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Transcriptional Profiling of Th2 Cells Identifies Pathogenic Features Associated with Asthma

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Allergic asthma and rhinitis are two common chronic allergic diseases that affect the lungs and nose, respectively. Both diseases share clinical and pathological features characteristic of excessive allergen-induced type 2 inflammation, orchestrated by memory CD4⁺ T cells that produce type 2 cytokines (Th2 cells). However, a large majority of subjects with allergic rhinitis do not develop asthma, suggesting divergence in disease mechanisms. Because Th2 cells play a pathogenic role in both these diseases and are also present in healthy nonallergic subjects, we performed global transcriptional profiling to determine whether there are qualitative differences in Th2 cells from subjects with allergic asthma, rhinitis, and healthy controls. Th2 cells from asthmatic subjects expressed higher levels of several genes that promote their survival as well as alter their metabolic pathways to favor persistence at sites of allergic inflammation. In addition, genes that enhanced Th2 polarization and Th2 cytokine production were also upregulated in asthma. Several genes that oppose T cell activation were downregulated in asthma, suggesting enhanced activation potential of Th2 cells from asthmatic subjects. Many novel genes with poorly defined functions were also differentially expressed in asthma. Thus, our transcriptomic analysis of circulating Th2 cells has identified several molecules that are likely to confer pathogenic features to Th2 cells that are either unique or common to both asthma and rhinitis. *The Journal of Immunology*, 2016, 197: 000–000.

Asthma and allergic rhinitis affect an estimated 400 million people worldwide (1). This extensive global health concern led to internationally collaborative studies such as Allergic Rhinitis and its Impact on Asthma, which found strong evidence for increased prevalence of asthma in patients with allergic rhinitis; however, asthma can also develop without prior instances of allergic rhinitis (2). Both allergic asthma and rhinitis are chronic inflammatory diseases with a high comorbidity in children and adults (2, 3). Currently, there is no cure for asthma, and newer therapies are only partially successful in certain sub-

types (4), underscoring the importance of a better understanding of molecular features of the disease.

The immunological hallmark of allergic asthma and rhinitis is excessive type 2 inflammation (5). Type 2 immune responses are orchestrated by T cells and innate immune cells that produce the signature Th2 cytokines IL-4, IL-5, and IL-13 (5). Type 2 immune responses are essential for the clearance of parasitic infections, but, when directed against harmless allergens, cause allergic diseases such as asthma, rhinitis, and atopic dermatitis (3, 5, 6). Multiple genetic and genome-wide association studies in asthma have consistently identified a disproportionately large number of disease-associated single-nucleotide polymorphisms in the Th2 cytokine locus as well as in loci of several other genes involved in type 2 immune responses (*IL-25*, *IL-33*, *TSLP*) (7, 8), further emphasizing the importance of the Th2 pathway in disease pathogenesis. Animal and human studies have shown that targeting the various arms of the type 2 (Th2) immune response has considerable promise in the control of asthma (9). More specifically, finding biological modulators of Th2 cytokines, Th2 immune pathways, and Th2 cells has emerged as a rational approach for developing new treatments for asthma (10).

Despite sharing several immunological hallmarks of Th2 cytokine-driven inflammation and environmental triggers for disease exacerbation (allergen exposure), asthma and rhinitis are very different diseases; asthma affects the lungs, and rhinitis the nose. The factors that drive this singular distinction are not known, but one factor could be differences in the nature of pathogenic Th2 cells that drive these two diseases. Another puzzling observation is that even healthy nonallergic subjects generate Th2 cells capable of producing the canonical Th2 cytokines in response to allergen exposure in vitro, although they do not cause allergic disease in vivo (i.e., are non-pathogenic). What factors make these Th2 cells pathogenic in some instances (healthy versus allergic asthma) and confer organ specificity (asthma versus rhinitis) in others? Some possible hypotheses are as follows: 1) qualitative differences in Th2 cells between

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Abbreviations used in this article: BDR, bronchodilator reversibility; DUSP, dual specificity phosphatase; FDR, false discovery rate; FEV1, forced expiratory volume in 1 s; LJI, La Jolla Institute; RNA-Seq, RNA-sequencing; SRC, spare respiratory capacity; WGCNA, weighted gene coexpression network analysis.

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healthy, rhinitis, and asthma subjects play a central role in driving differences in outcomes of allergen exposure; 2) Th2 cells are very similar in all states (healthy, rhinitis, asthma), but either simple quantitative changes (i.e., more numbers in asthma) or pathogenic alterations in the target organ that activate or reprogram these cells drive outcome differences; and/or 3) differences in homing potential, that is, Th2 cells in asthma and rhinitis are endowed with the ability to traffic to the lung and nose, respectively, and those from nonallergic healthy subjects lack target-organ homing potential.

To test these possibilities in an unbiased fashion, we performed genome-wide transcriptional profiling of a Th2 cell-enriched CD4 memory subset (CCR4⁺CD4⁺ T cells) (11, 12) from a large number of subjects with allergic asthma, rhinitis, and nonatopic healthy controls. CCR4⁺CD4⁺ T cells from asthmatic subjects overexpressed several genes that are likely to support excessive production of Th2 cytokines as well as promote persistence of Th2 cells at sites of inflammation, thus lending support to the hypothesis that circulating Th2 cells in asthmatic subjects differ qualitatively from those present in allergic rhinitis and healthy subjects.

Materials and Methods

Subject characteristics

Recruitment of subjects included in this study followed Institutional Review Board (La Jolla Institute for Allergy and Immunology, La Jolla, CA) approval, and study participants gave written informed consent. Thirty-seven nonsmoking subjects with allergic asthma (12 subjects were treated with inhaled corticosteroids, and 25 subjects were treated only with inhaled bronchodilators), 25 subjects with allergic rhinitis but no asthma, meeting established diagnostic criteria (13), and 15 healthy nonatopic (based on radioallergosorbent test) subjects were studied (Supplemental Table I). Subjects with asthma and rhinitis underwent pulmonary function tests and/or methacholine challenge to establish diagnosis (bronchodilator response of >12%, or >200 ml, and/or methacholine challenge with a provocative concentration causing a drop of the forced expiratory volume in 1 s [FEV1] of 20% <8 mg/ml). All asthmatic and rhinitis subjects were classified as allergic based on skin test reactivity to a panel of 32 common extracts, including seasonal allergens such as various trees, grasses, and wheat as well as perennial allergens, including cat dander, dog epithelia, and house dust mite (Supplemental Fig. 1, Supplemental Table I).

Cell isolation

For isolating Th2-enriched CD4⁺ cells from peripheral blood samples, PBMCs were stained with a mixture of fluorescently conjugated Abs, anti-CD25 FITC conjugated (R&D Systems; clone M-A251), anti-CCR4 PE conjugated (R&D Systems; clone 205410), anti-CCR7 PerCP-Cy5.5 conjugated (BioLegend; clone G043H7), anti-CD45RA allophycocyanin conjugated (BioLegend; clone HI100), anti-CD3 PE-Cy7 conjugated (BioLegend; clone UCHT1), anti-CD4 allophycocyanin-Cy7 conjugated (BioLegend; clone RPA-T4), and sorted on the FACS Aria to obtain a Th2 cell-enriched CD4⁺ subset: CD3⁺CD4⁺CD45RA⁺CD25⁺CCR4⁺. Sorted cells were centrifuged for 5 min at 400 × g (4°C) and then resuspended in 250 μL T cell medium and lysed with 750 μL TRIzol LS, as recommended by the manufacturer's instructions (ThermoFisher). Samples were stored at -80°C.

Dual ELISPOT assays

Cytokine production from cultured PBMCs in response to antigenic stimulation was assessed by dual ELISPOT assays (IFN-γ/IL-5 and IL-10/IL-17), as described previously (14). Flat-bottom 96-well plates were coated with either 10 μg/ml anti-human IFN-γ (clone 1-D1K; Mabtech, Cincinnati, OH) and anti-human IL-5 (clone TRFK5; Mabtech) or anti-human IL-10 (clone 9D7; Mabtech) and anti-human IL-17 Abs (clone 147178; eBioscience, San Diego, CA). Cells were then incubated at a density of 1 × 10⁵ cells/well with a pool of 20 predominant antigenic TG regions (P20) at a concentration of 50 and 5 μg/ml, respectively. PHA (10 μg/ml) and medium were referred to as controls. After 24 h, cells were removed and plates were incubated with 2 μg/ml biotinylated anti-human IL-5 Ab (Mabtech) and HRP-conjugated anti-human IFN-γ Ab or 2 μg/ml biotinylated anti-human IL-10 Ab (Mabtech) and FITC-conjugated anti-human IL-17 Ab (eBioscience) at 37°C for 2 h. Spots corresponding to the biotinylated Ab (IL-5, IL-10) were incubated with alkaline phosphatase complex (Vector Laboratories, Burlingame, CA) and developed using Vector Blue Alkaline Phosphatase Substrate Kit III, according to manu-

facturer instructions. Spots corresponding to the HRP-conjugated Ab (IFN-γ) were developed with 3-amino-9-ethylcarbazole solution (Sigma-Aldrich, St. Louis, MO). Spot-forming cells were counted by computer-assisted image analysis (KS-ELISPOT reader; Zeiss, Munich, Germany). Each assay was performed in triplicate. Student *t* test using the mean of triplicate values of the response against the extract, pool, or individual peptides, compared with the response against medium control, was applied to calculate statistical significance. As previously described, criteria for positivity were 100 spot-forming cells/10⁶ PBMCs, *p* < 0.05, and a stimulation index >2 (14).

RNA sequencing

Total RNA was purified using a miRNAeasy micro kit (Qiagen) and quantified, as described previously (12, 15). Purified total RNA (5 ng) was amplified following the Smart-seq2 protocol (16). cDNA was purified using AMPure XP beads (1:1 ratio; Beckman Coulter). From this step, 1 ng cDNA was used to prepare a standard Nextera XT sequencing library (Nextera XT DNA sample preparation kit and index kit; Illumina). Samples were sequenced using a HiSeq2500 (Illumina) to obtain 50-bp single-end reads. Both whole-transcriptome amplification and sequencing library preparations were performed in a 96-well format to reduce assay-to-assay variability. Quality control steps were included to determine total RNA quality and quantity, the optimal number of PCR preamplification cycles, and fragment size selection. Samples that failed quality control were eliminated from further downstream steps. Barcoded Illumina sequencing libraries (Nextera; Illumina) were generated utilizing the automated platform (Biomek FXp). Libraries were sequenced on the HiSeq2500 Illumina platform to obtain 50-bp single-end reads (TruSeq Rapid Kit; Illumina), generating a total of >700 million mapped reads (median of ~9 million mapped reads per sample).

RNA-sequencing analysis

RNA-sequencing (RNA-Seq) data were mapped against the hg19 reference using TopHat (17) (v1.4.1, library-type fr-secondstrand-C) and the RefSeq gene annotation downloaded from the University of California – Santa Cruz Genome Bioinformatics site. Sequencing read coverage per gene was counted using HTSeq-count (-m union -s yes -t exon -i gene_id, <http://www.huber.embl.de/users/anders/HTSeq/>). To identify genes differentially expressed between allergic asthma, rhinitis, and healthy control groups, we performed negative binomial tests for pairwise comparisons employing the Bioconductor package DESeq2, disabling the default options for independent filtering and Cooks cutoff (18). We considered genes differentially expressed between any pairwise comparison when the DESeq2 analysis resulted in a Benjamini-Hochberg-adjusted *p* value <0.2, false discovery rate (FDR) <0.2 (Supplemental Table II). We used the software from the ConsensusPathDB interaction database (version 27) (19, 20) for the overrepresentation analysis and gene pathway analysis (Supplemental Table II).

Weighted gene coexpression network analysis

We performed a weighted correlation network analysis using the weighted gene coexpression network analysis (WGCNA) package version 1.46 (21) for R version 3.2.0 (22). First, we removed those genes whose expression values were in the bottom third of all genes for at least 80% of the samples, as established in the third phase of the MAQC project (MAQC-III), also called Sequencing Quality Control (23). We then applied the regularized log transformation from the DESeq2 package (18) to obtain homoscedastic data, which can be used as input to the network analysis.

The first step for building the coexpression network was to calculate a Spearman correlation matrix using all of the genes that passed the filter. This correlation matrix was transformed into a signed adjacency matrix using the following power function:

$$a_{ij} = |0.5 + 0.5 \times \text{cor}(x_i, x_j)|^\beta$$

A $\beta = 5$ was selected following the scale-free topology criterion (24). To measure the interconnectedness in the network, we computed the following topological overlap matrix:

$$\text{TOM}_{ij} = \frac{\sum_u a_{iu} a_{uj} + a_{ij}}{\min(k_i, k_j) + 1 - a_{ij}}$$

$$k_i = \sum_u a_{iu}$$

A dissimilarity measure calculated from this topological overlap matrix ($\text{TOM}_{\text{diss}} = 1 - \text{TOM}$) was then clustered using the standard R hierarchical clustering function *hclust*. Modules were identified as branches of the hierarchical clustering dendrogram after using the dynamic tree cut

algorithm (25–28) and named using colors (Fig. 1A, Supplemental Table II).

For each module, the first principal component was defined as the module eigengene. Modules with highly correlated eigengenes (>0.8) were merged together, as they were deemed similar (Fig. 1A). We also used these eigengenes to correlate the importance of each module to the disease groups. To this end, we performed a series of *t* tests comparing the eigengene sample values according to their condition (allergic rhinitis, allergic asthmatic, or healthy control). The sign of the *t*-statistic indicates which condition has higher mean expression for the genes contained within the module, and the value indicates the magnitude of the difference between conditions (Fig. 1B, Supplemental Table II).

Accession code

Sequencing data have been deposited into the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE75011.

Results

Transcriptomic analysis of Th2 cells in asthma

We performed global transcriptional profiling to identify molecular features that distinguish subjects with allergic asthma from allergic rhinitis or healthy nonatopic subjects. Previous transcriptomic studies to unravel the molecular features of asthma were performed in heterogeneous cell populations (26–30). To ensure that asthma-specific molecular features were captured, or at least not lost, across the wide range of circulating T cell subtypes present in vivo, we devised a simple sorting strategy to enrich for human Th2 cells, a subset of CD4⁺ T cells that is highly relevant to asthma pathogenesis (3, 5). Furthermore, to avoid mistaking differences in the relative abundance of Th2 cells for asthma-specific transcriptional changes, and to capture changes that specifically occur in circulating Th2 cells, we performed transcriptional profiling in pure populations of CCR4-expressing memory CD4⁺ T cells (CCR4⁺CD4⁺CD45RA[−]; for simplicity, hereafter referred to as Th2 cells), as human Th2 cells are enriched in CD4⁺ memory T cells that express the chemokine receptor CCR4⁺, and are depleted in CCR4[−] cells (11, 12, 31). By this approach we also expected to capture asthma-specific changes resulting from T cell activation and differentiation in the host, for example, during the programming of naive T cells to different types of pathogenic memory Th2 cells in asthma (32).

We performed RNA-Seq in Th2 cells from a total of 80 samples from 77 patients, including 3 biological replicates (Supplemental Table I). Gene expression values were normalized, and pairwise comparisons were performed between subjects with allergic asthma, rhinitis, and healthy nonatopic controls using two approaches, as follows: WGCNA (21) and differential expression analysis using DESeq2 (18). In WGCNA, emphasis is placed on gene modules (hubs of genes that show a similar pattern of expression across all samples), whereas DESeq2 analysis looks for differences in average expression gene by gene. We identified a total of 15 distinct gene modules based on Th2 cell gene expression data from all donors in our cohort (Fig. 1A, Supplemental Table II). When examining the expression of different gene modules across disease groups, the most significant differences were observed in the comparison of asthmatic and healthy subjects (two modules significantly upregulated and three downregulated in asthma, blue bars; Fig. 1B), suggesting that the greatest divergence occurs between the molecular program of Th2 cells in asthma compared with healthy subjects. Four of these modules were also significantly different between allergic asthmatics versus rhinitis subjects, albeit to a much lesser extent, suggesting relatively smaller differences in the molecular program of Th2 cells in asthma compared with rhinitis subjects. Genes in the midnight blue module showed modest differences between asthma and rhinitis, but showed significant differences in allergic asthma

and rhinitis subjects when compared with nonatopic healthy subjects, suggesting these genes are linked to allergy in general (i.e., common to both allergic asthma and rhinitis).

Next, to identify specific genes that were differentially expressed across disease groups, we used DESeq analysis to perform pairwise comparisons of RNA-Seq profiles of Th2 cells among the three following subject groups: allergic asthma, rhinitis, and nonatopic healthy subjects. Given that asthma patients are a highly heterogeneous group with potentially several molecular subtypes as well as differences in medication usage, we expected large variations in gene expression among asthma patients, and thus set a less stringent threshold to define differentially expressed genes (FDR, <0.2 using the Benjamini-Hockberg procedure; see *Materials and Methods*) by the DESeq method. At this FDR, we found a strong overlap ($\sim 90\%$) between differentially expressed genes detected by DESeq method and genes in modules that were significantly different between asthma and rhinitis (detected by WGCNA method), implying that both analytical approaches yield concordant results (Fig. 1C).

IL17RB and genes linked to cellular longevity are increased in asthma

DESeq analysis identified >500 genes that were differentially expressed in asthmatic subjects when compared with healthy subjects (Fig. 1, Supplemental Table II). Pathway analysis of these genes revealed significant overrepresentation of genes involved in apoptosis, zinc transporters, MAPKs, NF- κ B, Notch, and TNF signaling (Supplemental Table II). Principal component analysis of these genes revealed a clearly distinct gene expression pattern in healthy subjects, although a significant overlap existed between asthmatic and rhinitis subjects (Fig. 2A). Expression of most of these genes was similar between allergic rhinitis and asthmatic subjects, implying that regulation of these genes is not strictly asthma specific, but rather allergy dependent (Fig. 2B). To specifically address whether allergic rhinitis had a distinct transcriptional signature or was an intermediate phenotype in the spectrum from healthy to asthmatic subjects, we examined the 548 genes that had significantly different expression in healthy versus asthmatic donors, and reviewed their expression levels in subjects with allergic rhinitis. To easily compare them, we rescaled the logarithmic expression values so that 0.0 corresponded to the average expression in healthy donors, and 1.0 corresponded to the average expression in asthmatic (Supplemental Fig. 2). For subjects with allergic rhinitis, the majority of the genes ($n = 374$; 68%) showed an intermediate expression level between 0.0 and 1.0, the range defined by asthmatic and healthy subjects. A smaller fraction of genes ($n = 135$; 25%) was expressed with values >1.0 , implying an even more pronounced difference from healthy than the asthmatics. Fewer genes ($n = 39$; 7%) showed expression values <0.0 , meaning they had the opposite difference from healthy donors than what was observed in asthmatics. Overall, this suggests that the genes that differentiate asthmatic from healthy subjects show an intermediate phenotype in allergic rhinitis subjects, which is leaning more toward the asthmatic phenotype. Furthermore, comparison of steroid-naïve and steroid-treated asthmatic subjects revealed no significant differences in gene expression (Supplemental Table II).

Expression of *IL17RB*, encoding IL-25R (also known as IL17RB), was most significantly different (~ 3.35 -fold increase) in Th2 cells from asthmatic subjects compared with healthy subjects; a similar increase in expression, although less significant, was also seen in allergic rhinitis subjects (Fig. 2C). Notably, we found a good correlation between the levels of *IL17RB* transcripts and the magnitude and breadth of wheal responses to common allergens

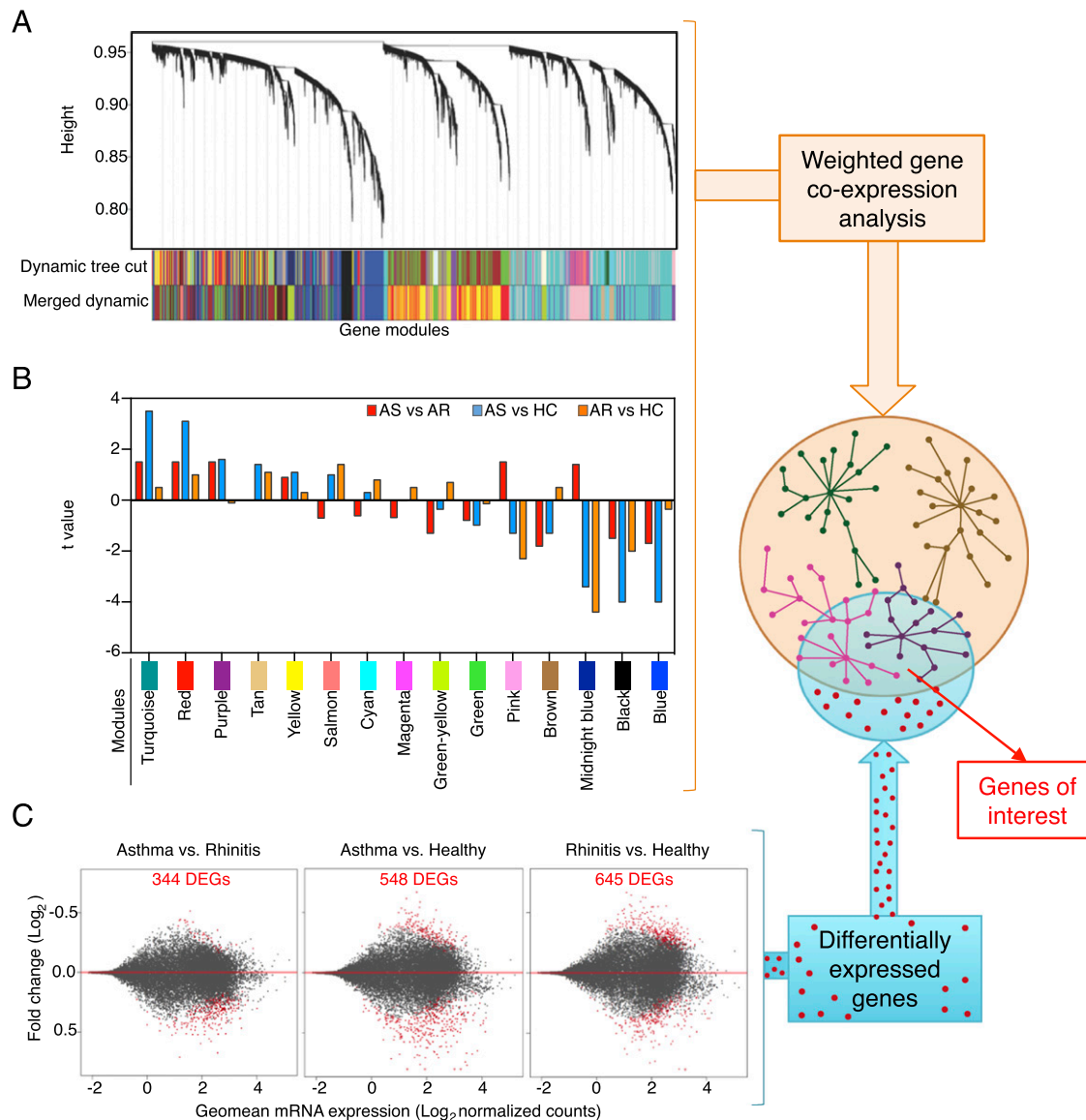


FIGURE 1. RNA-Seq analysis of Th2 cells. **(A)** WGCNA showing gene modules identified in Th2 cells. Hierarchical clustering of genes with topological similarity is shown. The color bands underneath the dendrogram show the module assignments after applying the dynamic tree cut algorithm (*top band*) and after merging modules with highly correlated eigengenes (*bottom band*; see *Materials and Methods*). **(B)** List of identified modules ordered by their *t* statistics. The *t* values were calculated by performing a pairwise comparison of eigenvalues for each module (*t* test) between the three subject groups: asthmatic, rhinitis, and healthy controls (see *Materials and Methods*). **(C)** DESeq2 analysis showing pairwise comparison of gene expression between Th2 cells from healthy, rhinitis, and asthmatic subjects. Minus-average plots show the geometric mean of expression between the samples (*x*-axis) plotted against the fold change in expression between the samples (*y*-axis).

(Fig. 2D), suggesting a potential link to the severity of allergic responses. Circulating human CD4⁺ CRTH2⁺ Th2 cells have been shown to express low levels of surface IL17RB; however, following activation, they rapidly upregulate expression of *IL17RB* transcripts and respond to IL-25 (33). IL-25 has been shown to stimulate their proliferation as well as enhance Th2 polarization and cytokine production by upregulating the expression of transcription factors such as GATA-3 and c-MAF (34, 35). Increased *IL17RB* transcripts were also reported in CD4⁺ T cells isolated from nasal polyps of patients with eosinophilic chronic rhinosinusitis (36) and in circulating CD4⁺ T cells following nasal allergen challenge (37). Several studies in animal models have consistently shown that IL-25, like the other prototypical type 2 cytokines IL-5, IL-13, and IL-4, promotes pathogenic Th2 cell-mediated inflammatory responses in the lung (34, 38). Thus, the increased expression of *IL17RB* in Th2 cells from subjects with

asthma and rhinitis could enhance their longevity and pathogenicity at sites of allergic inflammation.

Several genes involved in the regulation of apoptosis (*MAN1A1*, *CASP2*, *GIMAP7*) and cellular energy metabolism (*CPT1A*) were preferentially upregulated in Th2 memory cells from asthmatic subjects (Fig. 2C, Supplemental Table II). Of note, carnitine palmitoyl transferase 1a (CPT1a) is a mitochondrial protein (rate-limiting enzyme) that aids in the utilization of fatty acids as an alternative energy source, which can substantially enhance spare respiratory capacity (SRC) (39). SRC is the extra mitochondrial capacity available in a cell to produce energy under conditions of increased work or stress and is thought to be important for long-term cellular survival and function. CPT1a, by regulating fatty acid metabolism and SRC, has been shown to contribute to the formation of long-lived protective CD8⁺ memory T cells postinfection or vaccination (39). Similarly, the increased expression of

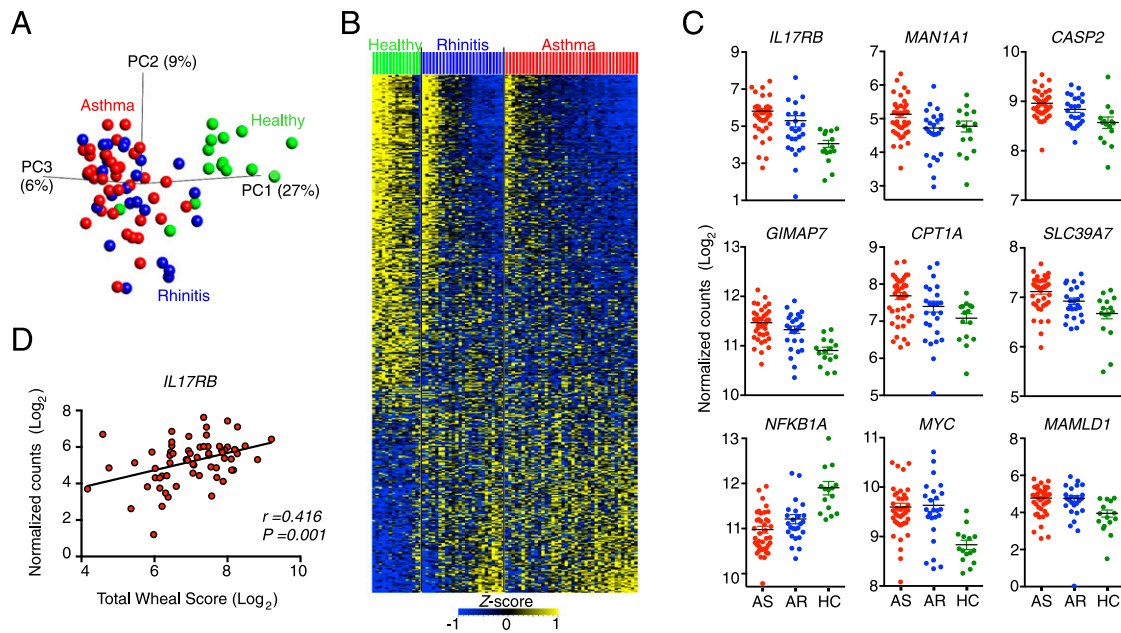


FIGURE 2. Differentially expressed genes in asthma compared with healthy subjects. **(A)** Principal component analysis of gene expression patterns based on differentially expressed genes obtained by pairwise comparisons of Th2 cells from healthy versus asthmatic subjects (DESeq2 analysis, Benjamini-Hochberg method FDR <0.2; see *Materials and Methods*). **(B)** Row-wise Z-scores of library size-normalized read counts for each differentially expressed gene. RNA-Seq data from each independent sample ($n = 80$) are shown in the columns. **(C)** Dot plots showing expression values (read counts) of indicated genes for each individual sample analyzed by RNA-Seq; each dot shown in green (healthy subject = HC), blue (allergic rhinitis = AR), and red (asthma = AS) are data from a single RNA-Seq assay, and error bars are mean $\pm$ SEM. **(D)** Correlation between *IL17RB* transcripts levels and sum of wheal score (see *Materials and Methods*) of allergic asthma and rhinitis subjects; r value indicates the Spearman correlation coefficient.

CPT1A seen in Th2 cells may promote the persistence of long-lived pathogenic allergen-specific Th2 cells that could sustain allergic inflammation in asthma and rhinitis.

Transcripts encoding for a zinc channel protein, ZIP7 (*SLC39A7*), were increased in Th2 cells from asthmatic subjects. ZIP7 is the only zinc channel known to reside in the membrane of the endoplasmic reticulum, where it acts as a gatekeeper for the release of intracellular zinc stores into the cytoplasm (40). Various external stimuli, including TCR activation, can activate a ubiquitous protein kinase (casein kinase 2) to phosphorylate serine residues in ZIP7 and open the channel gate (40, 41). The consequent rise in cytosolic Zn^{2+} concentration activates protein kinases, particularly the MAPKs ZAP70 and AKT, which can promote T cell proliferation, activation, and migration (40, 41). Furthermore, studies in T cells have shown that Zn^{2+} is required for T cell proliferation and for the production of cytokines such as IL-2 and IFN- γ (41, 42).

In addition to ZIP7, transcripts of positive (*TBLIXR1*) and negative regulators (*NFKB1A* and *NFKB1E*, encoding I κ B α and I κ B ϵ) of NF- κ B activation (43–46) were increased and decreased, respectively, in Th2 cells from asthmatic and rhinitis subjects; this is likely to have important functional consequences, given the established role of NF- κ B in preventing activation-induced cell death (protection from apoptosis) and promoting IL-2 release to support proliferation and expansion of CD4⁺ T cells (46). We also detected increased transcripts for *MYC*, which is known to regulate T cell proliferation through transcriptional control of cell cycle regulators and by inducing metabolic reprogramming to support cell growth and effector differentiation (47), and increased transcripts for genes in the Notch pathway (*MAMLD1*, *TBLIXR1*), also known to regulate proliferation and cytokine release by peripheral T cells (48, 49). Overall, these data suggest that Th2 cells from allergic asthmatic and to a lesser degree from rhinitis subjects display molecular features that support the generation of

long-lived and highly pathogenic Th2 cells that can sustain allergic inflammation.

Downregulation of negative regulators of T cell activation in asthma

We identified 344 genes that were differentially expressed in the pairwise comparison of allergic asthma and rhinitis subjects (DESeq analysis, FDR <0.2); pathway analysis of these genes revealed significant overrepresentation of genes involved in activating transcription factor 2, forkhead box O, and activator protein-1 transcription factor networks, cytokine (IL-4, IL-6, IL-1, IL-12, TNF- α), and MAPK signaling (Supplemental Table II). The majority of the differentially expressed genes were expressed at lower levels in Th2 cells from asthmatic subjects (Fig. 3A), including transcripts for genes (*DUSP10*, *DUSP8*, *PELI1*) that are known negative regulators of T cell activation (Fig. 3B). *DUSP10* encodes for MAPK phosphatase 5 (50) (also known as dual specificity phosphatase [DUSP] 10), which is known to inhibit the JNK signaling pathway and reduce the activity of activator protein-1 transcription factor (50, 51). Th1 and Th2 cells generated in vitro from *DUSP10*-deficient mice produce increased levels of IFN- γ and IL-4, respectively (51). Excessive production of these cytokines, as well as IL-2 and TNF- α , in *DUSP10*-deficient mice results in immune-mediated death following infection with lymphocytic choriomeningitis virus, confirming that *DUSP10* is a negative regulator of effector T cell cytokine expression (51). *DUSP8* is another member of the DUSP family that is also known to specifically target JNK, although its function in T cells has not been fully defined. The pellino (PELI) proteins, in contrast, are E3 ubiquitin ligases known to catalyze ubiquitination and degradation of c-Rel, a member of the NF- κ B family of transcription factors, and IL-1R-associated kinase 1 (52). In mice, *PELI1*-deficient T cells were shown to be hyperresponsive to stimulation via TCR and CD28, producing excess proinflammatory

cytokines, as well as resistant to suppression by regulatory T cells and TGF- β ; consequently, *PELI1* has been shown to prevent the development of autoimmunity in mice (52). Overall, the reduced levels of several critical negative regulators of T cell activation could result in excessive release of proinflammatory cytokines by the Th2 cells from asthmatic subjects, thus exacerbating airway inflammation following allergen exposure.

Modulators of Th1 differentiation and autophagy are downregulated in asthma

Several genes that modulate Th differentiation were also downregulated in asthma (Fig. 3A, 3B), notably *PTGER4*, encoding PG receptor E4 (EP4), a G protein-coupled receptor that induces intracellular cAMP generation upon stimulation by PGE₂ (53). The PGE₂-EP4-cAMP pathway has been shown to promote the transcription of *IL12RB2* and *IFNGR1*, receptors for the critical cytokines IL-12 and IFN- γ that are required for Th1 differentiation (54, 55). Studies in EP4-deficient mice have shown that loss of EP4 in T cells restrains Th1 differentiation and attenuates Th1 cell-mediated inflammation in vivo (53, 55). Furthermore, transcripts of the Rel/NF- κ B family of transcription factors *REL* and *RELB* were reduced in Th2 cells from asthmatic subjects. Consistent with the presence of c-REL (encoded by *REL*) binding sites within the *IFNG* gene, T cells from mice lacking Rel or RelB show major defects in IFN- γ production and a failure to differentiate into Th1 cells even under Th1-inducing conditions; on the contrary, Th2 responses were enhanced in Rel-deficient mice (56). T cell-intrinsic expression of *REL* has been shown to be required for the optimal activation and expansion of Th1 effector cells; thus, REL transcription factors can promote Th1 differentiation as well as restrain Th2 differentiation (57). Given the plasticity of human Th cells, the reduced expression of several molecules that promote the Th1 program in CCR4⁺ cells (Th2 cell-enriched memory subset) is most likely to play an important role in promoting the Th2/Th1 imbalance in asthma.

Other genes involved in Th differentiation whose expression was lower in Th2 cells from asthmatic subjects were *SGK1* and *ZBTB10*. *SGK1*, a glucocorticoid-regulated kinase, was recently shown to positively regulate the differentiation of Th17 and Th2 cells (58, 59). The reduction in *SGK1* levels may be due to negative feedback mechanisms that counteract excessive Th17/Th2 differentiation in asthma. *ZBTB10*, a gene linked to asthma susceptibility, is a zinc finger transcription factor that negatively regulates Sp1, a transcriptional activator that has been shown to regulate the expression of IL-2R β (*IL2RB*) and several cytokines such as IL-21, TNF- α , and IL-10 (60, 61).

Two transcripts for genes involved in the autophagy pathway (*GABARAPL1*, *ATG2A*) (62) were also downregulated in asthma (Fig. 3B). When autophagy is blocked, Th2 cells are more resistant to cell death following growth factor withdrawal (63), suggesting that a similar survival advantage may also be present for Th2 cells from asthmatic subjects.

Finally, we examined the correlation between expression of asthma-specific genes (discussed above) and lung physiological measures linked to asthma severity such as FEV1, bronchodilator reversibility (BDR) following short-acting β 2 agonist treatment, and methacholine challenge. We found a moderately good correlation between BDR and transcript levels for *ZBTB10*, *SGK1*, and *GABARAPL1*, suggesting that the molecular program in circulating Th2 cells may influence BDR in asthmatic subjects (Fig. 3C).

GPR55 and ELAVL1 transcripts are increased in asthma compared with rhinitis

In this work, we first wanted to experimentally address whether Th2 cells from allergic asthma and rhinitis differ in the number or nature of allergen-specific T cells, which are likely to account for a minority of the total Th2 population. For this reason, we estimated the magnitude of allergen-specific T cell responses by ELISPOT assays following 2-wk in vitro expansion of T cells with a pool of timothy grass-specific peptides, as described previously (14). The

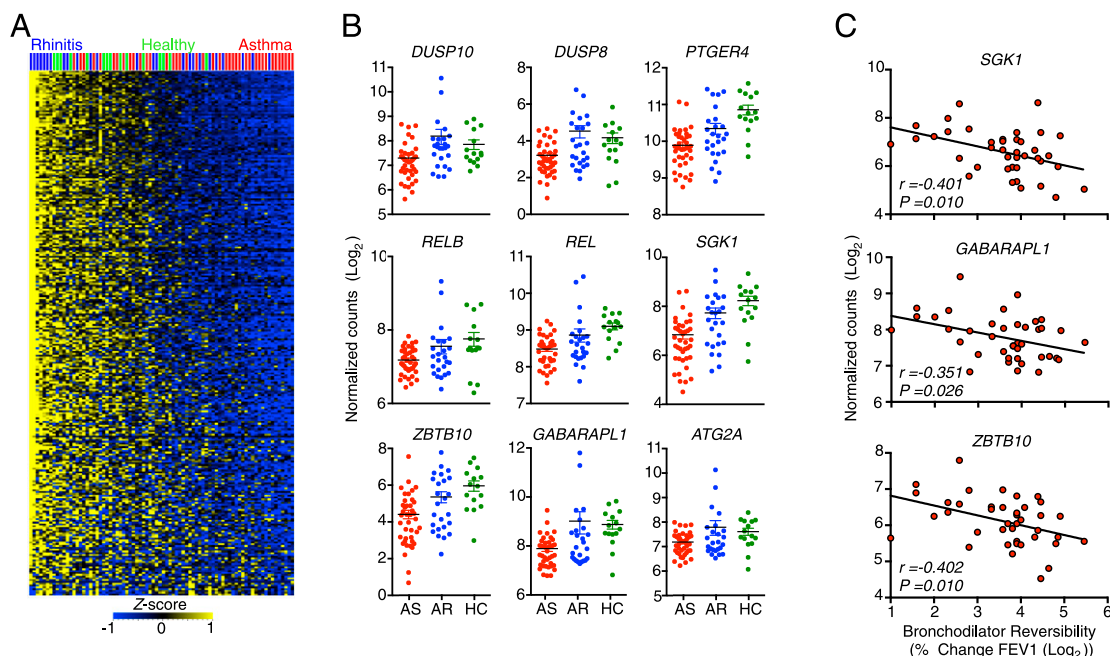


FIGURE 3. Downregulated genes in asthma compared with allergic rhinitis. **(A)** Row-wise Z-scores of library size-normalized read counts for each differentially expressed gene downregulated in asthmatic subjects compared with rhinitis subjects (DESeq2 analysis, Benjamini-Hochberg method FDR <0.2; see *Materials and Methods*). RNA-Seq data from each independent sample ($n = 80$) are shown in the columns. **(B)** Dot plots showing expression values (read counts) of indicated genes for each individual sample analyzed by RNA-Seq; each dot shown in green (healthy subject = HC), blue (allergic rhinitis = AR), and red (asthma = AS) are data from a single RNA-Seq assay, and error bars are mean $\pm$ SEM. **(C)** Shows correlation between *ZBTB10*, *SGK1*, and *GABARAPL1* transcript levels and BDR values (expressed as percentage change in FEV1) from asthmatic and rhinitis subjects; r value indicates the Spearman correlation coefficient.

number of IL-5–producing allergen-specific Th2 cells was not significantly different between allergic asthma and rhinitis subjects (Fig. 4A); we detected a much smaller number of IL-17–producing allergen-specific T cells, again at similar numbers in allergic asthma and rhinitis subjects. Thus, we observed no difference in the number of timothy grass–specific Th2 cells between allergic asthma and rhinitis subjects.

Our transcriptional analysis revealed >50 genes that were expressed at significantly higher levels in Th2 cells from subjects with allergic asthma compared with rhinitis (DESeq analysis, FDR <0.2; Fig. 4B). Notably, we did not observe any significant differences in the expression of chemokine receptors and adhesion molecules involved in trafficking of T cells, suggesting that Th2 cells from asthmatic subjects do not differ in their homing potential when compared with Th2 cells from allergic rhinitis subjects or healthy controls (Supplemental Table II).

Several genes whose functions are poorly defined in T cells were prominently increased in asthma when compared with allergic rhinitis and healthy controls (Fig. 4C). These include *DUSP7*, encoding a poorly characterized dual specificity phosphatase (50); *CLIP3*, cytoplasmic linker protein 3, which has recently been identified as a microtubule-binding protein that associates with lipid rafts and regulates intracellular organelle movement and cellular apoptosis (64); *ACAD8*, encoding a member of the acyl-CoA dehydrogenase family of enzymes that catalyze the dehydrogenation of acyl-CoA derivatives in the metabolism of fatty acids or branched amino acids (65); and *EGLN3* (PHD3) (66), encoding a protein involved in the regulation of hypoxia-induced apoptosis.

Most interestingly, Th2 cells from asthmatic subjects had significantly increased transcripts for *GPR55*, which encodes a recently described G protein–coupled receptor (Fig. 4C) whose ligand is the endogenous lipid mediator phospholipid L- α -lysophosphatidylinositol (67). In cancer cell lines, activation of *GPR55* results in RhoA-dependent increases in Ca^{2+} signaling and NFAT activation as well as activation of both pERK and pAKT, leading to increased cell proliferation and migration (67). In monocytes and NK cells, activation of this receptor leads to increased production of proinflammatory cytokines such as TNF- α (68). Together these findings imply that increased expression of *GPR55* in Th2 cells may influence their survival and activation, as well as the nature of cytokines released following allergen stimulation.

Finally, Th2 cells from asthmatic subjects had increased transcripts for *ELAVL1* (Fig. 4C), which encodes a RNA-binding protein (*ELAVL1*, also known as HUR) known to selectively bind to AU-rich elements in the 3' untranslated regions of mRNA and regulate transcript stability and translation. *ELAVL1* regulates several genes involved in the cell cycle and stress response and thus promotes cell survival (69). Most interestingly, and relevant to asthma pathogenesis, *ELAVL1* has been shown to posttranscriptionally regulate (increase) the expression of Th2 cytokines (IL-4, IL-13) as well as the master Th2 transcription factor GATA3, and thus play an important role in the differentiation and function of Th2 cells (70). Therefore, increased levels of *ELAVL1* transcripts could enhance the capacity of Th2 cells from asthmatic subjects to produce Th2 cytokines.

Overall, our data point to several qualitative differences in Th2 cells from asthmatic subjects compared with those from allergic rhinitis or healthy control subjects. These differences, especially those linked to enhanced survival, activation, and Th2 functions, could play an important role in driving unrestrained and sustained Th2 inflammatory responses in asthmatic airways.

Discussion

The existence of pathogenic Th2 cells has been described in mouse models of allergic asthma and in human allergic diseases (71–75); Th2 cells have been defined as either pathogenic or protective based on their ability to produce additional proinflammatory cytokines such as TNF- α , IFN- γ , and IL-17 or an anti-inflammatory cytokine, such as IL-10 (71, 72, 74, 75). Furthermore, Th2 cells that are endowed with the ability to produce excessive amounts of IL-5, in addition to IL-4 and IL-13, have recently been shown to play a pathogenic role in driving allergic airway inflammation and chronic allergic dermatitis in mouse models (73). Such IL-5–producing Th2 cells, which express the chemokine receptor CCR2, have also been described in humans (32), but have not been fully characterized in patients with allergic asthma. In our large study of subjects with well-phenotyped asthma and rhinitis, we undertook an unbiased characterization of the full spectrum of molecules and gene expression programs that discriminate the pathogenic features of Th2 cells, if any, present in asthmatic subjects; specifically, we asked whether Th2 cells from subjects with asthma have a pathogenic molecular program when compared with Th2 cells from subjects with allergic rhinitis or non-atopic healthy subjects.

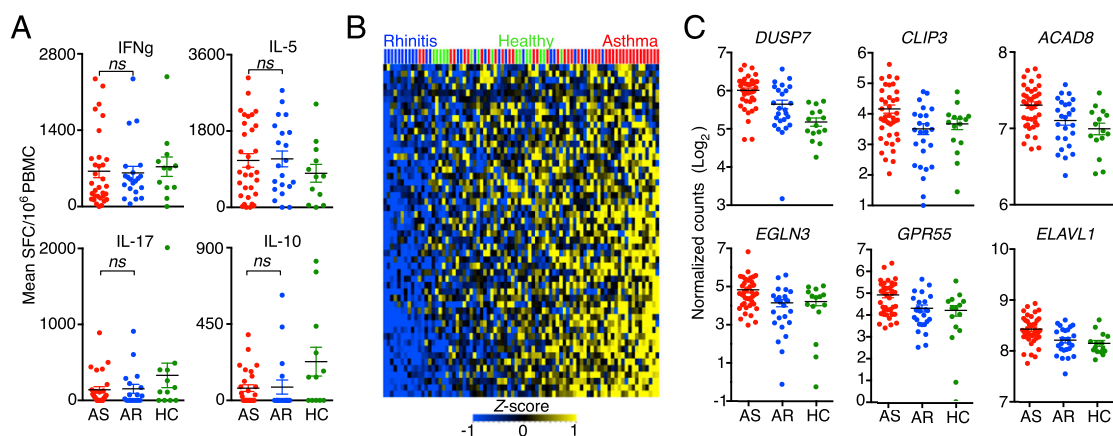


FIGURE 4. Upregulated genes in asthma compared with allergic rhinitis. **(A)** Magnitude of cytokine production in allergic rhinitis, asthmatic, and nonatopic healthy control subjects. IFN- γ , IL-5, IL-17, and IL-10 responses elicited by timothy grass peptide pool in a 14-d in vitro restimulation assay. **(B)** Row-wise Z-scores of library size-normalized read counts for each differentially expressed gene overexpressed in asthmatic compared with rhinitis subjects (DESeq2 analysis, Benjamini-Hochberg method FDR <0.2; see *Materials and Methods*). RNA-Seq data from each independent sample ($n = 80$) are shown in the columns. **(C)** Dot plots showing expression values (read counts) of indicated genes for each individual sample analyzed by RNA-Seq; each dot shown in green (healthy subject = HC), blue (allergic rhinitis = AR), and red (asthma = AS) are data from a single RNA-Seq assay, and error bars are mean $\pm$ SEM.

We found many molecular features that may contribute to the pathogenicity of Th2 cells in asthma. These include 1) long-term persistence due to altered expression of several genes involved in the IL-25, MAPK, AKT, NF- κ B, Notch, and c-Myc pathways that are known to promote survival, resist apoptosis, and enhance proliferation (34, 35, 46–49). Interestingly, we found that intracellular zinc signaling is likely to be perturbed in Th2 cells from asthma subjects, and, given the role of zinc in T cell proliferation and activation (40–42), this could be a new pathway that is important in asthma. Another possible contributor is 2) altered energy metabolism due to increased expression of *CPT1A* and *MYC* (39, 47), which could switch metabolic activity to favor long-term survival in nutrient-deprived environments such as inflamed airways. 3) Excessive Th2 polarization and Th2 cytokine release may result from the observed increased expression of the receptor for IL-25 (*IL17RB*) (33) and HUR (*ELAV1*), a RNA-binding protein known to posttranscriptionally regulate GATA3, IL-4, and IL-13 (70), and decreased expression of genes that promote alternative fates, that is, Th1 differentiation, such as *PTGER4* (involved in PGE₂ signaling), *REL*, and *RELB* (NK- κ B transcription factors) (54–57). Finally, 4) enhanced activation potential is implied by decreased expression of several members of the DUSP family of negative regulators of T cell activation (50, 51). Overall, our observations suggest that there are qualitative differences in Th2 cells from subjects with asthma, and that these features could confer Th2 cells with pathogenic properties required to initiate and/or sustain allergic airway inflammation in asthma. At the same time, we found no differences in the expression of chemokine receptors and adhesion molecules involved in trafficking of T cells, suggesting that alterations in homing potential are an unlikely explanation for the pathogenicity of Th2 cells in asthma. Notably, the transcriptional profile of Th2 cells from allergic rhinitis subjects revealed an intermediate phenotype between asthma and healthy subjects that is more similar to asthma. Our findings suggest that Th2 cells from allergic rhinitis subjects are also likely to exhibit pathogenic properties, albeit less pronounced compared with asthma.

Further studies should focus on Th2 cell heterogeneity, as transcriptomic experiments that examine only population-level characteristics can often obscure crucial biological differences observed in single cells within that population. Importantly, the differential expression of genes observed in the Th2 population (i.e., CCR4⁺CD4⁺ T cells that are enriched in Th2 cells) from asthma subjects may be due to the expansion of some novel Th2 subsets within this population that selectively express these genes. For example, the increased expression of *IL17RB* or *CPT1A* in Th2 cells from asthmatic subjects may well be due to expansion of a small new subset of Th2 cells that exclusively express these genes. This possibility can be addressed at a genome-wide level by employing cutting-edge single-cell RNA-sequencing techniques, which can both identify the cells expressing target genes as well as determine the global gene expression profile of these cells, thus helping define new distinct subsets in a given population of cells (76–79). Notably, we have identified a number of new genes (e.g., *GPR55*, *SLC39A7*, *CLIP3*, *TTC38*) that are selectively enriched in the Th2 population from asthmatic subjects; before embarking on studies to determine their functional significance in Th2 cells, it will be helpful to establish whether additional new Th2 subsets selectively expressing these genes are present within the Th2 population from asthmatic subjects.

Our study has defined a spectrum of molecules that are likely to confer pathogenic properties to Th2 cells from subjects with asthma. Exploring the function of these molecules and the cells that are expressing them is likely to reveal new targets for controlling

Th2-driven inflammation in asthma. Furthermore, it will improve our understanding of the diversity present in the Th2 cell population from healthy controls as well as subjects with allergic diseases.

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Disclosures

The authors have no financial conflicts of interest.

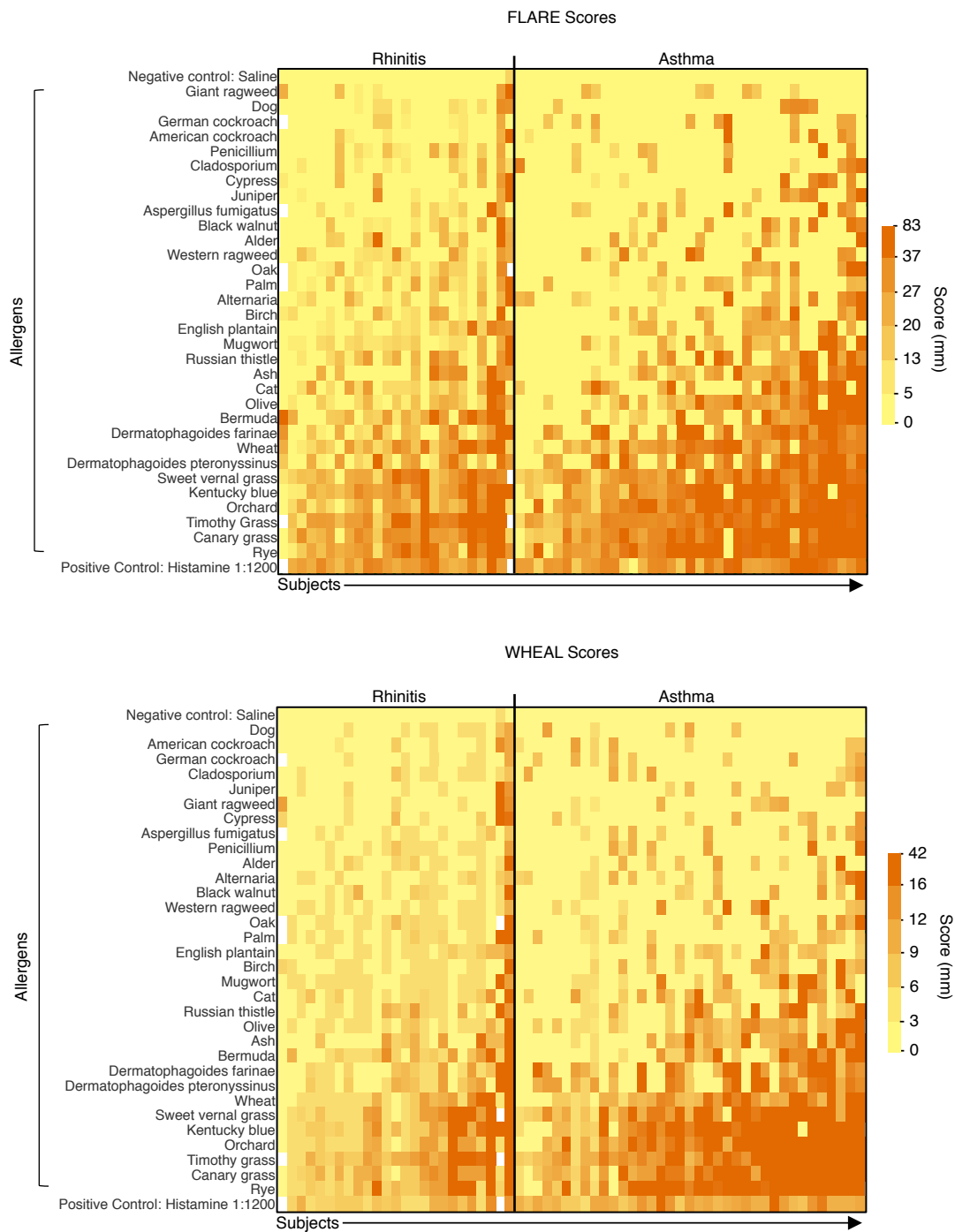
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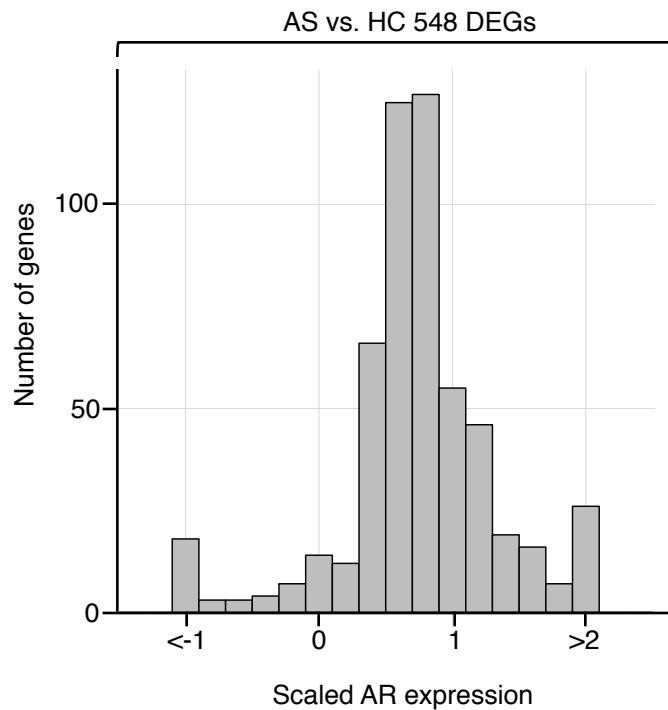
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Supplemental Figure 1. Heatmaps showing the flare and wheal scores for every allergic subjects, for 32 common allergens.



Supplemental Figure 2. Genes that differentiate asthmatic from healthy subjects show an intermediate phenotype in allergic rhinitis subjects.



Supplemental Figure 2. Histogram showing the distribution of gene expression levels for subjects with Allergic Rhinitis (AR) for the 548 differentially expressed genes identified when comparing healthy controls (HC) vs. asthmatic (AS) subjects. The gene expression levels in allergic rhinitis subjects were log transformed and rescaled so that 0.0 corresponds to the average expression in healthy donors, and 1.0 corresponds to the average expression in subjects with asthma. Values greater than 1.0 imply an even more pronounced difference in rhinitis from healthy than the asthmatics. Values lower than 0.0 mean they had the opposite difference from healthy controls than what was observed in asthmatic subjects.