilcoxon signed rank test/paired t test (baseline versus 1 h after NAC in the healthy subjects).

neutrophil frequency and NLR at early time points after NAC may reflect the highly activated innate immune response against the allergen, but their values at 6 h after NAC were likely related to the interaction between the innate and adaptive immune systems.

We cannot know the subtype frequencies of lymphocytes using CBC data, but the identified immune gene cluster, Q4/M4, corresponding to lymphocyte frequency, was composed of genes related to lymphocyte function: *FCER2* (low-affinity receptor for

IgE) and *CD180* for B cell; *TCF7*, *LY9*, and *FLT3LG* for T cells. Although peripheral whole blood has not been previously well studied in the NAC model, recent papers reported significant ($p < 0.05$) changes in the proportion of $CD4^+$ T cell subsets and type 2 innate lymphoid cells in purified PBMCs after NAC (8, 49). Whereas $CD4^+CCR4^+$ T cells decreased, $CD4^+CD25^{lo}$ T cells, $CD4^+CD152^+$ T cells, and $CD4^+CRTh2^+$ T cells increased at 6 h after NAC compared with before NAC or control (diluent) (8). Type 2 innate lymphoid cells increased at 4 h after NAC compared with baseline (49). These are consistent with our results and may be associated with LPR and amplification of the IgE response. Levels of the Th2 cytokines IL-4, IL-5, IL-9, and IL-13 significantly increased in nasal fluid at 8 h after NAC (50). The microenvironment of the nasal mucosa after NAC is thought to be modified by recruitment of leukocytes causing tissue remodeling and providing germinal center-like reactions, which facilitate the isotype switching to IgE $^+$ B cells and their proliferation and maturation to IgE-producing plasmablasts/plasma (2, 10, 44, 51–53). These responses may also be related to nonspecific nasal hyperresponsiveness (4, 45).

Furthermore, the seven clusters of immune genes had deconvoluted information that demonstrated significantly differentially expressed immune genes that were associated with specific leukocyte subtypes and immune responses (Fig. 4). For example, cluster 3 (Q3/M3) corresponded to neutrophils and NLR, and was significantly ($FDR < 0.01$) related to TLR-, IL-1-, and IL-4-mediated signaling pathways in an enrichment analysis (Enrichr). Its geometric mean of the ratio at 6 h after NAC was negatively associated with EPR (Fig. 6, sum of TNSS in EPR): to minimize effect of extreme values, we used the geometric mean instead of the arithmetic mean because the ratio of immune gene expression was not standardized.

Transcriptional changes in immune genes are not always highly correlated with protein levels and specific cell frequencies. Nevertheless, clustering of gene expression correlated with corresponding cell frequency may allow identification of traceable pattern signatures (54) that may provide more objective biomarkers for diagnosis, prognosis, and pathophysiological or pharmacological understanding. For example, although phenotyping using cell surface markers is difficult for classification of acute myeloid leukemia, the gene expression signature using the consistent difference of signaling patterns between primitive and mature leukemia subpopulations, the mean expression of which was correlated with its corresponding subpopulation frequency, was useful for prognosis of overall patient survival (55).

AR is a complex disease involving interactions between the local inflammatory site and the systemic immune system (e.g., lymphoid organs and peripheral blood). Systemic characteristics of AR have previously been demonstrated in alterations of the nervous system caused by immune responses of AR such as eosinophil recruitment to nasal nerves after NAC (56, 57). Systemic immune responses of AR triggered by the allergen challenge may also be influenced by many factors such as stress, diurnal variation, and dehydration. The ratio of cluster 4 in the 5 healthy nonallergic subjects demonstrated that they may have a pattern in the normal diurnal condition, which was significantly different from the 13 AR subjects (Fig. 7B). Future studies comparing allergic with healthy nonallergic subjects at later time points will be needed to definitively rule out, as well as rule in, any elements of diurnal variability.

Although further large-scale studies may be necessary to elaborate these systemic immune responses, we suggest that our immune gene signature approach using peripheral blood may be a useful tool to understand complex aspects of the pathophysiology of AR.

In conclusion, investigation of peripheral blood under the NAC model provided traceable signatures of immune gene expression clusters corresponding to immune cell frequencies, representing a novel cross-sectional view of the pathophysiological systemic immune response of AR. These signatures may also be useful to diagnose AR and investigate efficacy and mechanisms of AR treatments as a potential objective measurement method.

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Disclosures

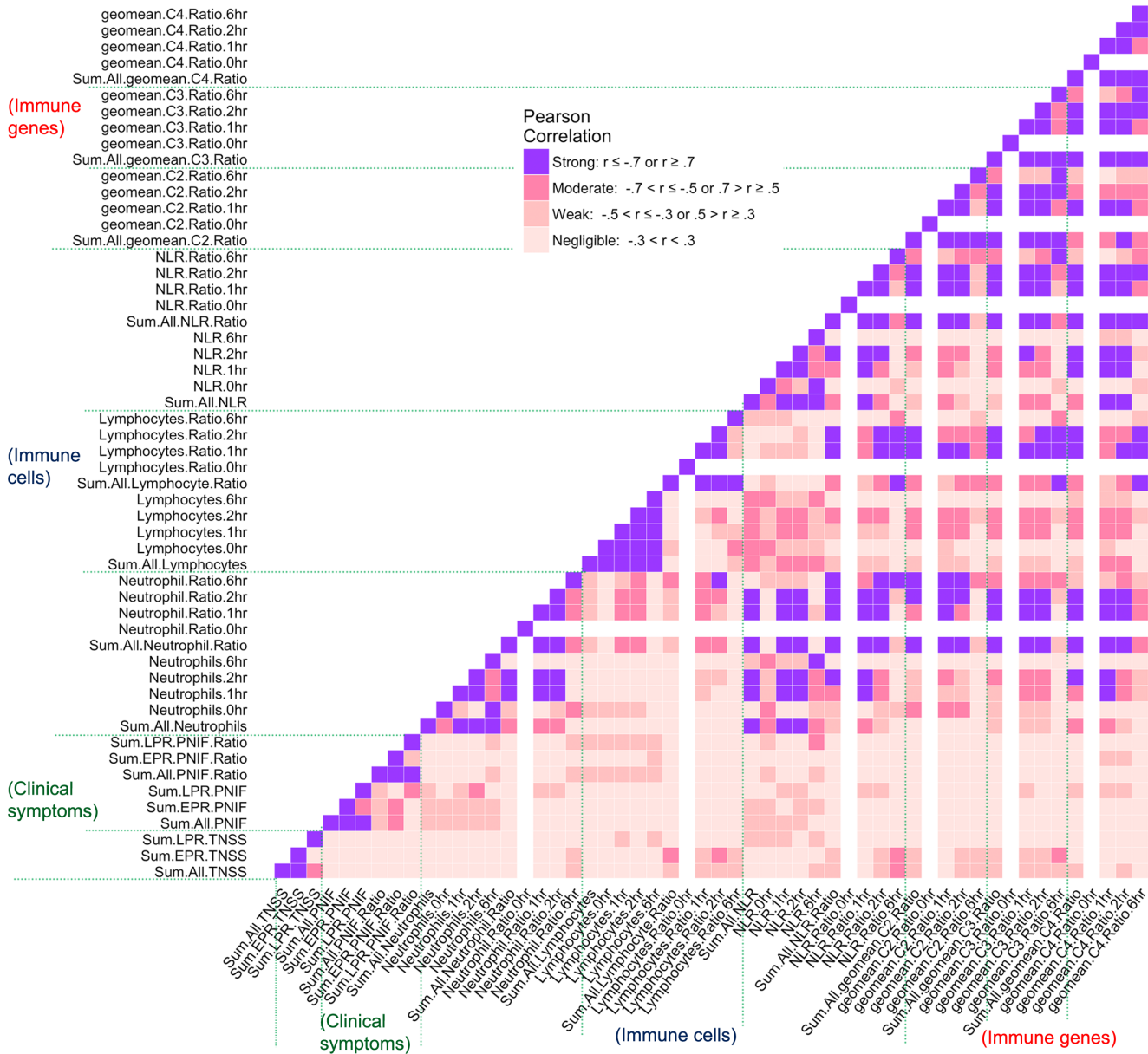
The authors have no financial conflicts of interest.

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Supplemental Figure 1.



Correlation between clinical symptoms and systemic immune response in 13 subjects who had no missing data. The Pearson correlation between sum of clinical symptom scores (TNSS, PNIF and PNIF.Ratio) and immune cell frequencies (lymphocytes, neutrophils and NLR; lymphocytes.Ratio, neutrophils.Ratio and NLR.Ratio) or immune gene clusters (geometric mean of the ratio of Cluster 2, 3 and 4 using shared genes in both Q and M cohrots). n = 13, Sum.ALL: a sum of score over all measured time points (baseline to 12hr post-NAC in clinical symptoms; baseline to 6hr post-NAC in immune cell frequencies), Sum.EPR: a sum of score over time points in EPR (baseline to 6hr post-NAC), Sum.LPR: a sum of score over time points in LPR (7 to 12hr post-NAC).