

Short communication

Analysis of CD4+ T-cell gene expression in allergic subjects using two different microarray platforms

Background: Allergic diseases are thought to involve dysregulated activation of T cells including CD4+ lymphocytes. T-cell activation results in changes in gene expression, but the optimal method to study gene expression profiles in T cells, and how this changes over time, are not known.

Methods: Circulating CD4+ T cells were obtained from subjects with atopic asthma, nonatopic asthma or nonallergic controls, and total mRNA was rapidly isolated. Atopy was defined as positive skin prick test to one of nine allergens. Gene expression was analyzed using hybridization and Affymetrix® oligonucleotide arrays (Hu133A and Hu133B chips, $n = 84$), or by reverse transcription-polymerase chain reaction (RT-PCR) with a pathway-targeted array (Human Th1–Th2–Th3 RT² Profiler™ PCR Array, Superarray, $n = 16$).

Results: Using Affymetrix arrays, it was difficult to discern a dominant allergy-associated profile because of heterogeneity in gene expression profiles. In contrast, a Th2-like signature was evident using RT-PCR arrays with increased expression of expected genes (e.g. IL-4, 5, 9, and 13, all $P < 0.05$) as well as unexpected gene transcripts (e.g. osteopontin). Gene expression profiles were relatively stable over time in circulating CD4+ T cells from two subjects using both platforms.

Conclusions: Unstimulated CD4+ T cells isolated from allergic subjects express a characteristic profile of genes when analyzed using RT-PCR based microarrays.

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Allergic diseases are considered to reflect a 'Th2 biased' immune response against allergens and other environmental exposures, which involves both genetic and environmental factors (1, 2). Microarrays have been used to identify new allergy-associated genes in different tissues including peripheral blood mononuclear cells (PBMCs) (3, 4), even though PBMCs are a heterogeneous mixture of different cell types. We recently analyzed the gene expression in circulating CD4+ T cells from allergic asthmatics, using Affymetrix oligonucleotide arrays. In preliminary studies, we found that 30 genes were either up- or down-regulated in CD4+ T cells from five subjects with severe allergic asthma, including about 10 genes not previously implicated in asthma pathogenesis (5). In subsequent studies, we analyzed CD4+ gene expression in more than 80 allergic subjects characterized according to skin test reactivity. Interestingly, in these analysis we did not detect a dominant allergy-associated profile in CD4+ T cells from allergic or nonallergic subjects (unpublished data, $n = 84$). However in these studies, the expression

of interleukins, including the Th2 signature cytokines IL-4, 5, and 13, was at or near the detection limit of the arrays (data not shown).

One possibility was that circulating allergen-specific Th2 cells are very rare, and that Th2-associated genes would therefore be of low abundance. Therefore, we hypothesized that Th2-associated genes may be more easily detectable using assays involving an amplification step [e.g. with reverse transcription-polymerase chain reaction (RT-PCR)]. To test this possibility, nine subjects with atopy (A) and seven subjects without atopy (NA) were studied. All subjects were female, and were equally matched in terms of age (37 vs 34 years, A vs NA), race (6 vs 7 European American, A vs NA), and medication use (four in each group on inhaled corticosteroids). Atopy was defined as a positive skin prick test (≥ 3 mm) to one of nine common aero-allergens (Greer Laboratories, Lenoir, NC, USA) including *Penicillium notatum*, *Alternaria tenuis*, Cockroach mix (German and American), Dust mite mix (*D. farinae* and *D. pteronyssinus*), Dog Hair, Cat Hair, Grass pollen mix (Kentucky,

Orchard, Red Top Timothy), eastern Oak Pollen mix (Black, Red and White Oak), Ragweed Pollen (Giant, Short), compared with negative control (glycerine). Subjects were defined as nonatopic, if they had a negative skin prick test to the nine allergens as well as positive reaction to histamine. Eight of the nine atopic subjects and five of the seven nonatopic subjects had asthma. Active smokers and subjects on oral corticosteroids were excluded. Each subject was provided written informed consent, and the study was approved by the Western Institutional Review Board (WIRB), Olympia, WA, USA. Sixty milliliter of peripheral blood was collected and CD4+ mRNA was isolated within 2 h of the initial phlebotomy as described (5). The purity of the CD4+ T cells was >95% as determined by flow cytometry (not shown). Quantitative RT-PCR was performed using manufacturer's protocols for the RT² ProfilerTM PCR Array (Human Th1–Th2–Th3, Superarray, Gaithersburg, MD, USA). Relative gene expressions were calculated by using the $2^{-\Delta\Delta C_t}$ method, in which C_t indicates the fractional cycle number where the fluorescent signal reaches detection threshold. The 'delta–delta' method (which is described in Ref. (6) uses the normalized ΔC_t value of each sample, calculated using a total of five endogenous control genes (18S rRNA, *HPRT1*, *RPL13A*, *GAPDH*, and *ACTB*). Fold change values are then presented as average fold change = $2^{-(\text{average } \Delta\Delta C_t)}$ for genes in treated relative to control samples. Clinical variables were characterized using descriptive statistics, and the statistical significance of differences in gene expression between groups was calculated using the student's *t*-test.

Detectable PCR products were obtained for 74/83 genes in most subjects (defined as requiring <35 cycles, Fig. 1). Seven genes were not detected including *CCL7*,

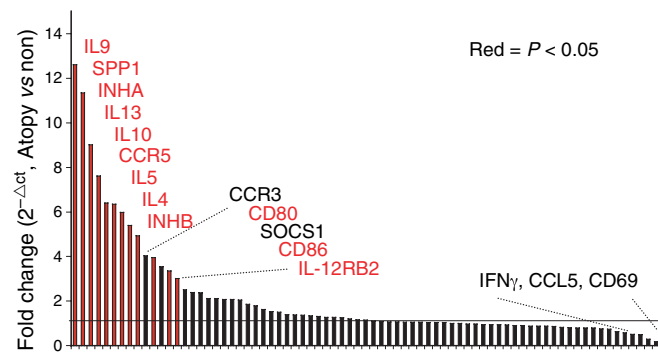


Figure 1. CD4+ T-cell gene expression profiles obtained from allergic vs nonallergic subjects were determined using RT-PCR-based arrays. Fold-changes of 74 transcripts is shown expressed as fold-change according to the $2^{\Delta\Delta C_t}$ method. Genes that were significantly changed are indicated in red ($P < 0.05$). The *x*-axis is not fully annotated for clarity, but the 14 most highly increased and three most decreased genes are indicated. See also Table 1.

Table 1. Up-regulated genes between atopic and nonatopic individuals

Gene name	Gene symbol	Fold change	P-value
Up-regulated genes			
Interleukin-9	IL9	12.6	0.013
Osteopontin	SPP1	11.4	0.019
Inhibin, alpha	INH1A	9.0	0.029
Interleukin-13	IL13	7.6	0.018
Interleukin-10	IL10	6.4	0.014
Chemokine receptor 5	CCR5	6.4	0.023
Interleukin-5	IL5	6.0	0.030
Interleukin-4	IL4	5.4	0.033
Activin AB alpha polypeptide	INHBA	4.9	0.005
CD80 antigen	CD80	3.9	0.024
CD86 antigen	CD86	3.4	0.021
Interleukin 12 receptor, beta 2	IL12RB2	3.0	0.025
Housekeeping control genes			
	18sRNA	−0.24	NS
	HPRT1	−0.18	NS
	RPL13A	−0.41	NS
	GAPDH	−0.29	NS
	ACTB	−0.33	NS

NS, non significant.

IL12B, *IL6*, *PCGF2*, *SOCS5*, *TLR4*, and *TNFRSF9*. Two genes gave more than one peak on dissociation curves (*CSF2* and *GFI1*), indicative of nonspecific priming: this was reported to the array manufacturer and the data was excluded from subsequent analysis. Expression of the five housekeeping genes (18SrRNA, *HPRT1*, *RPL13A*, *GAPDH*, and *ACTB*) was similar between allergic and nonallergic subjects (Table 1). Twelve genes were expressed at significantly higher levels in CD4+ T cells from allergic compared with nonallergic subjects including the Th2 cytokines *IL4*, *IL5*, *IL9*, and *IL13* (Table 1). Expression of these cytokines was not detected by Affymetrix arrays using the same mRNA pool derived from 11 subjects (data not shown). The Th2-inducing transcription factors *STAT6* and *GATA3* were not differentially expressed (data not shown). Other genes more highly expressed included *osteopontin* (11.3-fold), *CCR5* (6.4-fold) and *inhibin A* and *B* (9.0- and 4.9-fold, respectively). The most highly down-regulated genes were *IFNG*, *CCL5*, and *CD69* (0.5, 0.3, and 0.2-fold, respectively), although these results did not reach statistical significance.

To determine if the CD4+ gene expression profiles were stable over time, CD4+ cells were obtained on two separate occasions from two individuals and mRNA analyzed using both the RT-PCR array platform (Fig. 2) and Affymetrix arrays (Fig. 3). These revealed that gene expression profiles were generally stable over time, with a few outlying genes. Most of the outliers in Fig. 3A are from the hemoglobin family, likely reflecting low level contamination with erythrocytes in this particular CD4 sample.

Taken together, our data show that: (i) RT-PCR arrays are a sensitive platform that can detect Th2 and other cytokine transcripts in unstimulated circulating CD4+

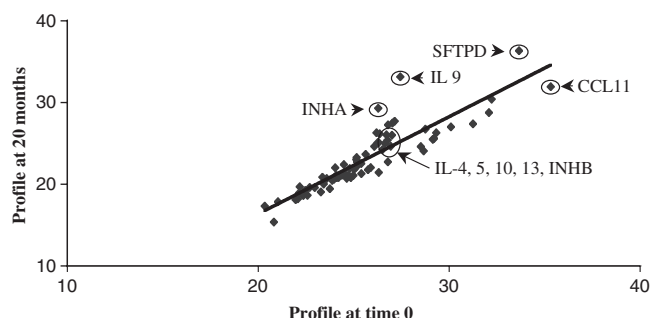


Figure 2. Stability of CD4+ gene expression profile over time using RT-PCR arrays. Gene expression profiles were obtained in one allergic subject 20 months apart and expressed as Ct values for each transcript. Outlying genes as well as a cluster of cytokines are indicated. Abbreviations are as in Table 1. CCL, C-C chemokine ligand; SFTPD, protein D.

T cells; and (ii) gene expression patterns in circulating CD4+ T cells are relatively stable over time. Increased sensitivity of the Superarray platform likely reflects the amplification of low abundance transcripts during RT-PCR. The fundamental problem with microarray sensitivity is that it must accommodate a huge dynamic range of gene expression in a single processing event. Low abundance mRNA species may have only a few copies per cell for some important genes, yet the array platform must simultaneously accommodate actin, ribosomal genes, and other high abundance 'housekeeping' genes. Amplification of the mRNA to levels which permit detection of low abundance genes results in saturation of the microarray response for high abundance genes; this

in turn complicates or prevents analysis by skewing array data normalization. Because RT-PCR is based upon quantifying the amplification step where the gene is first detectable, saturation of the response is not an analysis issue. Consequently, microarray dynamic range is typically 3–4 log units of expression while high quality RT-PCR can achieve 6–8 log units (7).

Our study supports that idea which unstimulated circulating CD4+ T cells can provide a window into dysregulated immune responses in allergy (8), but a clear cautionary note is indicated because of the fact that most of the subjects we analyzed using the RT-PCR platform had asthma. The immunopathology of atopic and nonatopic asthma are quite similar, and we only detected significant differences when we retrospectively stratified subjects according to skin test reactivity. It should be of interest in future studies; however, to use a similar approach in prospective analyses of well characterized subjects with and without different atopic diseases including asthma.

Despite these limitations of our study, we did observe high expression of Th2 cytokines (*IL4*, *5*, *9* and *13*) and low expression of *IFNG* in CD4+ T cells from atopic vs nonatopic asthmatics. This may reflect constitutive gene expression in circulating allergen-specific T cells, but it is currently not possible to identify these cells without re-stimulation in the circulating pool. We also identified increased expression of several genes not typically associated with Th2 immune responses including *osteopontin* (*SPP1*), *CCR5* and *IL12RB2*. Osteopontin regulates apoptosis and may influence the Th1/Th2 balance. Polymorphisms in the osteopontin gene have been associated with IgE levels (9), and osteopontin was recently

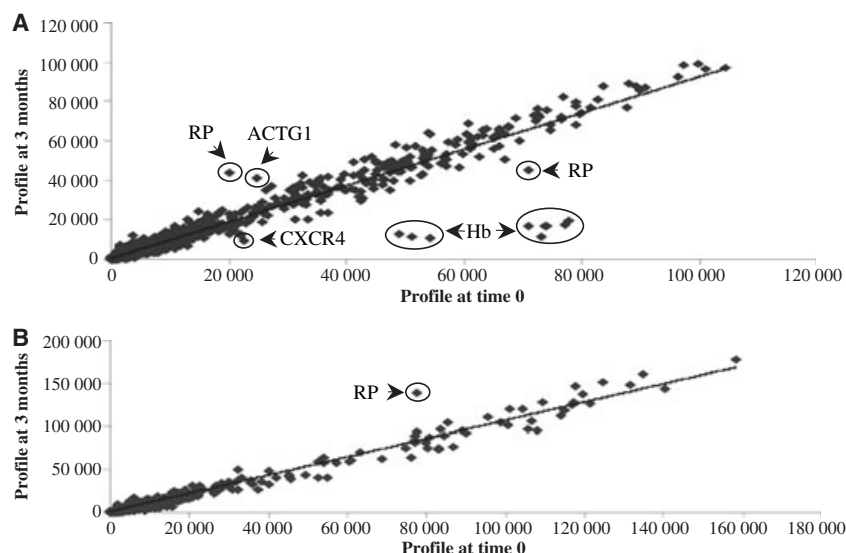


Figure 3. Stability of CD4+ gene expression profile over time using Affymetrix arrays. Gene expression profiles were obtained in one nonallergic subject 3 months apart and expressed as relative expression levels values for each transcript for the Hu133A chip (A) and Hu133B chip (B). ACTG1, actin gamma 1; CXCR4, C-X-C chemokine receptor 4; Hb, hemoglobin; RP, ribosome protein.

shown to be expressed in the lungs of asthmatic subjects and influence dendritic cell differentiation (10). We also found that activin A (*INHBA*) and its naturally occurring antagonist, inhibin- α (*INHHA*) were also upregulated in CD4+ T cells from atopic individuals. Interestingly, activin A was recently identified as a Th2-associated cytokine (11), and our results support the observation of Karagiannidis et al. (12) who showed that activin A mRNA expression was increased in CD4+ T cells from subjects with asthma. It will be important in future studies to compare expression of these different cytokines and mediators in CD4+ T cells from subject groups with

and without different atopic diseases including asthma. Furthermore, analysis of the CD4+ T cell transcriptome using RT-PCR based arrays may prove useful in following disease activity or response to therapy.

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