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Genome-wide expression profiling of B lymphocytes reveals IL4R increase in allergic asthma

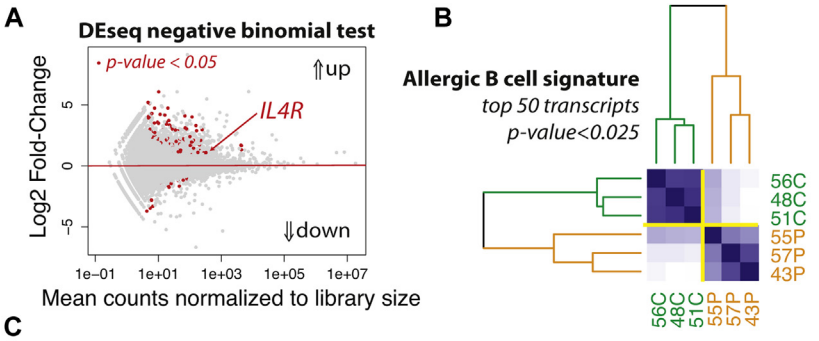
To the Editor:

Allergic asthma is a multifactorial disorder in which activation and differentiation of B lymphocytes toward the production of IgE play an essential role. In these B cells, molecular mechanisms to generate IgE antibodies from IgM depend on a complex network of molecules that trigger class-switch recombination specifically at the Sε region of the IgH locus. Several transmembrane receptors and activating signaling cascades are known to physiologically participate in these processes, including the IL4R signaling pathway. In this regard, our group has studied in the past genetic polymorphisms in the IL4/IL4R pathway that might hold clinical correlations with atopy and asthma.[1,2] Because IgE antibodies play a key role as allergen recognizers, understanding the deregulation of IgE synthesis in B cells is crucial to elucidate the pathogenesis of allergic asthma and to identify B-cell–specific targets with therapeutic potential. In this sense, genome-wide association studies have been proven useful to identify some associated gene candidates and pathways involved in the pathogenesis of the disease.[3] Indeed, we have previously suggested that a more intricate interaction of genetic and epigenetic factors probably leads to a specific transcriptome, characteristic of the allergic B cell.[4,5] For this reason, we decided to use "next-generation" massively parallel sequencing technologies for quantitative transcriptomics (RNA-seq) of circulating allergic human B cells. The aim of this study was to explore for the first time by using RNA-seq whether aberrant gene expression of B cells could reveal pathological hallmarks of allergy. To achieve this, we explored the transcriptome of $CD19^+$ circulating lymphocytes from patients with house dust mite (HDM) allergic asthma. This group was selected because, unlike pollens, HDMs are permanently present in the environment, allowing us to minimize the effect of seasonal variations.

A total of 41 samples corresponding to unrelated white individuals were analyzed in this study, with 24 samples from patients with HDM allergic asthma and 17 from control individuals. Each subject signed an informed written consent, and the local ethics committee approved this study. Controls had to fulfill the following criteria: (1) absence of symptoms or history of asthma or other pulmonary diseases, (2) no symptoms or history of allergy, (3) negative skin prick test results to a battery of common aeroallergens,[1] and (4) absence of familial history of asthma or allergic diseases. Because both allergy and asthma were ruled out, they could be considered as control subjects. However, patients having a diagnosis of HDM allergic asthma had a clinical history compatible to HDM sensitization, a positive skin prick test result to HDM and specific IgE to HDM above class 2, and either a positive bronchodilator test result or a positive metacholine test result.

Peripheral blood $CD19^+$ B lymphocytes were isolated using immunomagnetic procedures, and fluorescence-activated cell sorting analysis was used to confirm a purity above 90% of the sorted cellular populations. Total RNA was isolated from purified $CD19^+$ B lymphocytes, and bar-coded directional libraries were prepared using the dUTP/UNG system from 6 individuals (3 patients and 3 controls). The generated libraries were sequenced on the Illumina HiSeq 2000 platform (100 bp single-end reads), providing an average library size of approximately 30 million reads per sample (GEO accession no. GSE52742). Results were generated using the WASP system pipeline (http://wasp.einstein.yu.edu), which integrates the quart, gsnap, and htseq-count strategies for managing raw data, supervising quality

FIG 1. A, DESeq analysis of RNA-seq data revealed a number of Ensembl transcripts to be differentially expressed between allergic and nonallergic groups. A preliminary low-stringency filter for $P < .05$ was used to highlight significant transcripts in *red*. The logarithm (base 2) of the fold change from control to allergic patients is represented by the *y-axis*. Normalized abundance of the transcripts (overall base mean values, ie, overall mean of read counts normalized to the library size of each sample) is shown on the *x-axis*. **B,** Heatmap showing the Euclidean distances between the samples to illustrate hierarchical clustering by using the distance matrix of the top 50 differentially expressed transcripts ($P < .025$). **C,** Significant IPA Top Diseases and BioFunctions associated with the top 50 transcripts. **D,** Fold increase (mean ± SEM) in the expression of candidate genes in allergic $CD19^+$ cells *(left y-axis)* as measured by RNA-seq or qRT-PCR. Abundance of transcripts (base mean reads) in allergic $CD19^+$ cells as measured by RNA-seq (right y-axis). **E** and **F,** Expression of *IL4R* transcripts relative to *GAPDH* and a standard calibrator by qRT-PCR in purified $CD19^+$ cells (Fig 1, *E*) or in bulk PBMCs (Fig 1, *F*). Each dot represents a sample from a human subject. **G,** Description of the control and allergic groups of subjects included in this study. **H,** Linear regression of serum IgE levels and *IL4R* expression in the peripheral blood of 26 subjects. *The slope significantly deviates from zero ($P = .0342$) when constrained across the median of controls. **I,** Genotype frequencies of 576Q>R*IL4R* single-nucleotide variant (SNV: CAG>CGG), as explored by PCR amplification followed by Sanger sequencing, and their correlation with *IL4R* expression by qRT-PCR in PBMCs or $CD19^+$ cells. **J,** Histograms of methylation data (reanalysis from Pascual et al[4]) in the approximately 70-kb region comprising the *IL4R* human locus and visualized using the UCSC Genome Browser. Deflections below the zero line represent increased methylation, whereas deflections above the line represent relative hypomethylation. A single CpG island is highlighted at the promoter region. *GAPDH*, Glyceraldehyde 3-phosphate dehydrogenase.



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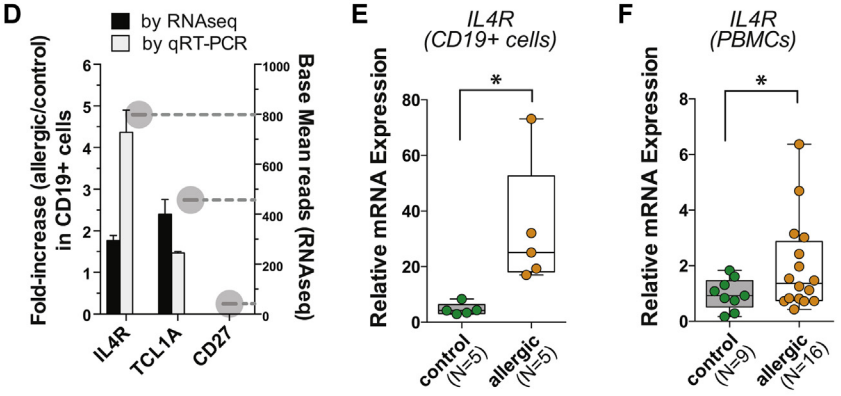
Top IPA® Diseases and Bio Functions	p-value	Molecules
Hematological/Immunological Disease	1.04E-03 - 4.96E-02	IL4R, TCL1A, CD27
- ***hyper-IgE syndrome***	***6.20E-03***	***IL4R***
- proliferation of B lymphocytes & eosinophilia	*4.38E-02 - 4.96E-02*	*IL4R, TCL1A*
- B-cell non-Hodgkin's disease & lymphoma	*1.04E-03 - 2.96E-02*	*TCL1A, CD27*



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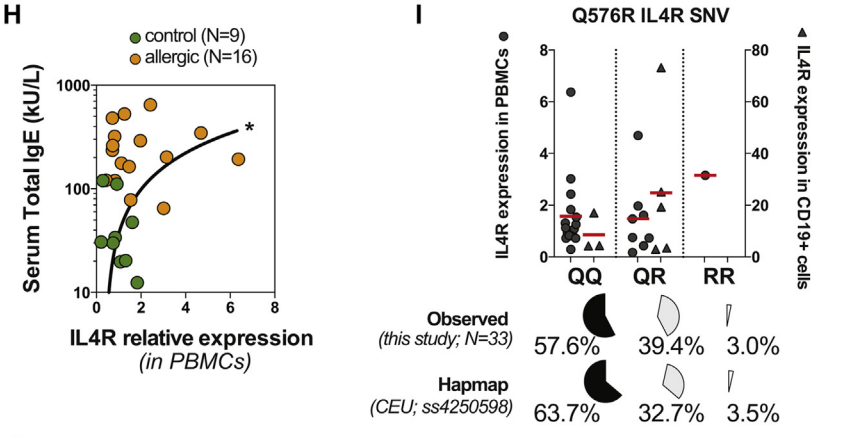
	CD19+ (screening group)		*CD19+ (validation group)*		*PBMCs (validation group)*	
	Control	Allergic	Control	Allergic	Control	Allergic
N (total 41)	3	3	5	5	9	16
Age (mean)	38.3	31.3	34.0	37.2	48.9	33.8
Gender (%M)	33.3	66.7	40.0	60.0	44.4	62.5



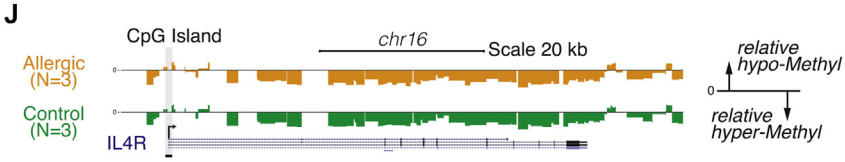


TABLE I. Protein-coding transcripts most differentially expressed between the group of patients with allergic asthma and the controls, listed by their base mean value in the control group

Ensemble ID	External gene ID	Base mean		Fold change	*P* value
		Controls	Patients		
ENSG00000077238	*IL4R*	454.8	798.5	1.8	.024
ENSG00000100721	*TCL1A*	189.7	457.4	2.4	.001
ENSG00000187231	*SESTD1*	127.9	311.6	2.4	.002
ENSG00000139193	*CD27*	37.7	96.9	2.6	.025
ENSG00000196374	*HIST1H2BM*	15.3	45.7	3.0	.002
ENSG00000150625	*GPM6A*	85.7	41.2	0.5	.003
ENSG00000134909	*ARHGAP32*	1.5	38.8	26.7	.011
ENSG00000064886	*CHI3L2*	70.8	22.3	0.3	.012
ENSG00000107104	*KANK1*	3.7	21.1	5.7	.023
ENSG00000185015	*CA13*	0.6	14.6	26.6	.014
ENSG00000042317	*SPATA7*	43.9	14.6	0.3	.012
ENSG00000154380	*ENAH*	2.5	13.4	5.5	.003
ENSG00000122970	*IFT81*	0.3	10.4	34.5	.024
ENSG00000173210	*ABLIM3*	31.5	10.1	0.3	.010
ENSG00000185666	*SYN3*	33.8	10.1	0.3	.006
ENSG00000105507	*CABP5*	0.6	9.2	15.2	.012
ENSG00000151838	*CCDC175*	0.0	5.2	Inf	.021
ENSG00000174599	*TRAM1L1*	12.1	1.2	0.1	.023

metrics, and assigning the reads to known transcripts in the human ENSEMBL GTF annotation file. Differential expression analysis between control and allergic samples was performed by using DESeq, a Bioconductor package specifically designed for the analysis of replicated count-based expression data. DESeq calculates *P* values using a negative binomial distribution to test whether for a given transcript, the observed difference in read counts normalized to library sizes (base values) is greater than would be expected from random variation.

We observed transcriptional differences between control and allergic groups (Fig 1, *A*; $P < .05$), which were most evident for a subgroup comprising the top 50 transcripts ($P < .025$). Indeed, when supervised hierarchical clustering analysis was applied to this set of transcripts, it succeeded in clustering allergic and nonallergic groups (Fig 1, *B*), suggesting that these genes are recurrently deregulated in B cells during the allergic response and might hold some translational value. The coding transcripts more differentially expressed between patients and controls are listed in Table I. Other noncoding transcripts and pseudogenes are listed in Table E1 in this article's Online Repository at www.jacionline.org. The interleukin 4 receptor (*IL4R*) attracted our attention because of its broadly studied role in B-cell activation, IgE production, and hypersensitivity reactions, as highlighted by Ingenuity Pathway Analysis (www.ingenuity.com) (Fig 1, *C*). Although other genes such as *TCL1A* or *CD27* are also associated with hematologic and immunologic disease, we focused on *IL4R* as the best candidate allergic biomarker because it exhibited higher base mean reads by RNA-seq (Fig 1, *D*, right y-axis), which indeed supported a more robust detection when compared with the moderate fold increase in *TCL1A* or the subtle decrease in *CD27* (Table I and Fig 1, *D*, left y-axis). To further explore whether the deregulation of *IL4R* could be validated in a larger cohort of independent allergic patients (full list in Fig 1, *G*), *IL4R* gene expression was analyzed by using qRT-PCR in 10 additional $CD19^+$ samples. Consistent with the RNA-seq data, upregulation of the expression of *IL4R* was confirmed in purified B cells (unpaired *t* test, $P = .024$) (Fig 1, *E*). In this study, we extended the validation to 25 PBMC samples to explore *IL4R* as an easily detected marker by using a cheap, noninvasive, and widely applicable approach. The results were also confirmed in bulk PBMCs (unpaired *t* test, $P = .043$) (Fig 1, *F*), validating the discovery potential of our genome-wide approach in patients with allergic asthma. Interestingly, the relative *IL4R* expression levels were significantly higher in the sorted B cells than in peripheral blood (33.3 ± 23 vs 1.9 ± 1.6; $P = .038$). Although both cellular populations are not directly comparable because cells were obtained from different subjects, this observation could also be due to a masking effect from other cell types in the PBMCs. This could also explain why differences observed between patients and controls were statistically more significant in $CD19^+$ than in PBMCs.

In summary, our study revealed an increased expression of *IL4R* in B cells of patients with HDM allergic asthma. It is well known that human B cells undergo isotype class switching and secrete IgE when stimulated with IL-4 and anti-CD40.[6] Consequently, it had been speculated that upregulation of IL4R would facilitate signaling through IL-4 and favor class-switch recombination to increase synthesis of IgE,[7] which indeed is in accordance with our observation that allergic patients with higher *IL4R* levels tend to also exhibit high levels of serum IgE (>100 KU/L) (Fig 1, *H*). Previous genome-wide studies in allergic asthma were developed using primarily microarrays in peripheral blood, highlighting that additional important information would be obtained from specifically studying the lymphoid tissue where IgE is produced. Here, we strictly selected control subjects to identify 50 transcripts differentially expressed in peripheral $CD19^+$ cells from allergic patients. Although only a few of those ($n = 7$) appeared downregulated in the allergic group, the majority of the identified transcripts appeared upregulated when compared with control subjects ($n = 43$). Many of these deregulated genes could be ascribed to biological pathways involved in hematologic and immunologic functions, suggesting a potential implication of those genes in the pathophysiology of allergic diseases. Our results revealed *IL4R* as a robust protein-coding biomarker of allergic B cells, which is consistent with previous work suggesting a potential role of genetic variations of the IL4/IL4R pathway in allergy.[1,2,8] In our population, however, the exact effect of *IL4R* polymorphisms on the allergic phenotype has been difficult to elucidate because of complex significance in meta-analysis.[3] Importantly, the fact that the increase in IL4R can also be detected in peripheral blood, although with an attenuated effect, could provide a more easily detected, noninvasive, and widely applicable diagnostic approach. The translational significance of our results is further strengthened by the fact that several therapeutic strategies to target the IL4R pathway have recently being proposed. For example, a soluble form of the IL4 receptor (shIL-4Ralpha) is able to block B-cell binding of IL-4 or other IL-4R antagonists[9] and it has been recently reported that omalizumab may have an effect over the *in vitro* expression of *IL4R* in B cells.[10]

Although genetic variations and aberrant epigenetic mechanisms might play a role in *IL4R* overexpression in allergic B cells, we could not prove a clear correlation between missense mutations, such as the Q576R,[1,2] and final *IL4R* expression levels (Fig 1, *I*). Reanalysis of analogous epigenetic studies on the B-cell methylome of HDM allergic patients[4] also failed to identify a distinct DNA methylation signature at the *IL4R* locus of allergic samples (Fig 1, *J*). To conclude, although further studies are

required to explore the causes for its transcriptional deregulation, it is plausible to suggest that the *IL4R* might hold translational potential as both a biomarker and a therapeutic target in patients with allergic asthma to HDM.

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TNF-α/IL-17 synergy inhibits IL-13 bioactivity via IL-13Rα2 induction

To the Editor:

IL-13 is a pleiotropic cytokine that provokes diverse pathophysiological outcomes. Although its effect during gastrointestinal helminth infection is prototypic of a protective T_H2 response (increased peristalsis, goblet cell hyperplasia and mucus secretion, eosinophil recruitment, fibroblast activation, and wound repair), temporal or spatial dysregulation of this response is thought to underlie diseases such as asthma, allergic hyperreactivity, and organ fibrosis.[1] IL-13 signals via the IL-13Rα1/IL-4Rα heterodimer to induce several genes specific to T_H2 inflammation including *CCL26*, *CCL11*, *POSTN*, and *MUC5AC*.[2] IL-13Rα2 binds IL-13 with significantly higher affinity, and the secreted form found in mice acts as a decoy receptor, protecting mice from IL-13–induced immunopathology. However, humans do not alternatively splice the *IL13RA2* transcript and therefore IL-13Rα2 is expressed only as a cell surface protein.[3] The factors that regulate the expression of IL-13Rα2 are unclear, and the biological function of the endogenously expressed IL-13Rα2 remains controversial.[4,5] In this study, we show that the inflammatory cytokines TNF-α and IL-17, often associated with severe asthma, synergize to induce IL-13Rα2 in primary human lung fibroblasts and mouse lungs, and evaluate its biological role using a novel IL-13Rα2–blocking antibody.

Primary human neonatal lung fibroblasts (NLFs) were grown to confluence and incubated with TNF-α, IL-4, and IL-17 for 72 hours, at which time cell lysates were analyzed by using quantitative PCR with primers specific for *IL13RA2* (see the Methods section in this article's Online Repository at www.jacionline.org). Although TNF-α and IL-4 synergistically induced *IL13RA2* as described,[6] a combination of TNF-α and IL-17 induced higher expression (Fig 1, *A*). In contrast, *IL13RA1* decreased in the presence of TNF-α (Fig 1, *B*). IL-13Rα2 mRNA progressively increased over time, peaking at 72 hours (Fig 1, *C*), and longer incubations did not consistently result in any further increase (data not shown); therefore, 72 hours was chosen as the ideal end point for subsequent experiments. To determine whether IL-13Rα2 expression was sustainable, NLFs were incubated with a combination of TNF/IL-17 for 72 hours, after which the supernatants were removed and the wells rinsed and replenished with fresh media without added cytokines. On removal of TNF-α and IL-17, IL-13Rα2 expression declined between 24 hours and 72 hours postwash, though it remained elevated (Fig 1, *D*). We confirmed that primary lung fibroblasts derived from healthy adult donors (adult lung fibroblasts) also manifested similar synergy to TNF-α and IL-17 (see Fig E1 in this article's Online Repository at www.jacionline.org). Using flow cytometry, we verified that the transcriptional induction of IL-13Rα2 was associated with augmented surface expression of protein (Fig 1, *E* and *F*). To ascertain whether this phenomenon can be recapitulated *in vivo*, we administered multiple doses of the cytokines into mouse airways. IL-13Rα2 transcripts were strikingly higher in the lungs of mice that received both