

## Highly conserved gene expression profiles in humans with allergic rhinitis altered by immunotherapy

Z. Liu<sup>\*†</sup>, R. W. Yelverton<sup>\*</sup>, B. Kraft<sup>\*</sup>, S. B. Tanner<sup>\*†</sup>, N. J. Olsen<sup>\*†</sup> and T. M. Aune<sup>\*†</sup>

<sup>\*</sup>Department of Medicine, Division of Rheumatology, <sup>†</sup>Department of Medicine, Division of Allergy & Immunology and <sup>‡</sup>Department of Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, TN, USA

### Summary

**Background** Atopic diseases, resulting from hypersensitivity to a wide variety of allergens, affect 10–20% of the population. Immunotherapy is an effective treatment for atopic diseases, but its mechanisms are not fully understood.

**Objective** We studied gene expression profiles in the peripheral blood mononuclear cells (PBMC) and examined whether the individuals with allergic rhinitis (AR) have a unique gene expression profile and how the immunotherapy affect the gene expression profiles.

**Methods** We used cDNA microarray and 'expression analysis systemic explorer' to examine the gene expression profiles in the PBMC of atopic subjects and other groups.

**Results** We identified a highly conserved gene expression profile in atopic subjects that permitted their accurate segregation from control or autoimmune subjects. A major feature of this profile was the under-expression of a variety of genes that encode proteins required for apoptosis and over-expression of genes that encode proteins critical for stress responses and signal transduction. We also identified 563 genes that can segregate individuals with AR based upon receipt of immunotherapy.

**Conclusion** There is a highly conserved gene expression profile in the PBMC of individuals with AR. This profile can be used to identify individuals with AR and to evaluate responses to immunotherapy. Quantitative endpoints, such as gene expression, may assist clinicians faced with clinical decisions in the diagnosis of patients and the evaluation of response to therapy. The knowledge of the possible genetic basis for immunotherapy efficacy may also lead to novel therapeutic approaches for atopic diseases.

**Keywords** allergic rhinitis, apoptosis, atopic diseases, cDNA microarray, gene expression profiles

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### Introduction

Atopic disease represents one of the major categories of immune-mediated disease in the human population [1, 2]. Both environmental and genetic factors contribute to disease onset and severity [3, 4]. One common type of atopic disease, allergic rhinitis (AR), is a consequence of immediate hypersensitivity to common airborne allergens localized to the upper respiratory tract [5, 6]. De-sensitization by immunotherapy ('allergy shot') is a common clinical treatment for atopic diseases [7] and can provide a possible permanent improvement in the clinical disease. However, its precise mechanism is not fully understood.

Gene expression profiling offers a powerful tool to classify human diseases, identify common features of clinically distinct diseases, and contribute to disease diagnoses. Alterations in gene expression profiles in a common cellular or

tissue source may also permit more accurate monitoring of changes in disease activity after therapy, may predict patient responses to a given type of treatment, or may predict risks of unwanted side-effects in response to treatments [8–11]. Identification of genes whose expression levels fulfil these goals could have significant impact on the decisions made by clinicians in patient treatment.

Several groups including our own have recently reported initial results of these gene expression studies in human autoimmune diseases [12–14]. These results clearly demonstrate that it is possible to use gene expression profiling to classify human diseases and to identify common features present in clinically distinct autoimmune diseases. Therefore, in this study, using similar approaches, we aimed to determine if we also found conserved gene expression profiles in peripheral blood mononuclear cells (PBMC) in individuals with AR and if these profiles shared common features with those of human autoimmune disease. We also wanted to determine if and how gene expression profiles in these individuals might change after immunotherapy. Here, we demonstrate that individuals with AR have unique gene

Correspondence: Thomas M. Aune, MCN T3219, Vanderbilt University Medical Center, 1161 21st Ave. S., Nashville, TN 37232, USA.  
E-mail: thomas.aune@vanderbilt.edu

**Table 1.** Patients information

| Age | Gender | Disease duration* | Skin test+ <sup>†</sup> | Specific IT <sup>‡</sup> | Pre-IT ID # score <sup>‡</sup> | Post-IT ID # score <sup>‡</sup> | Interval between microarray (days) |
|-----|--------|-------------------|-------------------------|--------------------------|--------------------------------|---------------------------------|------------------------------------|
| 22  | M      | >10               | b                       | b                        | 001 3                          | —                               |                                    |
| 58  | F      | >10               | a                       | a                        | 002 3                          | —                               |                                    |
| 32  | M      | >10               | a                       | a                        | 004 3                          | —                               |                                    |
| 34  | F      | 16                | b                       | b                        | 007 3                          | —                               |                                    |
| 23  | F      | 13                | a                       | a                        | 006 3                          | 015 0                           | 167                                |
| 30  | F      | >10               | a                       | a                        | 010 3                          | 016 1                           | 41                                 |
| 31  | F      | >10               | b                       | b                        | 013 3                          | 017 0                           | 119                                |
| 34  | F      | >30               | b                       | b                        | 009 3                          | 019 0                           | 188                                |

\*Disease duration in years.

<sup>†</sup>Skin test activity: a, cat, moulds, grasses, trees, weeds; b, dust mite, cat, dog, moulds, grasses, trees, weeds.

<sup>‡</sup>Symptoms score.

IT, immunotherapy.

expression profiles in their PBMC compared with those who are not allergic or have autoimmune diseases without AR. We also demonstrate that these gene expression profiles change in a characteristic way after immunotherapy. Thus, we provide direct support for the concept that identification of 'biomarkers' in human disease is possible and that they can be used to monitor responses to therapy. The identification of which gene expression pathway may underlie atopic diseases and is altered with immunotherapy, an effective clinical treatment, may provide insight into development of novel approaches to disease management.

## Methods

### Patients

Patients with AR ( $N=8$ ; 22–58 years of age) had blood drawn at their first clinic visit before receiving allergy immunotherapy in spring and summer of 2003, none of them had received immunotherapy previously. Four patients (female; three Caucasians one African American; mean age 30, range 23–34 years) had blood collected on the day of RUSH immunotherapy [15, 16] and mean 129 days after RUSH (range 41–188 days) when the maintenance dose was achieved. Concentration of RUSH (w/v) at last sample is 1:100. None of these four patients have asthma, all skin tested positive to a broad range of perennial and seasonal allergens. Allergy extracts used in skin tests and immunotherapy were as follows:

Greer Laboratories<sup>TM</sup> (Lenior, NC, USA). Mould mixes: *Alternaria*, *aspergillus*, *helminthosporium*, *ormodendrum*, *penicillium*, *curvularia*, *fusarium*, *mucor*, *pullularia*, *rhizopus*. Weed mixes: Cocklebur, lambs quarter, rough pigweed, giant ragweed, short ragweed, sage mix (prairie sage, common sagebrush), dock sorrel mix (yellow dock, sheep sorrel), kochia, English plantain, nettle. Tree mixes: Eastern sycamore, sweet gum, white mulberry, elm mix (American, Chinese), eastern cottonwood, oak mix (black, red, white), sugar maple-box elder mix, birch mix (black red, white), red cedar, pecan pollen-shagbark hickory mix, ash mix (green, white), acacia, mountain cedar, olive. Hollister-Stier (Spokane, WA, USA)<sup>TM</sup>. Dust mites (*D. Farinae* and *D. Pteronyssinus* mites), cat hair, dog hair & dander. Cockroach mix (American & German). Grass mixes: Kentucky Bluegrass, Orchard Grass, Redtop,

Timothy, Sweet Vernal, Meadow Fescue, Perennial Rye, Johnson Grass, Bermuda Grass.

All of these four patients had nasal (sneezing, running, blocked), ocular (itching, tear flow, redness), ear, headache symptoms prior to immunotherapy and reported symptom improvement 1–3 months after immunotherapy. Symptoms intensity was documented as 0 = no symptoms, 1 = mild, 2 = moderate, and 3 = severe. All these patient's symptoms were improved and medication usages were reduced after immunotherapy. The detailed information of the patients is summarized in Table 1.

As a control population, we employed eight individuals without a clinical diagnosis and no known family history of atopic or autoimmune disease. Our analysis also included samples from our established cohort of autoimmune individuals which has been described before [12]. These studies were approved by the Vanderbilt University Institutional Review Board. All individuals in diseases groups and control group gave written informed consent.

**Sample preparation and microarray procedures** Analysis procedures presented here comply with minimal information about a microarray experiment (MIAME) guidelines established by the Microarray Gene Expression Data Society ([www.mged.org](http://www.mged.org)). PBMC were isolated from 20 mL heparinized blood on a Ficoll-Hypaque gradient. All samples were processed within 1 h of blood collection. Total RNA was isolated with Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH, USA), 5 µg RNA was reverse-transcribed by reverse transcriptase (Superscript II, Invitrogen Corporation, Carlsbad, CA, USA) in the presence of <sup>33</sup>P-dCTP. Labelled probes were purified using a Bio-Spin 6 Chromatography Column (Bio-Rad Laboratories Inc., Hercules, CA, USA). Before hybridization, GeneFilters membranes (GF-211, Research Genetics/Invitrogen Corporation) were washed in boiled 0.5% SDS, saturated with 5.0 mL Microhyb solution (HYB125.GF, Research Genetics/Invitrogen Corporation). Filters were treated with pre-hybridization reagents (5.0 µg Human Cot-1 DNA and 5.0 µg Poly dA, Invitrogen Corporation) in a hybridization roller tube (Midwest Scientific, St Louis, MO, USA) for 2 h at 42 °C. Purified, labelled probes were denatured and added to roller bottles containing filters and pre-hybridization solution. GeneFilters membranes were hybridized overnight at 42 °C. After hybridization, membranes were washed (first and second

wash:  $2 \times$  SSC, 1% SDS at 50 °C for 20 min; third wash:  $0.5 \times$  SSC, 1% SDS at 55 °C for 15 min). After washing, membranes were exposed to imaging screens for 24 h and the screens were scanned by a phosphorimager (Molecular Dynamics/Amersham Biosciences, Piscataway, NJ, USA). Acquired images by the phosphorimager were loaded into Pathways 4.0 software (Research Genetics/Invitrogen Corporation). The relative intensity of each spot on the membrane was determined and the microarray dataset was subjected to further analysis using the different analytical platforms.

Microarray data have been deposited into GEO database, accession number GSE1964 and series 1–5 (GSM35074–GSM35142).

**Data analysis** Cluster (version 3.0) [17] and JAVA Treeview [18] software programs were used to identify similarities among individual samples. Datasets were analysed using hierarchical clustering algorithms. As a separate approach, we treated expression levels of individual genes from the microarray datasets as separate variables. We calculated averages and standard deviations. Because of its familiarity, we used the Student's two-tailed, unpaired *t*-test to determine statistical significance, *P*-value <0.05.

**Biological process categorization by gene ontology** EASE: The Expression Analysis Systemic Explorer (<http://david.niaid.nih.gov/david/ease.htm>, EASE version 2.0) was used to search for common biological themes within gene lists generated by our microarray analysis. EASE assigns identified genes to 'GO: Biological Process' categories of the Gene Ontology Consortium ([www.geneontology.org](http://www.geneontology.org)) [19–21] and categories are tested statistically (EASE: Fisher's exact test) to identify over-represented categories of identified genes within the biological process system. Significant functional categories are those with the number of list hits (LH) of at least 2 with a *P*-value (EASE) <0.05 (Fisher's exact test).

### Statistical analysis

Data were normalized to yield an average intensity of 1.0 for each clone (4132) represented on the microarray. Reproducibility of the method was established by performing replicate hybridizations to separate microarrays. Linear regression analysis demonstrated that separate hybridizations yielded  $R^2$  values ranging from 0.87 to 0.96. Different exposure lengths of identical filters also produced high  $R^2$  values (0.99). The Student's *t*-test was used to determine statistical significance.

## Results

### Hierarchical clustering of gene expression profiles

We selected individuals with AR and performed gene expression profiling. Application of hierarchical clustering to gene expression profiles permitted accurate segregation of the AR group from control subjects and autoimmune subjects by using the entire microarray dataset that included 4132 genes. We also identified 68 over-expressed genes, and 165 under-expressed genes that exhibited a greater than threefold difference in expression between control individuals and

individuals with AR (before immunotherapy) ( $P < 0.05$ , Student's *t*-test). Using this smaller dataset, we were also able to perfectly segregate the AR group, independent of receipt of immunotherapy, from the other groups (Figs 1a, b, and c). These results demonstrate that each individual with AR has a common gene expression profile that is distinct from control individuals and individuals with autoimmune diseases. The ratio of expression levels (allergic/control) of genes that were differentially expressed in individuals with AR (before immunotherapy) compared with controls varied from a minimum of  $-4.64$  (log2) to a maximum of  $5.36$  (log2).

Given the small sample size of our base of subjects, we also wanted to use standard methods to evaluate if these differences between allergic and control subjects would achieve statistical significance. To do this, we treated the expression level of each gene as an individual variable and used, because of its familiarity, the student's *t*-test to determine statistical significance. We found a number of under-expressed and over-expressed genes that exhibited a high degree of difference between the two subject groups with a high level of statistical significance (Table 2). These same genes were also identified by the hierarchical clustering methods in the heat maps in Fig. 1.

According to recent discussions [22–28], we searched our microarray dataset for genes of possible relevance to AR, such as cytokines: *IL-3*, *IL-4*, *IL-5*, *IL-9*, *IL-10*, *IL-12*, *IL-13*, *GM-CSF*; cytokine receptors: *IL-4R*, *IL-10RA*, *IL-10RB*, *IL-13RA1*, *IL-13RA2*; chemokines and chemokine receptors: *T-bet*, *GATA3*; all the chemokines and chemokine receptors; immunoglobulin genes: *IgE*, *IgG*, *IgA*; cell surface markers: *CD4*, *CD8*, etc. However, these gene expression levels only exhibit modest differences between AR patients and healthy controls, except *IL-8* and *IL-15*. (GEO database: GSE1964: GSM35074–GSM35142)

Our criteria for a differentially expressed gene is that this gene exhibited a greater than threefold difference in expression between control individuals and individuals with AR (before immunotherapy) with  $P < 0.05$  (Student's *t*-test). Therefore, applying these criteria, the genes I discussed above cannot be considered as differentially expressed genes. In fact, in a previous report, Benson et al. [29] used microarrays to study gene expression in nasal biopsies from AR patients, they analysed genes of these cytokines, growth factors and their receptors, but the differences in gene expression between patients and controls were generally modest. However, even the gene expression difference between allergic patients and healthy controls is modest; it can still have a significant effect on pathogenesis of AR.

We also searched our dataset to identify the changes of these allergy-related genes before and after immunotherapy, only a few of their expressions are significantly changed ( $P < 0.05$ , student's *t*-test) after immunotherapy, such as *CCL16*, *CXCL13*, *CCL4*, *CCL25*, *CCL7*, but the fold changes are less than 2, and *TGF- $\beta$*  exhibit a twofold difference between before and after immunotherapy, however, the *P*-value of the statistical calculation for *TGF- $\beta$*  between before therapy and after therapy is 0.09.

In our dataset, several genes that have not been previously associated with atopic disease showed markedly different levels of expression between the two groups. These included *MARCH-VI*, *CASP6*, *RAB6*, and *MAN2A2*.

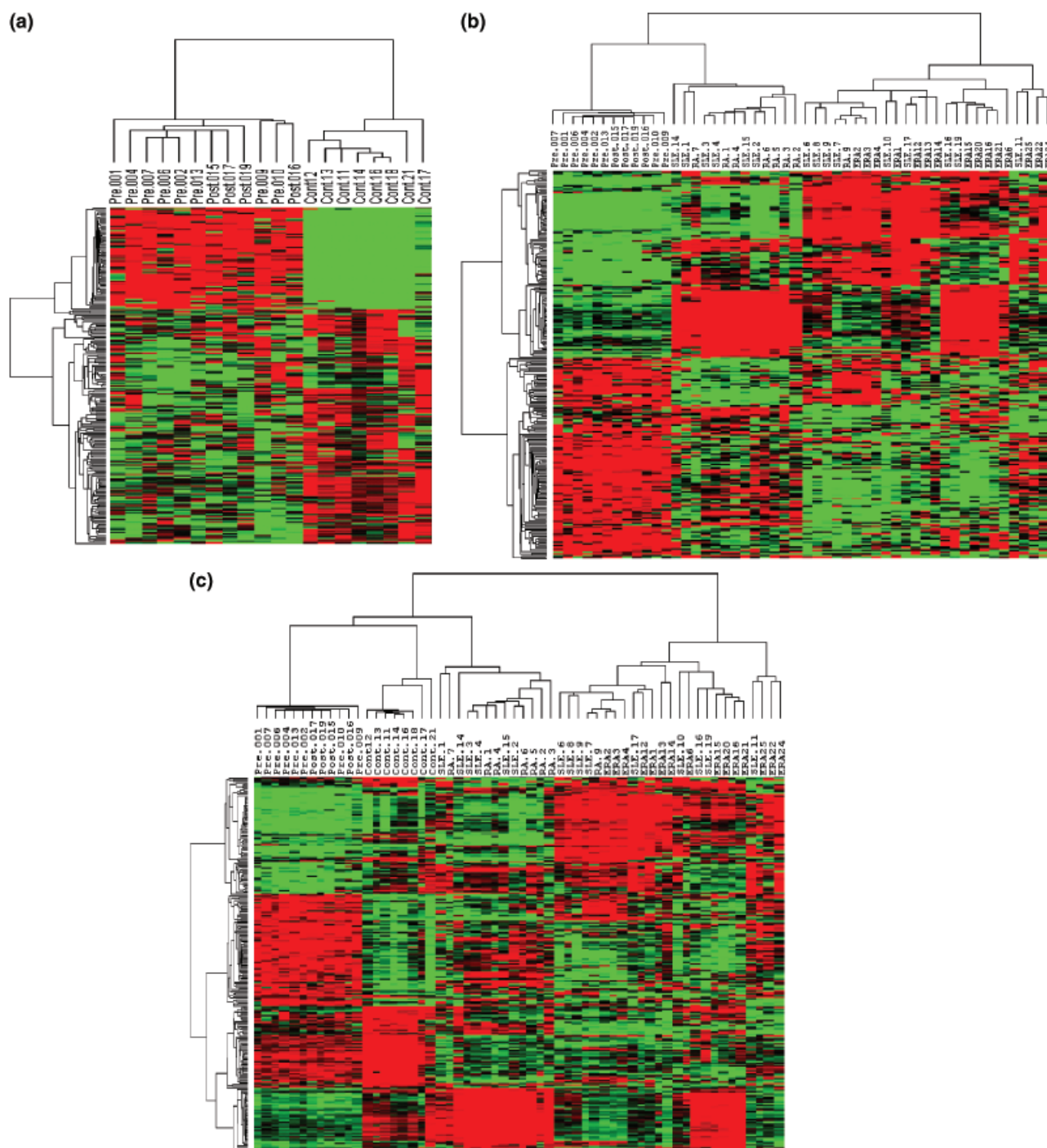


**Fig. 1.** Hierarchical clustering of gene expression profiles separates the allergic rhinitis (AR) group from other groups—control and autoimmune. Hierarchical clustering of microarray data using subjects with AR before (pre-) and after (post-) receipt of immunotherapy and age-matched controls (Cont) (a), and subjects with AR and autoimmune disease (SLE, RA, and ERA (early RA)) (b), and subjects with AR, control subjects and subjects with autoimmune disease (c).

#### Individual variability in differential gene expression

We selected the 10 most under- and over-expressed genes in the atopic population compared with non-atopic control individuals for further analysis and grouped control individuals (white bars), atopic individuals before therapy (black bars), and atopic individuals after therapy (hatched bars). We

found that each under-expressed gene exhibited a similar expression level in each atopic individual independent of therapy (Fig. 2a). *P*-values for each of these differences in gene expression between the allergic and control groups treated as an individual variable (Student's *t*-test), ranged between 0.01 and 0.0001. Over-expressed genes, as a group, exhibited somewhat greater variability in the atopic popula-

**Table 2.** Quantitative gene expression differences between the allergic and control groups

| Gene                          | Control     | Allergy     | P-value |
|-------------------------------|-------------|-------------|---------|
| <i>Under-expressed genes:</i> |             |             |         |
| CPNE1                         | 11.5 ± 3.9  | 0.5 ± 0.2   | <0.001  |
| MARCH-VI                      | 9.2 ± 6.0   | 0.5 ± 0.2   | 0.005   |
| CASP6                         | 9.4 ± 2.1   | 0.6 ± 0.2   | <0.001  |
| DIPA                          | 7.1 ± 1.8   | 0.5 ± 0.2   | <0.001  |
| CDKN1B                        | 8.9 ± 2.4   | 0.6 ± 0.2   | <0.001  |
| BPHL                          | 6.2 ± 2.0   | 0.5 ± 0.2   | <0.001  |
| SST                           | 8.0 ± 5.8   | 0.6 ± 0.2   | 0.009   |
| EFNB1                         | 7.8 ± 3.9   | 0.6 ± 0.2   | 0.001   |
| RIOK3                         | 5.2 ± 1.0   | 0.4 ± 0.2   | <0.001  |
| SLC1A5                        | 8.2 ± 4.3   | 0.7 ± 0.2   | 0.002   |
| PON3                          | 6.7 ± 4.0   | 0.6 ± 0.3   | 0.003   |
| GGTLA1                        | 4.5 ± 2.5   | 0.4 ± 0.2   | 0.002   |
| POR                           | 4.1 ± 2.2   | 0.4 ± 0.2   | 0.002   |
| PAR5                          | 5.0 ± 2.3   | 0.5 ± 0.2   | 0.001   |
| LMAN1                         | 4.2 ± 2.8   | 0.5 ± 0.2   | 0.006   |
| RAB7                          | 4.4 ± 2.3   | 0.5 ± 0.2   | 0.002   |
| UBE2I                         | 15.8 ± 12.4 | 1.8 ± 0.8   | 0.015   |
| GNAO1                         | 4.3 ± 2.0   | 0.5 ± 0.2   | 0.001   |
| TAF1C                         | 6.1 ± 2.7   | 0.7 ± 0.4   | 0.001   |
| <i>Over-expressed genes</i>   |             |             |         |
| RAB6                          | 0.3 ± 0.1   | 13.3 ± 11.7 | 0.016   |
| RPL10                         | 0.5 ± 0.2   | 4.5 ± 3.0   | 0.007   |
| APEH                          | 0.3 ± 0.1   | 2.4 ± 0.7   | <0.001  |
| CETN1                         | 0.3 ± 0.1   | 2.2 ± 1.7   | 0.014   |
| CD22                          | 0.3 ± 0.1   | 2.1 ± 0.9   | 0.001   |
| ESR1                          | 0.3 ± 0.1   | 1.7 ± 1.4   | 0.024   |
| CD53                          | 0.3 ± 0.1   | 1.4 ± 0.6   | 0.001   |
| MCSP                          | 1.4 ± 0.4   | 7.8 ± 4.2   | 0.003   |
| IL8                           | 0.3 ± 0.1   | 1.8 ± 0.6   | <0.001  |
| RPML3                         | 0.3 ± 0.1   | 1.2 ± 0.6   | 0.003   |
| PLGL                          | 0.4 ± 0.2   | 2.0 ± 0.7   | 0.001   |
| PDGFB                         | 0.2 ± 0.1   | 0.9 ± 0.4   | 0.002   |
| RGS10                         | 0.3 ± 0.1   | 1.4 ± 0.6   | 0.001   |
| HTR3A                         | 0.3 ± 0.1   | 1.2 ± 0.5   | 0.001   |
| PTP4A1                        | 0.3 ± 0.1   | 1.2 ± 0.6   | 0.005   |
| PROS1                         | 0.2 ± 0.1   | 0.8 ± 0.1   | <0.001  |
| RPL7A                         | 0.9 ± 0.3   | 3.5 ± 1.5   | 0.002   |
| UTX                           | 0.3 ± 0.1   | 1.2 ± 0.5   | 0.002   |
| SEMA5A                        | 0.2 ± 0.0   | 0.6 ± 0.2   | <0.001  |

Results are expressed as the average expression level (control,  $n = 8$ ; allergic,  $n = 8$ ) ± standard deviation.  $P$ -values were determined by the student's  $t$ -test.

tion (Fig. 2b). Some of the variability in the group of over-expressed genes appeared to reflect responses to therapy. For example, expression levels of *HBE1* and *MSTP9* were relatively high in individuals before immunotherapy but were reduced to control levels after immunotherapy.

**Expression Analysis Systemic Explorer analysis of differentially expressed genes in individuals with allergic rhinitis** We performed EASE analysis to categorize the over- and under-expressed genes in the AR group. The gene ontology biological process categories in which over- or under-expressed genes were over-represented by EASE scores (Fisher's exact test,  $P < 0.05$ ) are shown in Table 3. The most over-represented categories in the over-expressed gene group

included inflammatory responses, signal transduction and stress responses. In the under-expressed gene group, we surprisingly found that the most over-represented category was the biological process of apoptosis and activation of the JNK cascade that is also critical for many pathways of apoptosis.

### Immunotherapy alters gene expression profiles

Immunotherapy is a common and effective treatment for atopic disease but its precise mode of action is not fully understood. We wanted to determine if immunotherapy affects the gene expression profile observed in the PBMC of subjects with AR. To do so, we performed hierarchical clustering analysis of subjects with AR pre- and post-immunotherapy using different standard deviations to evaluate different numbers of genes in the dataset. We were unable to perfectly discriminate between the pre- and post-immunotherapy groups after filtering the dataset by using 1, 1.5, or 2 standard deviations (447, 228, and 123 genes, respectively) (data not shown). However, by decreasing the standard deviation of the filtering condition we were able to include more data for analysis and found that expression levels of 563 genes could significantly segregate allergic individuals based upon receipt of immunotherapy ( $P < 0.05$ , Fisher's exact test) (Fig. 3a).

Next, we wanted to determine if immunotherapy resulted in detectable changes in gene expression in PBMC of individuals with AR. We searched our microarray dataset to identify changes in expression levels of specific genes in allergic individuals before and after receipt of immunotherapy. We identified a group of genes whose expression levels were high or low in allergic individuals (before immunotherapy,  $P < 0.05$ ) compared with controls that partially or completely returned to control levels after immunotherapy (Fig. 3b,  $P < 0.05$ ). In contrast, genes that were under-expressed in the allergic group before immunotherapy ( $P < 0.05$ ) remained under-expressed after immunotherapy (Fig. 3c, representative examples).

We subjected these 563 discriminatory genes to EASE analysis as shown in Table 4. The most over-represented category was 'response to external stimulus'. In fact, many of the gene ontology groups selected by the EASE analysis were groups that are typically considered to participate in the innate or adaptive immune response. These results suggest that immunotherapy may alter the activities of these pathways to alleviate the symptoms of allergy.

### Scoring system to classify atopic disease

As we have done with autoimmune disease [12, 30], we determined if we could devise a scoring system based upon expression levels of small numbers of genes that would classify individuals as allergic and distinguish these individuals from control individuals and those with autoimmune disease. We selected 15 under-expressed genes and five over-expressed genes that, based upon our analysis of the datasets, appeared to have the greatest discriminatory power. To give each gene equal weight in the scoring system, we summed the average expression value in the control ( $N = 8$ ) and allergic group ( $N = 6$ ) for each gene and divided by 2 ((control+allergy)/2). We inspected expression levels of each gene in each

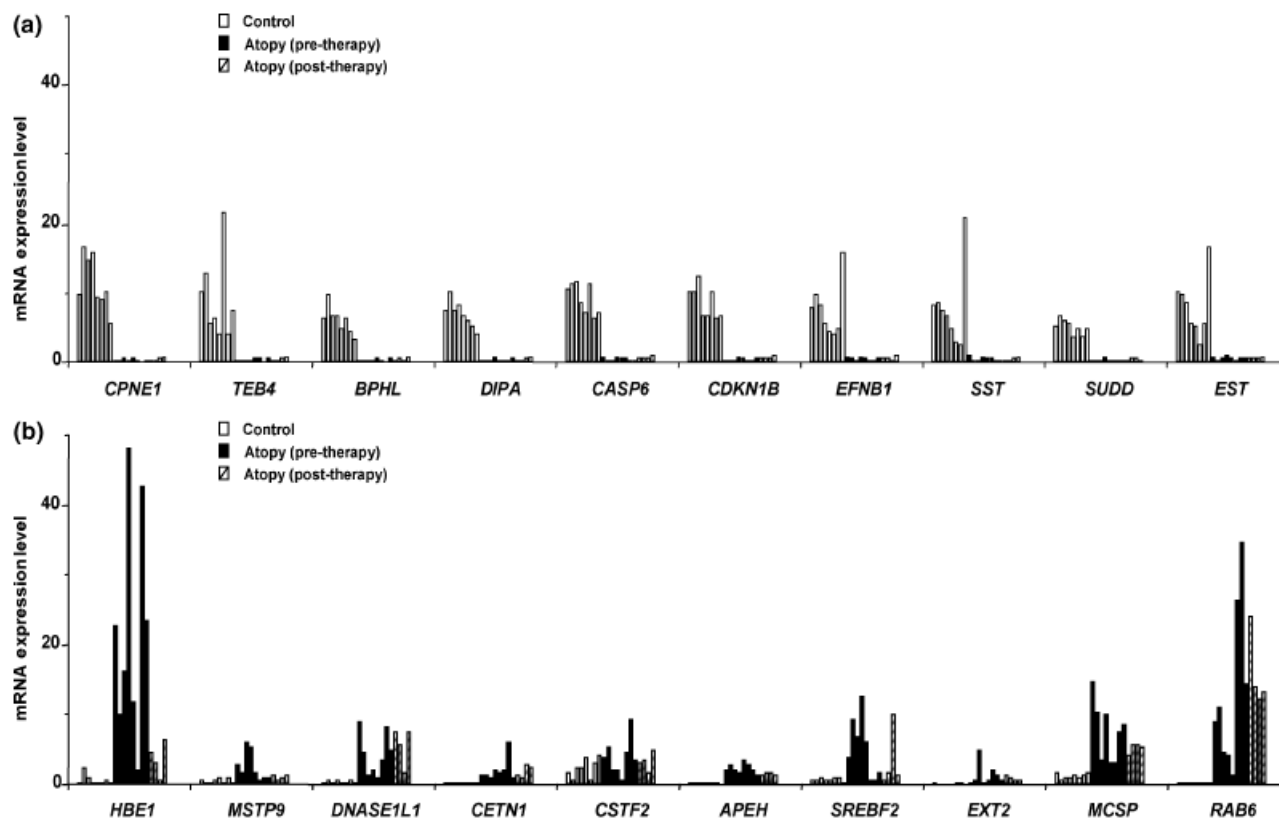


**Fig. 2.** Individual variation in gene expression in allergy. Expression levels of individual genes were compared in eight control individuals (white bars) and 12 allergic rhinitis individuals (eight pre-therapy, black bars, four post-therapy, hatched bars). Under-expressed (a) and over-expressed (b) genes were analysed.

sample and assigned it a value of 0 if it was less than the average and 1 if it was greater than the average. The maximum possible score was 15 or 5 for the under- or over-expressed gene categories, respectively, and the minimum possible score was 0 (Fig. 4a). For under-expressed genes, the range of scores for non-allergic individuals (control and autoimmune) ranged from 8 to 15. All allergic individuals used to derive the initial scoring system achieved a score of 0 in the test. We also analysed expression data from six additional allergic individuals who were not included to derive the initial scoring system. They also received a score of 0 in the test. Examination of over-expressed genes yielded similar results. Both groups of allergic individuals received scores of 4–5 while all other individuals received scores of 0–1. All these comparisons yielded high levels of statistical significance ( $P \leq 0.0001$ ). In addition, no individual was incorrectly assigned to or excluded from the allergy population based upon score results. Similarly, we designed scoring systems to discriminate the status based upon receipt of immunotherapy. We employed two groups of genes whose expression levels were high (group 1) or low (group 2) in allergic individuals (before immunotherapy,  $P < 0.05$ ) compared with controls and which partially or completely returned to control levels after immunotherapy. We can clearly discriminate pre- and post-therapy groups: by using group 1 genes, the pre-therapy group received scores of 3–7, the post-therapy group received scores 0–1; by using group 2 genes, the pre-therapy group received scores

of 0–2 and the post-therapy group received scores of 6–7 (Fig. 4b).

## Discussion

Our results indicate that there is a conserved gene expression profile in individuals with AR. In this study, the small sample size may be expected to limit our conclusions, especially as statistical methods for microarray analysis are oftentimes absent or unfamiliar. Therefore, we also wanted to evaluate statistical significance of the microarray results using more familiar methods. To do so, we also treated each gene as an individual variable and used the student's *t*-test to determine statistical significance. We found that this approach was sufficient to identify a number of genes that were differentially expressed between the control and allergic groups with a high degree of statistical significance, even for such a small sample size. This supports the idea that atopic individuals have a conserved gene expression profile in their PBMC. Using similar approaches, we have also found a conserved gene expression profile in individuals with autoimmune disease. As with autoimmune disease, we find that expression profiles in all individuals with AR are extremely consistent. There is <5% overlap between these two gene expression profiles. Taken together, these results demonstrate that the two major immune-mediated diseases in the human population are associated with two unique gene expression profiles

**Table 3.** Biological process categories significantly over-represented ( $P < 0.05$ ; Fisher's exact test) by differentially expressed genes of individuals with allergic rhinitis are shown

| Over-expressed genes |    |      | Under-expressed genes    |    |      |
|----------------------|----|------|--------------------------|----|------|
| Gene category        | LH | EASE | Gene category            | LH | EASE |
| Cellular process     | 31 | 0.03 | Induction of apoptosis   | 5  | 0.03 |
| Cell communication   | 19 | 0.02 | JNK cascade              | 3  | 0.01 |
| Signal transduction  | 15 | 0.04 | Activation of JUNK       | 2  | 0.01 |
| Response to stress   | 8  | 0.04 | Lipoprotein biosynthesis | 2  | 0.01 |
| Cell motility        | 5  | 0.03 | Cellular morphogenesis   | 2  | 0.02 |
| Angiogenesis         | 3  | 0.00 | Lipoprotein metabolism   | 2  | 0.02 |
| Cell growth          | 3  | 0.02 | Glycerophospholipids     | 2  | 0.03 |
| Chemotaxis           | 3  | 0.03 | DNA replication          | 2  | 0.04 |

LH, list hits, the number of identified differentially expressed genes within the specific category. EASE, Fisher's exact test.

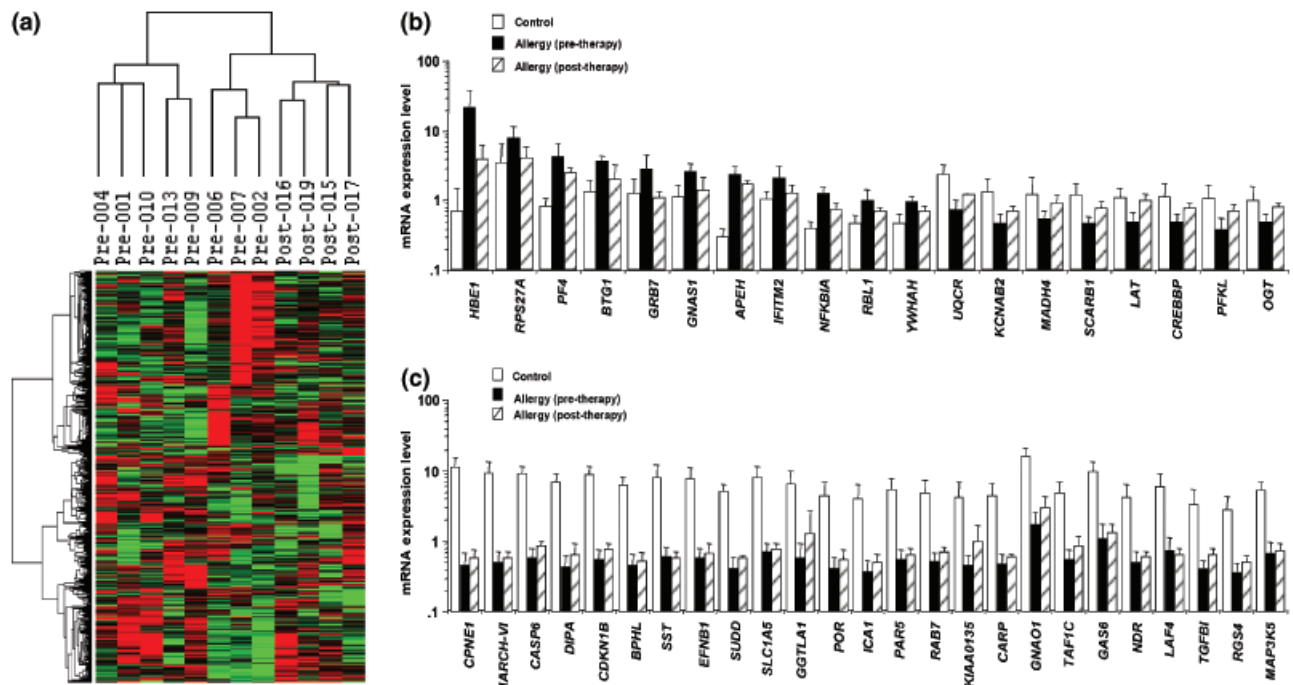


**Fig. 3.** Immunotherapy alters gene expression profiles of individuals with allergic rhinitis. (a) Hierarchical clustering analysis was performed with a group of atopic individuals before (pre-) or after (post-) immunotherapy. Expression levels of 563 genes accurately segregated the pre- and post-immunotherapy groups into separate branches. (b) Genes that change expression levels after immunotherapy are shown. Levels of gene expression for control (white bars) and pre- (black bars) and post-immunotherapy (hatched bars) (normalized average expression  $\pm$  SD) are shown, the changes between control and pre-immunotherapy, and changes between pre-immunotherapy and post-immunotherapy are significant ( $P < 0.05$ , Student's *t*-test). (c) As in (b), except that genes that do not change expression levels after immunotherapy are shown (changes between control and pre-immunotherapy are significant,  $P < 0.05$ , and changes between post-immunotherapy and pre-immunotherapy are not statistically significant).

in PBMC. It is our hypothesis that these alterations in gene expression result in alterations in cellular functions that confer a liability that an individual will develop atopic or autoimmune disease, respectively.

The source we used for this study is PBMC instead of affected tissues. One of the reasons to use PBMC is easy access. Also, surgically removed affected tissues are generally only in small size that cannot generate enough amount of RNA used for microarray study, and the positions to obtain the tissues may generate more variation compared with PBMC, because the changes of positions may result in variation of cell populations which can cause greater variations in microarray study, as there are increases in recruitment of certain inflammatory cells around affected

tissues, which can contaminate the source for microarray study [31]. In contrast, current protocol can generate enough RNA from PBMC and also the current protocol for isolating the PBMC from the whole blood can greatly decrease the contamination of other source.

In this study, we also designed scoring systems, based upon levels of expression of individual genes, one scoring system can correctly classify individuals as having AR and exclude controls and individuals with autoimmune disease from this category, similarly, and another system can correctly classify individuals based upon receipt of immunotherapy. We have taken a similar approach to the analysis of autoimmune disease and find that we can also design a scoring system, based upon expression levels of small numbers of genes,

**Table 4.** Biological process categories significantly over-represented ( $P < 0.05$ ; Fisher's exact test) in the 563 gene list that permits accurate separation of the pre- and post-immunotherapy groups are shown

| Gene category                    | LH | EASE   |
|----------------------------------|----|--------|
| Response to external stimulus    | 77 | 0.005  |
| Response to biotic stimulus      | 61 | <0.001 |
| Biosynthesis                     | 58 | 0.004  |
| Defense response                 | 56 | 0.001  |
| Macromolecule biosynthesis       | 50 | 0.002  |
| Immune response                  | 50 | 0.004  |
| Protein biosynthesis             | 41 | 0.000  |
| Cell motility                    | 26 | 0.015  |
| Muscle contraction               | 14 | 0.009  |
| Antigen presentation             | 8  | <0.001 |
| Ribosome biogenesis and assembly | 7  | 0.002  |
| Cell cycle checkpoint            | 7  | 0.004  |
| DNA recombination                | 7  | 0.034  |
| Smooth muscle contraction        | 4  | 0.029  |
| Centrosome cycle                 | 3  | 0.001  |
| DNA damage response              | 3  | 0.012  |

LH, list hits, the number of identified genes within the specific category. EASE, Fisher's exact test.

which can correctly classify individuals as autoimmune. These scoring systems may aid clinicians by offering analyses that can correctly classify or exclude an individual from the atopic or autoimmune category and can monitor the responses to immunotherapy. Therefore, predictive tests that absolutely classify individuals into these disease categories or monitor their responses to therapy may aid the decision-making process by the clinicians.

We have four patients who are monitored after immunotherapy, the limited sample size is always a issue needed to be handled in microarray study, generally complicated statistical methods are applied to validate the results [9], in our study, the hierarchical clustering algorithm can significantly separate the post-immunotherapy group from pre-immunotherapy group ( $P < 0.05$ , Fisher's exact test), also the gene expression profile of our scoring systems can discriminate the pre- and post-immunotherapy groups, this also cross validates our results.

We used a new computer program, EASE, to search for common biological themes represented by the genes that are differentially expressed in atopy. EASE assigns the identified genes to biological processes based upon the Gene Ontology Consortium and employs statistical methods (Fisher's exact test) to identify over-represented biological process categories in a given gene list. We separated our gene lists into two

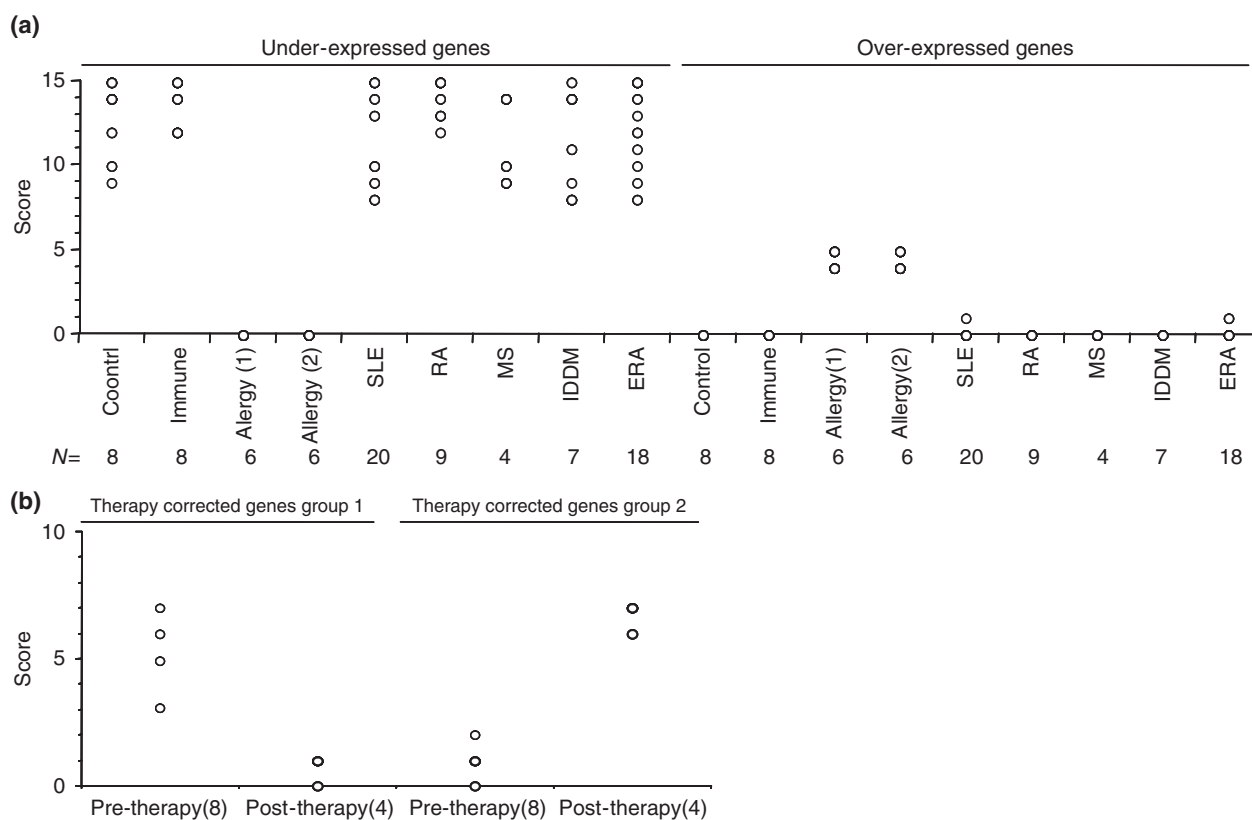


**Fig. 4.** Gene expression profiles accurately discriminate allergy from autoimmunity and pre-therapy from post-therapy. (a) The score (y-axis) is shown for each individual subject analysed from the different populations (x-axis). We used 15 under-expressed genes (left panel) and five over-expressed genes (right panel) to develop the scoring system. Numbers of samples (N) for each category are shown below the graphs. The allergy (1) group was used to derive the scoring system and the allergy (2) group was used to test the scoring system. Under-expressed genes: *MARCH-VI*, *SST*, *EFNB1*, *SLC1A5*, *GGTLA1*, *POR*, *TAF1C*, *PAR5*, *RAB7*, *CARP*, *MAP3K5*, *GNAO1*, *LAF4*, *LILRB2*, and *TGFBI*, over-expressed genes: *RPML3*, *CD53*, *CD22*, *APEH*, and *CETN1*. (b) As in (a), we used genes whose expression levels have been corrected after immunotherapy to design scoring system. We have 'therapy corrected genes group 1': *HBE1*, *PF4*, *BTG1*, *GRB7*, *APEH*, *NFKBIA*, *RBL1*, and *YWHAH*. And 'therapy corrected genes group 2': *UQCR*, *KCNAB2*, *MADH4*, *LAT*, *CREBBP*, *PFKL*, and *OGT*.

categories, over- and under-expressed genes. The over-expressed gene groups in atopy could be broadly categorized as contributing to specific aspects of the innate or adaptive immune response. Genes in the under-expressed group could be broadly categorized as genes that encode proteins required for apoptosis. Taken together, the observed alterations in these two groups suggest that both chronic activation (over-expressed gene group) and reduced apoptosis (under-expressed group) may contribute to the chronicity of AR. This is consistent with the suggestion that reduced apoptosis in inflammatory cells may contribute to the chronicity of inflammatory processes [32]. Many different pathways are involved in apoptosis, including signaling through death receptors (*FAS*, *TNFR1* [33]), the p53 pathway [34], the Bcl-2 family of genes [35] and the caspase cascade of effector proteases [35]. In our dataset, *CASP6* is under-expressed by about 20-fold and *CASP4* is under-expressed by about threefold. Components of other apoptosis pathways, such as, *FAS*, *TP53*, and *BCL2*, are not differentially expressed in AR PBMC.

A second goal of microarray-based research in human disease is the identification of genes whose expression levels change in response to a therapy. In this regard, these 'biomarkers' may provide quantitative measurements that demonstrate whether or not a given treatment is having its desired effect. Measurements of biomarker levels could augment clinician and patient assessments of therapeutic effectiveness of a given treatment. Desensitization by immunotherapy is a common effective treatment for AR although its precise mechanisms are not fully understood [7].

The immunological changes associated with immunotherapy have been discussed that specific immunoglobulin (Ig)E levels decrease and IgG titres often rise, perhaps further inhibiting IgE production by neutralizing the antigen and by antibody feedback [36]. It is also possible that immunotherapy may work by inducing specific T cell tolerance or by changing the predominant phenotype of antigen-specific T cells from T helper type 2 (Th2) to Th1 [37, 38]. However, most of these studies test the production of cytokines or immunoglobulins at proteins levels serologically, and it has also been described elsewhere that the Th2–Th1 shift represented by mRNA expression changes of cytokines happened in local nasal but not in peripheral blood [39, 40]. In our study, we used freshly isolated PBMC to examine the transcription levels of the genes that are suspected to have relevance in AR such as cytokines, chemokines, etc., these genes only exhibit modest differences at transcription levels between pre- and post- immunotherapy. However, our analysis of gene expression profiles of PBMC from atopic individuals before and after immunotherapy permitted accurate segregation of these two groups ( $P < 0.05$ , Fisher's exact test). Surprisingly, measurement of the contribution of expression levels of a large number of genes ( $> 500$ ) was required to achieve complete segregation of the atopic group into pre- and post-therapy subgroups. This result implicates that immunotherapy can alter the gene expression profile, but the genes that are conventionally considered to be related to AR did not exhibit substantial changes upon immunotherapy in PBMC. And the allergy signature was a relatively stronger signature imprinted in the patients before or after immunotherapy, we have to have more gene expression signals to

discriminate the gene expression profiles pre-immunotherapy from post-immunotherapy.

When we looked at changes in expression levels of individual genes, we found that we could identify a number of genes whose expression levels were significantly high or low in the atopic group compared with the control group and returned to control levels after immunotherapy. These genes are potential biomarkers that may augment the ability of a clinician to monitor responses to immunotherapy. Whether expression levels of these same genes would also change in response to other forms of therapy that reduce the symptoms of AR remains to be determined. In contrast to these results, expression levels of those genes that are low in atopy compared with control do not change after immunotherapy. A future goal of drug discovery may be to try to change expression levels of this group of genes, which may provide for novel approaches to treatment of these diseases. Nevertheless, these initial experiments demonstrate that it is feasible to use gene expression changes as biomarkers to monitor therapeutic responses to a complex disease such as AR.

We also applied EASE analysis to the group of genes chosen by computer that permitted accurate segregation of patients before and after immunotherapy. The most over-represented categories in this gene list may be broadly classified as those that contribute to an immune response. These categories include responses to external stimuli, defense responses, immune responses, biosynthesis, cell motility, and antigen presentation, all key elements of the innate and acquired immune response. Taken together, these results provide molecular support for the observed clinical role of immunotherapy, de-sensitization of the host.

In conclusion, there is a highly conserved gene expression profile among individuals with AR, and immunotherapy alters this gene expression profile. Knowledge of these gene lists and the changes following immunotherapy may identify potential therapeutic targets and potential biomarkers that may be used to monitor therapeutic responses. Both of these may contribute to better care of patients with atopic diseases.

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