

Oligonucleotide-microarray analysis of peripheral-blood lymphocytes in severe asthma

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CD4⁺ lymphocytes play a key role in asthma pathogenesis, but much remains unknown about the genetic mechanisms that affect disease severity. In this study we sought to investigate global patterns of gene expression in CD4⁺ lymphocytes isolated from subjects with severe asthma through the use of microarray technology. CD4⁺ lymphocytes were separated from peripheral blood, total RNA was purified, and biotinylated complementary RNA was prepared and hybridized to Affymetrix HU133 chips (Affymetrix, Santa Clara, Calif). Using the robust multi-chip average procedure, we compared the messenger RNA expression profiles of more than 33,000 genes of CD4⁺ lymphocytes in subjects with severe ($n = 5$) and mild ($n = 5$) asthma. Forty genes had 2-fold mean expression differences or greater. Thirty-seven genes were up-regulated, including transforming growth factor- β and those involved in T-cell activation, proliferation, and cytoskeletal changes. Three genes were down-regulated, including the T-cell-receptor delta locus. This study demonstrates a method by which CD4⁺ lymphocytes can be extracted from blood for the purpose of microarray analysis. Furthermore, we show that T-lymphocytes from the peripheral blood of subjects with severe and mild asthma differ in their gene-expression profiles, supporting the view that asthma is a systemic disease. These differentially expressed genes identify potential molecular targets for preventive and therapeutic options for severe asthma. (J Lab Clin Med 2005;145:263-274)

Abbreviations: cDNA = complementary DNA; cRNA = complementary RNA; EDTA = ethylenediaminetetraacetate; FEV₁ = 1-second expiratory volume; ICS = inhaled corticosteroid; IL = interleukin; mRNA = messenger RNA; PBMC = peripheral-blood mononuclear cell; RMA = robust multi-chip average procedure; RT-PCR = real-time polymerase chain reaction; TGF β = transforming growth factor; TNF = tumor necrosis factor

Asthma is a common chronic inflammatory disease of the lungs with serious public-health consequences. It affects more than 10 million adults in the United States alone, at a total estimated cost in 1998 of \$12.7 billion.¹ Asthma is a complex polygenic disease of variable severity. Patients with

more severe disease have disproportionately high rates of morbidity and mortality, and greater financial impact on the health-care system. Indeed, it has been estimated that 20% of the population with the most severe asthma accounts for 80% of asthma expenditures.²

Severe or poorly controlled asthma may lead to in-

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Table I. Asthma characteristics, by severity

Parameter	Mild asthma, no ICSs	Mild asthma requiring ICSs	Severe asthma
Diagnosis	Physician-diagnosed	Physician-diagnosed	Physician-diagnosed
ICS use	None	Daily	Daily
Criteria	Symptoms < 2 times/wk, nighttime symptoms < 2 times/mo, use of a short-acting β -agonist < 1 time/d	Symptoms < 2 times/wk, nighttime symptoms < 2 times/mo, use of a short-acting β -agonist < 1 time/d	At least 1 of the following: daily symptoms, nighttime symptoms > 1 time/wk, daily use of a short-acting β -agonist

creased tissue remodeling, which in turn can lead to irreversible disease and prolonged disability.³ However, data from research focused on genetic predisposition to severe asthma are scarce, and much remains unknown about the genetic and molecular mechanisms associated with asthma severity.

The severity of asthma has been shown to be based in heredity, with a familial correlation in clinical severity score and FEV₁.⁴ Aberrant regulation of gene expression resulting from genetic polymorphisms may influence asthma severity by augmenting proinflammatory-mediator expression or by reducing expression of antiinflammatory mediators. Studies in which asthma is examined as an “all-or-none” phenomenon may miss genes that modify disease severity or determine response to treatment. A few candidate genes associated with asthma severity have been identified.^{5–7} In particular, polymorphisms in the IL-4 and IL-4–receptor genes have been associated with asthma severity, in addition to susceptibility per se.^{6,8,9} IL-4 is secreted mainly by CD4⁺ T_H2 cells, lymphocytes thought to play a central role in airway inflammation and hyper-responsiveness.^{8,9} These cells are characteristic of helper T-cells in asthma and other allergic diseases, and they release cytokines, including IL-4, IL-5, IL-9, and IL-13, resulting in eosinophilic activation, IgE production, and subsequent mucosal inflammation of the airways.¹⁰ The relationship of lymphocytes and their effector function as they relate to the ongoing inflammation and airway remodeling seen in severe asthma needs to be explored in greater depth.

Understanding the pathophysiology of asthma and how severe asthma differs from mild and moderate disease is critical in the development of more effective treatments. In this study we sought to gain understanding of the pathogenetic process differentiating severe and mild asthma. With the use of DNA microarray techniques, we are able to assay the expression of thousands of genes quickly and simultaneously. We chose GeneChip microarrays (Affymetrix, Santa Clara, Calif), containing oligonucleotide hybridization probes representative of more than 33,000 genes, with which to perform a comprehensive examination of the expres-

sion profiles of CD4⁺ T-lymphocytes in patients with asthma of various levels of severity. This information will allow us to evaluate the genetic basis for the variability observed in asthma severity.

METHODS

Subjects. We recruited subjects with the use of mailings and posters. Patients seen at the Johns Hopkins Asthma and Allergy Center for asthma care, denoted by primary or secondary ICD-9-CM code 493.xx from claims data, were identified from billing records, and a random sample of these patients were sent a mailing. Posters describing the study were also displayed in Johns Hopkins University buildings. Eligibility criteria included (1) patient-reported, physician-diagnosed asthma; (2) symptoms of wheezing during the preceding year, (3) age 18 years or older, and (4) ability to provide informed consent. Exclusion criteria included (1) a history of other chronic lung disease, (2) human immunodeficiency virus–related disease, (3) current treatment with oral corticosteroids, (4) smoking during the preceding 2 weeks, and (5) asthma exacerbation during the preceding 4 weeks. Smokers were excluded because smoking can cause reversible alterations in immunoregulatory T-cells in peripheral blood.¹¹

One of the coauthors administered a questionnaire to elicit information on demographics, comorbid illnesses, respiratory symptoms, and medication use. Lung function and FEV₁ were assessed with the use of spirometry in accordance with American Thoracic Society standards.¹² Subjects were classified as having mild asthma not requiring ICSs ($n = 5$), mild asthma requiring ICSs ($n = 5$), or severe asthma ($n = 5$) (Table I).

Atopy status was assessed by means of skin-prick testing for 9 common aeroallergens (Greer Laboratories, Lenoir, NC): *Penicillium notatum*, *Alternaria tenuis*, cockroach mix (German and American), dust-mite mix (*Dermatophagoides farinae* and *Dermatophagoides 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), and 2 controls (histamine and glycerine). Atopy was defined as the formation of a wheal in response to at least 1 of the allergens at least 3 mm greater in diameter than that elicited by the negative control (glycerine).

The research was carried out in accordance with the principles of the Declaration of Helsinki, and each subject pro-

vided written informed consent. The study was approved by the Western Institutional Review Board (Olympia, Wash).

CD4⁺ lymphocyte and RNA extraction. Sixty milliliters of peripheral blood was collected in EDTA-treated tubes. All samples were processed within 2 hours of initial phlebotomy. CD4⁺ lymphocytes were subsequently isolated (>95% purity as assessed with the use of flow cytometry, data not shown) from anticoagulated whole blood by means of negative selection with the use of the RosetteSep procedure (Stem Cell Technologies, Seattle, Wash) in accordance with the manufacturer's instructions. CD4⁺ T-cells were washed once with phosphate-buffered saline solution plus 2% fetal-calf serum (Hyclone Inc., Logan, Utah), and total RNA was extracted with the use of TRIzol (Invitrogen Life Technologies, Carlsbad, Calif). RNA was quantitated by means of spectrophotometry, and mRNA integrity was confirmed with the use of agarose-gel electrophoresis and ethidium bromide staining. RNA was stored at -80°C until further use.

Microarray cRNA synthesis and labeling, hybridization, and expression profiling. Isolated CD4⁺ lymphocytes were homogenized in TRIzol reagent and total RNA was purified by means of extraction with chloroform followed by isopropanol precipitation. We synthesized double-stranded cDNA from 6 µg of total RNA using the SuperScript Choice system (Invitrogen Life Technologies) and the T7-Oligo(dT) promoter primer kit (Affymetrix). cDNA was purified with the use of Phase Lock Gels (Eppendorf 5-prime). Biotin-labeled cRNA was then synthesized from double-stranded cDNA with the use of the ENZO BioArray High Yield RNA transcript labeling kit (ENZO Life Sciences, Inc., Farmingdale, NY). cRNA was purified with the use of an RNeasy Mini kit (Qiagen, Chatsworth, Calif) and fragmented into 35- to 200-base-pair fragments through the use of metal-induced hydrolysis (200 mmol/L Tris acetate, pH 8.2; 500 mmol/L KOAc; 150 mmol/L MgOAc). Fifteen micrograms of biotin-labeled cRNA was hybridized onto Human Genome U133A and U133B GeneChips (Affymetrix) for 16 hours at 45°C and 60 rpm. GeneChips were then washed and stained in accordance with the instructions set forth in the Affymetrix GeneChip Expression Analysis manual. This procedure includes the removal of nonhybridized material, staining with phycoerythrin-streptavidin, and a laser scan to detect bound cRNA. We detected fluorescence using the Hewlett-Packard GS2500 Gene Array Scanner.

We followed strict quality-control measures to ensure the collection of high-quality data, including the requirement that scaling factors, percent of genes called present, average intensity, background values, housekeeping 3':5' ratios, and measured intensities of spiked-in controls fall within predefined limits. Additionally, only samples with "acceptable" amplification at the T7 RNA polymerase cRNA step were hybridized onto chips. A detailed description of all quality-control parameters, as well as all image files (.dat) corresponding to this work, is available on our Web site.¹³

Confirmatory studies. We conducted validation with the use of RT-PCR involving total RNA isolated from the same patient samples used in the microarray study. Fluorogenic LUX primers (Invitrogen Life Technologies) were used to

Table II. Primer sequences used for microarray validation studies (RT-PCR)

Gene	Primer sequence
Target	
TGFB1	Forward (FAM-labeled)* CAGCATCCACGGAAGAACTGCTG Reverse GAGGCAGAGTTGGCATGGT
TRD@	Forward (FAM-labeled) GACTTCTGTGTGACTGTGGAACC AAGAAGTC Reverse CCTTCACCAGACAAGCGACAT
JUND	Forward (FAM-labeled) CACTTTGCGCCTGGAAGAGAAAAG TG Reverse TTGAGCTGCGCCACCTG
PLEC1	Forward (FAM-labeled) CAGCGGATCTCCTATAAGGACGC GC5G Reverse GCTGTAGTAGCCCTTGGTGGAC
CD6	Forward (FAM-labeled) GACCTGATGCTGCCCATCCAGGT C Reverse CCGAGCCAGAGTCTGAGTCCT
Reference genes	
NMT1	Forward (JOE-labeled)* CTAACATGAAGGAGGGGAACCAG ATGT5AG Reverse CCATCAGAAGGCTGCCTCA
HLA-F	Forward (JOE-labeled) CAGCCAAGCACCAGATCAAACAG GCTG Reverse TGCTTCTCTCTCCATCAGAC

*Applied Biosystems, TaqMan, Foster City, Calif.

validate relative changes in mRNA levels by means of multiplex RT-PCR with the use of an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, Calif). To remove genomic DNA contamination, we treated total RNA with DNase I (Invitrogen Life Technologies) for 15 minutes at room temperature before cDNA synthesis with the use of the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies). First-strand cDNA was synthesized from 1 µg of total RNA. Multiplex PCR reactions were performed in triplicates with the use of Platinum Quantitative PCR SuperMix-UDG and ROX reference dye (Invitrogen Life Technologies). Reactions were conducted in 25-µL reaction volumes under the following conditions: 50°C for 2 minutes, 95°C for 2 minutes, and 45 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. Primer sequences used for the validation PCR are presented in Table II. A negative control reaction in the absence of template was performed routinely in triplicate for each primer pair as a means of ensuring that no signal was detected in the absence of amplification. Five genes with significant differences between patients with mild asthma

who were not taking ICSs and those with severe asthma were selected for another validation procedure, namely PLEC-1 (plectin-1), CD6 (CD6 antigen), TGFB1 (TGF- β_1), JUND (transcription factor jun-d), and TRD@ (T-cell-receptor delta locus).

Two genes (*N*-myristoyltransferase-1 and major histocompatibility complex class I, F) observed to have expression levels similar in magnitude to those observed for the genes of interest, as well as constitutive expression across all patient groups, were used as reference genes (Table II). Before their use in confirmation studies, we evaluated primer sets to determine that the efficiencies of the primers designed for the target and reference genes were approximately equal (data not shown). We analyzed results using the comparative Ct method.¹⁴

Statistical analysis. We characterized clinical variables using descriptive statistics, including proportions or means with ranges when appropriate. A sample size of 5 patients per group was chosen on the basis of the findings of previous studies successful in detecting differentially expressed genes with the use of microarray technology in patients with known genetic defects.¹⁵

We used an empirically motivated statistical approach, the RMA procedure, to estimate the expression of each gene.¹⁶ RMA comprises 4 stages. First, the mean background noise level is estimated and the intensity for each probe is adjusted to remove this. Next, probe-level data from the entire chipset are simultaneously normalized. This step eliminates systematic differences between chips without significantly altering the relative intensity of probes within a chip. Third, the normalized, background-corrected data is transformed to the log₂ scale. This step helps decrease the variance across the range of intensities. Finally a robust, median-polish procedure is used to combine multiple probes into a single measure of expression for each gene. Irizarry et al¹⁷ recently reported that the use of the RMA approach improves the ability to detect differential gene expression compared with standard methods.

We averaged expression estimates to obtain a single mean expression value for each asthma group and for each gene. Even though high confirmation rates for genes differentially expressed by less than 2-fold have been obtained in previous studies,¹⁸ the degree of differential expression representing biologically important changes in asthma is unknown. Therefore, as a means of reducing the false-positive rate, we selected genes with arbitrary fold changes greater than 2 between subjects with severe asthma and subjects with mild asthma not requiring ICSs as candidates for further study. To address the possible effect of ICSs on gene expression, we recruited 5 additional subjects with mild asthma symptoms despite daily ICS use. Gene expression in the subjects with mild asthma who were taking ICSs was compared with that of patients with mild asthma who were not taking ICSs as a means of isolating the possible effect of ICS use on gene expression. In addition, because 2 of the groups were heterogeneous with regard to race and because previous studies have revealed racial genetic heterogeneity,¹⁹ we recalculated fold change while excluding the nonwhite subjects. Significance analysis for microarray²⁰ was performed on each can-

didate gene as a means of testing for significant differential expression. Significance is reported according to the false-discovery rate with the use of the q-value measure.²¹

RESULTS

The clinical characteristics of the participants are described in Table III. All subjects had physician-diagnosed asthma, and 14 of the 15 subjects were atopic. All subjects denied a history of other chronic or inflammatory medical conditions, except for 1 subject with mild asthma using ICSs, who reported a history of chronic sinusitis. Among the subjects with severe asthma, 1 reported having undergone resection of the left upper lobe for the treatment of mucous plugging and abscess formation and another reported a history of osteoarthritis. Among the patients with mild asthma, a history of hypothyroidism, atrial tachycardia, and gastroesophageal reflux disease were reported by 1 subject each. All patients used albuterol on an as-needed basis. Additional asthma-medication use is reported in Table III. Three subjects reported using oral antihistamines (2 with severe asthma, 1 with mild asthma who was taking ICSs). Participants denied recent use of other antiinflammatory medications, except for 1 subject with mild asthma who was taking ICSs who reported occasional use (<2 times per month) of a nonsteroidal antiinflammatory medication. The subjects with severe asthma had a lower mean FEV₁ (percent of the predicted value) and were older. All of the patients were white except for 2 black participants with severe asthma.

Sixty milliliters of blood was drawn from each subject between 9 and 11 AM and processed immediately for CD4⁺ T-cell isolation. Sufficient RNA was obtained from each subject for microarray analysis, and all samples met subsequent strict quality-control measures (see Methods).

Using the RMA approach with a 2-fold cutoff, we identified a total of 40 candidate, differentially regulated genes between subjects with severe asthma and those with mild asthma who were not taking ICSs (Fig 1). These included 37 up-regulated (Table IV) and 3 down-regulated (Table V) genes in subjects with severe asthma (43 probe sets). Differentially expressed genes included those encoding transcription factors, cell-surface molecules, and genes involved in T-cell activation, proliferation, and migration. A plot showing the absolute intensity (expression) of the 10 most differentially expressed genes is shown in Fig 2, a graphic image of the variance in gene expression. The results of 5 of these genes (TGFB1, CD6, JUND, TRD@, and PLEC1) were confirmed on RT-PCR (Fig 3).

The groups of patients with severe and mild asthma were balanced with regard to sex but not race. Two of the subjects in the severe-asthma group were black.

Table III. Characteristics of the study subjects

Parameter	Mild asthma (n = 5)	Mild asthma, no ICSs (n = 5)	Severe asthma (n = 5)
Age (yr)*	27.8 (22–34)	38 (26–49)	43.6 (39–50)
Sex (F/M)	4:1	4:1	4:1
Race	All white	All white	3 white, 2 black
FEV ₁ (% predicted)*	91.7 (76.4–104.8)	98.5 (83.9–126.3)	57.3 (44.5–93.5)
Atopy†	4	5	5
Medications	Albuterol‡ (n = 5), cromolyn sodium (n = 1), salmeterol (n = 1)	ICSs (n = 5), albuterol‡ (n = 5), salmeterol (n = 2), leukotriene-receptor antagonist (n = 3), theophylline (n = 1)	ICSs (n = 5), albuterol‡ (n = 5), salmeterol (n = 4), leukotriene-receptor antagonist (n = 3), theophylline (n = 1)
Frequency of albuterol use	0–3 times/wk	0–3 times/wk	Daily
Frequency of wheezing	1–3 times/wk	0–3 times/wk	1 time/wk–daily
Nighttime symptoms in preceding 4 wk	0	1	4
No problems breathing between attacks	4	3	0
Patient rates asthma as being under good or excellent control	5	3	1
Emergency-department visits in preceding yr	1	0	3
Hospitalized for asthma at any point	0	3	4

*Data expressed as mean (range).

†Positive results on more than 1 skin test.

‡Subject reported taking medication on an as-needed basis.

Two tests suggested that the racial imbalance of the study did not contribute significantly to the differences in expression observed in the candidate genes. A simple *t* test showed little significant difference in expression between blacks and whites in the severe-asthma group (data not shown). Furthermore, we recalculated fold changes between subjects with severe asthma and mild asthma who were not using ICSs while excluding the 2 black subjects. Most identified genes demonstrated increased fold changes, indicating that the differential expression observed for those genes was not driven by racial differences. Twenty-nine of the genes continued to exhibit 2-fold differential expression when black subjects were excluded from the analysis. The genes that no longer demonstrated 2-fold differential expression, which may therefore have been influenced by racial effects, are shown in Table IV.

DISCUSSION

CD4⁺ lymphocytes play a central role in the inflammation and hyperresponsiveness of the airways of peo-

ple with asthma. Previous studies have revealed that peripheral-blood lymphocytes from subjects with asthma are activated,²² but this is the first study, to our knowledge, in which global patterns of gene expression in peripheral CD4⁺ T-cells from subjects with severe vs. mild asthma have been systematically investigated. In this analysis, we found substantial differences in the expression of a relatively small number of genes, some of which have been previously implicated in asthma pathogenesis (eg, TGF- β), a few with plausible links to asthma (eg, urotensin), and some not previously associated with asthma pathophysiology (eg protein kinase C substrate 80K-H [PRKCSH]). RT-PCR results correlated well with the differential gene-expression data produced with the use of Affymetrix GeneChips. The up-regulation of genes involved in T-cell activation, proliferation, and migration in the peripheral-blood T-cells of subjects with severe asthma supports the notion that asthma is a systemic disease and that activated CD4⁺ T-cells recirculate from the blood to the airways during inflammatory reactions. Furthermore, the up-

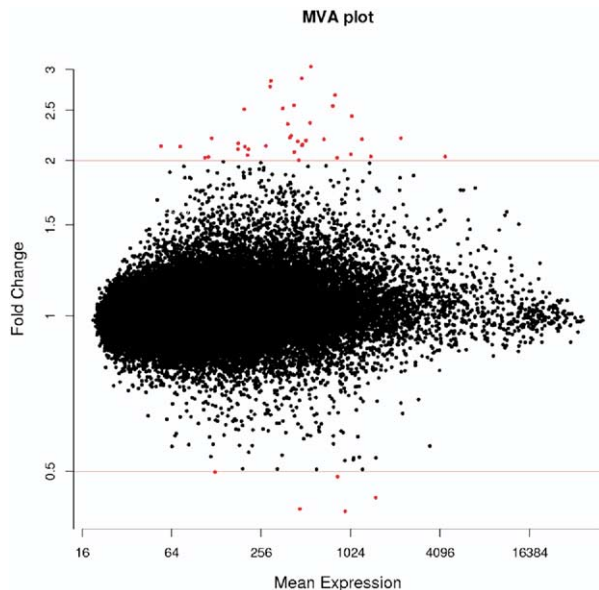


Fig 1. Each gene is represented by 1 or several probe sets on the Affymetrix GeneChip. The mean log intensity (*expression*) for all subjects of each probe set, represented by a *dot*, is plotted along the *x-axis*. The *y-axis* represents the log fold change between subjects with severe and mild asthma. Probe sets above the *upper horizontal line* and below the *lower horizontal line* correspond to genes with greater than 2-fold differential expression (listed in *Tables III and IV*, respectively). These probe sets are highlighted in *blue*.

regulation of genes involved in signal transduction, gene transcription, and extracellular-matrix modulation implicates mechanisms involved in airway remodeling.

Genes involved in T-cell activation (CD6, CD3E)^{23,24} and proliferation were up-regulated in subjects with severe asthma. Specifically, Ras genes are thought to be involved in the regulation of cell proliferation. Activation of Ras has been shown to be involved in eosinophilic inflammation and airway hyper-responsiveness, promoting T_H2 differentiation in a murine model.²⁵ There were 4 up-regulated genes (RAB14, RAB2, KRAS2, CAPRI) of the Ras oncogene family, indicating that this GTPase signaling may be increased in the T-cells of subjects with severe asthma. Activation of the Ras-mitogen-activated protein kinase (MAPK) pathway may help explain increased expression of JunD, a member of the AP-1 family of transcription factors. JunD, it should be noted, is involved in the transcriptional regulation of the T_H2 cytokines IL-4 and IL-5.²⁶

We also observed differential expression of several genes that may be involved in T-cell migration in our subjects with severe asthma. These findings included the up-regulation of genes implicated in cell adhesion (NK4, B4GALT1) and cytoskeletal organization (ARPC4, PLEC1, RAC2, SEPT1), as well as the down-

regulation of a gene (SPEC2) that may have inhibitory effects on cytoskeletal changes.²⁷ In particular, PLEC1 has been shown previously to be an integral component of the lymphocyte cytoskeleton and likely plays an important role in the regulation of shape changes that occur during lymphocyte migration from circulation to tissue.²⁸ The role of the cytoskeleton in T-cell activation and migration is an emerging area of research,²⁹ and our findings suggest that future study in this area of asthma immunology would be worthwhile.

Until recently asthma was considered essentially reversible disorder, but it is now acknowledged that chronic inflammation in asthma may lead to structural alterations. Airway remodeling involves a complex series of changes, including airway-wall thickening, sub-epithelial fibrosis, an increase in smooth muscle, vascular proliferation, and mucous-gland hyperplasia.³⁰ Remodeling of the lung architecture, subepithelial thickness,³ and collagen type III deposition³¹ are increased in severe asthma. Up-regulated genes in our study that may be involved in modification of the extracellular matrix included FCN1, LRAP, and TGF β . The function of FCN1 is unclear; however, it has been shown to bind elastin,³² an integral component contributing to lung elasticity. LRAP is an aminopeptidase that has been shown to be increased in the airways of asthmatic patients, and it is thought to be involved in the degradation of peptides and to modulate peptide-mediated inflammation in asthma.³³ TGF- β has been implicated in multiple chronic lung diseases, including asthma, acute respiratory-distress syndrome, and interstitial lung diseases.^{34,35} TGF- β plays a dual role. It acts as an antiinflammatory cytokine by inhibiting T-cell activation yet also induces formation of extracellular matrix and fibrosis. Polymorphisms in its promoter region have been associated with increased levels of total IgE³⁶ and with severe asthma.³⁷ Furthermore, TGF- β signaling, measured on the basis of phosphorylated Smad2, has recently been linked to the degree of airway remodeling and airway hyperresponsiveness.³⁸ TGF- β may also act, in part, through the Ras-MAPK pathway,³⁹ and we identified up-regulation of several genes in the Ras-MAPK pathway. The potential role of this signaling pathway for TGF- β in asthma merits further investigation. Our study supports the notion that increased gene expression of TGF- β plays a central role in the modification of asthma severity. It may act as a negative regulator of the ongoing inflammation while contributing to the irreversible structural changes that occur with long-standing asthma.

To our knowledge information on the importance of urotensin-2 on airway function is limited. Human urotensin-2 has been shown to contract tracheal and bronchial smooth-muscle preparations from the primate re-

Table IV. Up-regulated genes in severe and mild asthma

Gene	Affmetrix probe	Gene name/function	q
Activation/cell-surface markers			
CD3E	205456_at	ϵ -Subunit of the CD3 T-cell-receptor complex; signals T-cell activation ²³	.064
CD6	211893_x_at 208602_x_at	T-cell costimulation and adhesion; crosslinking of CD6 and CD28 induces IL-2-dependent T-cell proliferation	.058 .061
CD7	214551_s_at	T-cell interaction	.077
Proliferation (Ras gene family)			
RAB14*	211503_s_at	GTPase, signaling; function remains to be elucidated	.061
RAB2*	208730_x_at	Required for vesicle transport to Golgi complex	.353
KRAS2*	214352_s_at	v-Ki-ras2, Kirsten rate sarcoma 2 viral oncogene homolog; GTPase signaling; activation of Ras involved in eosinophilic inflammation and airway hyperresponsiveness by promoting Th2 differentiation in murine model ²⁵ , modifies proliferative response of rheumatoid synovial cells to TNF- α and TGF- α ⁵⁹	.499
CAPRI	208534_s_at	Ca ²⁺ promoted Ras inactivator; switches off the Ras-MAPK pathway after G protein-coupled receptor-stimulated intracellular-calcium increase	.058
Cytoskeleton			
PLEC1*	216971_s_at	Plectin-1, intermediate filament-binding protein, 500 kD; crosslinks the cytoskeleton and plasma membrane; integral part of the lymphocyte cytoskeleton; binds to lamin B, fodrin, actin, and vimentin; plays a role in the regulated shape changes occurring during lymphocyte recruitment and polarization of peripheral T-lymphocytes to migrate from circulation to tissue ²⁸	.093
ARPC4*	211672_s_at	Actin-related protein 2/3 complex, subunit 4, 20 kD; control of actin polymerization ⁶⁰	.105
RAC2	207419_s_at	Regulates cytoskeletal reorganization and axon guidance; regulates signaling pathways; enhancement of IL-2-promoter activity in response to TCR stimulation ⁶¹	.106
SEPT1	227552_at	Septin 1; involved in cytokinesis, cytoskeletal organization ⁶²	.269
Cell adhesion			
NK4*	203828_s_at	Natural killer-cell transcript 4; cell adhesion; expression increased after activation of T-cells	.055
B4GALT1	228498_at	UDP-Gal: β GLcNAc β 1,4-galactosyltransferase; lactose biosynthesis; possible role in cell trafficking; increased expression and activity in peripheral-blood T-cells activated in vitro (T-cells subsequently had increased carbohydrates with a high galactose-residue content and increased adhesion properties) ⁶³	.223
Transcription			
GTF2F1	202354_s_at	General transcription factor	.055
SP110	209761_s_at	May function as a nuclear hormone receptor transcriptional coactivator ⁶⁴	.102
ZFP36L2	201367_s_at	Zinc finger protein 36, C3H type-like-2; regulates response to growth factors	.055
JunD	203751_x_at	JunD protooncogene; involved in activation of IL-4 promoter after CD28 stimulation ²⁶	.055
Signaling			
MFNG	204152_s_at	Manic-fringe homolog; may control activation of notch signal transduction during embryonic development	.055
PRKCSH	200707_at	Protein kinase C substrate 80K-H; function unknown but linked to polycystic liver disease	.113
DGKA	211272_s_at	Diacylglycerol kinase- α , 80 Kd; converts diacylglycerol to phosphatidic acid; regulatory roles include cell proliferation and movement; activation is essential step in IL-2-mediated lymphocyte proliferation ⁶⁵	.369

Table IV. (continued)

Gene	Affmetrix probe	Gene name/function	q
Extracellular matrix			
TGFB1	203085_s_at	TGF- β_1 ; associated with degree of airway remodeling and hyperresponsiveness in asthma ³⁸	.055
FCN1*	205237_at	Ficolin-1; binds elastin and may function as opsonin ³²	.658
LRAP	219759_at	Leukocyte-derived arginine aminopeptidase; may modulate peptide-mediated inflammation in asthma; increased levels found in the airways of individuals with asthma ³³	.424
Miscellaneous			
EIF5A	213757_at	Eukaryotic translation initiation factor-5A; protein translation and mRNA decay	.121
PTBP1	212016_s_at	Polypyrimidine tract-binding protein-1; RNA-binding protein; required for pre-mRNA splicing	.061
MFHAS1*	213457_at	Malignant fibrous histiocytoma amplified sequence-1; may be related to cell cycle	.383
SRRM2*	208610_s_at	Serine/arginine repetitive matrix-2; splicing-coactivator subunit	.444
UTS2*	220785_at	Urotensin-2; contracts tracheal and bronchial smooth-muscle preparations from the primate respiratory tract ⁴⁰	.238
POLR2E	213887_s_at	DNA-dependent RNA polymerase	.055
Function unknown			
WDR18	212574_x_at	WD repeat domain 18; may mediate protein-protein interaction	.077
MGC10986	218600_at		.167
PRO1073	231735_s_at		.545
EST*	213015_at		.284
EST*	210172_at		.421
EST	224568_x_at		.562
EST	227462_at		.318
EST	231866_at		.566

*No longer up-regulated 2-fold when blacks were excluded from the comparison.

spiratory tract.⁴⁰ Its role in the pathogenesis of asthma is yet unknown. However, given its biologically plausible role in asthma, it deserves further study as a potential modifying gene in asthma severity.

The findings of previous independent studies have suggested that chromosome 19p13-13.3 harbors susceptibility loci associated with atopic phenotypes, IgE-related traits, and inflammatory conditions.⁴¹⁻⁴³ Six of the 37 up-regulated genes localized to this region, including B4GALT1, GTF2F1, JunD, PRKCSH, PTBP1, POLR2E and WDR18. The results of this study highlight potential candidate genes responsible for this susceptibility that might not have been otherwise targeted for investigation.

Only 3 genes were down-regulated in subjects with severe asthma, including the T-cell receptor delta locus. The biological function of $\gamma\delta$ T-cells is poorly understood, and debate continues as to whether they are essential or protective in asthma. In a murine model, $\gamma\delta$ T-cell deficiency was found to lead to increased airway hyperresponsiveness in both larger and smaller airways without change in inflammatory markers and despite a

decrease in eosinophilic inflammation.⁴⁴ The relative and absolute numbers of $\gamma\delta$ T-cells have been found to be lower in the peripheral blood of human beings with asthma than in normal controls in several studies.^{45,46} Their lower level in peripheral blood may be explained by their enhanced migration through endothelium to the inflamed respiratory tract. However, the findings of studies in which the level of $\gamma\delta$ T-cells has been measured in the bronchoalveolar fluid of patients with asthma are inconsistent. Krug et al found $\gamma\delta$ T-cells to be similarly present in bronchoalveolar fluid in patients with mild asthma and normal controls (although they found a functional difference, with a higher percentage of $\gamma\delta$ T-cells staining positive for the T_H2 cytokines IL-5 and IL-13).⁴⁷ On the other hand, Spinozzi et al found $\gamma\delta$ T cells to be increased in the bronchoalveolar fluid of asthma patients compared with that of normal controls.⁴⁸ Linkage of polymorphisms of the TCR-A/D locus on chromosome 14 and serum IgE levels has previously been reported.⁴⁹ These results suggest that $\gamma\delta$ T-cells are involved in the pathogenesis of inflam-

Table V. Down-regulated genes in severe and mild asthma

Gene	Affymetrix probe	Name/function	q
TRD@	213830_at	T-cell-receptor delta locus $\gamma\delta$ T-cell deficiency leads to increased airway hyperresponsiveness in (TCR delta-/- mice in both larger and smaller airways ⁴⁴ , previous studies have shown linkage of specific IgE responses to the TCR-A/D locus on chromosome 14 ⁴⁹ ; both percentage and absolute cell number of $\gamma\delta$ T-cells were lower in the peripheral blood of patients with asthma than in that of controls ⁴⁵ ; no difference in $\gamma\delta$ T-cell levels in the bronchoalveolar fluid of normal subjects and patients with asthma, but the percentage of the subset secreting IL-5 and IL-13 was increased ^{46,47}	.378
	216191_s_at		.428
	217143_s_at		.374
SPEC2	229026_at	Nonkinase Cdc42 effector protein SPEC2; cell-shape changes; modification (inhibition) of Cdc42 signaling involved in membrane trafficking, cytokinesis, and kinase-signaling pathways	.178
EST	236379_at		.357

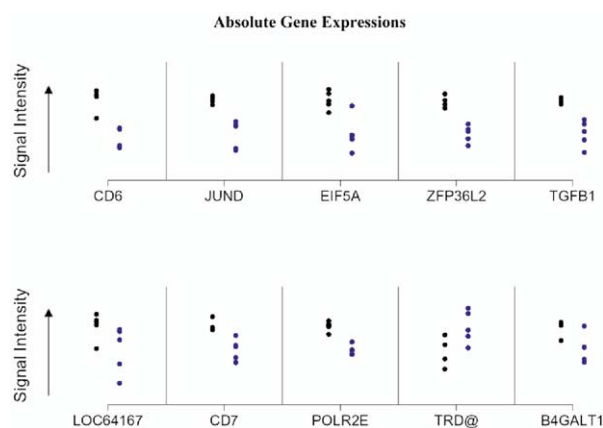


Fig 2. Absolute intensity values are plotted on the y-axis for the 10 genes with the greatest fold changes. Separate genes are plotted along the x-axis, as labeled. Each subject is represented by a dot: black for subjects with severe asthma, blue for those with mild asthma.

mation involved in asthma. Further research is necessary for understanding of their role.

A previous study by Brutsche and colleagues involving the use of PBMCs revealed differential patterns of gene expression in subjects with atopic asthma compared with controls.⁵⁰ There was no overlap between that study and our own. This difference may be related to the different characteristics of subjects studied (subjects with severe asthma compared with mild asthma in our study), different cell types (PBMCs vs CD4⁺ T-cells) or different methods used (custom DNA microarray vs oligonucleotide microarrays). Furthermore, despite the fact that asthma is considered a T_H2-biased disease, we did not detect differential expression of classic T_H2 markers such as GATA-3, NFATc, or STAT6 in subjects with severe asthma compared with subjects with mild asthma (data not shown). This finding may be a result of the fact that Th2 cells generally

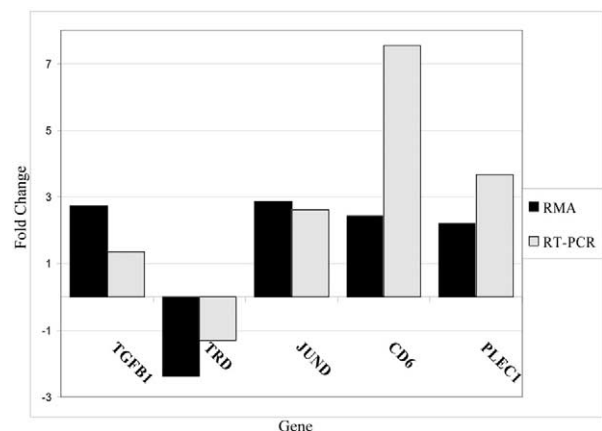


Fig 3. The y-axis represents fold change between subjects with severe and mild asthma for the 5 genes (plotted along the x-axis) confirmed with the use of RT-PCR. The results obtained from microarray analysis (black bars) and RT-PCR (gray bars) are shown for each gene. RT-PCR results confirmed the direction and approximate magnitude of fold change for each gene.

circulate in low numbers and the cells we studied were not activated. Furthermore, these genes may have been up-regulated but did not satisfy our predetermined minimum 2-fold differential expression criterion. For instance, STAT6 demonstrated 1.74-fold increased expression in subjects with severe asthma, not enough to meet our predetermined threshold.

Ethnic differences may influence genetic susceptibility to asthma.¹⁹ In this study we examined an ethnically heterogeneous group of subjects. When we excluded the 2 black subjects from our analysis, our results did not change substantially. All genes that were up-regulated in the initial severe-asthma group continued to demonstrate increased expression compared with that in the subjects with mild asthma, although in a few cases the association was less strong. Ethnic diversity

in our samples did not appear to have a substantial influence on our results.

Important distinctions between asthma severity and asthma control exist. "Asthma severity" describes the underlying disease, whereas "asthma control" describes the clinical status of disease in the face of intervention. Therefore, to ensure that we were analyzing asthma severity and underlying disease and not simply asthma control, we compared subjects defined as having severe asthma with subjects with mild asthma that did not require the use of ICS. The subjects with severe asthma experienced frequent symptoms despite controller therapy and were therefore likely to have severe underlying disease. The subjects with mild asthma that did not require controller medication likely had mild underlying disease that was not confounded by treatment. We did not conduct a direct comparison between the severe-asthma group and the mild-asthma group requiring ICSs because this may be confounded by treatment and influenced by the degree of asthma control.

Some of the asthma medications taken by our subjects with severe asthma, including ICSs and theophylline, may decrease the activation of peripheral lymphocytes and modulate gene expression.^{51–54} The effect of the use of ICSs or other asthma medications on the gene expression of peripheral lymphocytes is, at best, incompletely understood. Some of the differences in gene expression noted in our study may thus have been the result of systemic effects of medications taken by our subjects with severe asthma. Given the severity of the symptoms of our subjects, removing medications for the purposes of gene-expression analysis would have been unethical, in our view, and could have posed unnecessary risk to the participants with difficult-to-control asthma. However, despite the use of medications, the subjects with severe asthma continue to experience frequent symptoms and decreased FEV₁, and therefore the resultant gene expression likely represents a severe-asthma phenotype.

As a means of further addressing the possible effect of ICS on gene expression, we recruited 5 additional subjects with mild asthma symptoms despite daily ICS use. Gene expression among the subjects with mild asthma who were taking ICSs was compared with that in subjects with mild asthma who were not taking ICSs (data not shown) to isolate the possible effect of ICS use on gene expression. Ten of the genes from our initial analysis (FCN1, TFGβ₁, POLR2E, EIF5A, RAC2, PRKCSH, DGKA, RAB14, CD6, CD3E) were up-regulated when subjects with mild asthma and taking ICSs were compared with those who were not taking ICSs. The other 30 genes identified from our initial analysis were not differentially expressed between those with mild asthma and taking ICS and those

who were not taking ICSs, making it less likely that the expression of these genes is affected by the use of ICSs.

There is no standard agreement on the classification of asthma severity, and asthma phenotype/stability may vary over time. We analyzed subjects who reported no recent acute asthma exacerbation, and, to capture the subjects' current disease status, we classified asthma severity on the basis of symptoms experienced in the preceding 4 weeks. All subjects with severe asthma had moderate persistent or severe persistent symptoms, and all subjects with mild asthma had mild intermittent symptoms, according to guidelines set forth by the National Asthma Education and Prevention Program. Furthermore, we corroborated symptom frequency with spirometry results and health care–utilization data. Though pulmonary-function data were not used in the classification of asthma severity, 4 of the 5 subjects with severe asthma had an FEV₁ (percent predicted) of less than 55%. Furthermore, 9 of the 10 subjects with mild asthma had an FEV₁ (percent predicted) greater than 80%, and all subjects had FEV₁ (percent predicted) greater than 75%. To increase the chances that our findings could be compared with those in other studies, we provided rich phenotypic data, including symptoms and medication use as characterized by the National Heart, Lung and Blood Institute, spirometry according to American Thoracic Society criteria,¹² and allergen skin testing to establish atopic status.

RT-PCR results correlated well with the differential gene-expression data produced with the use of the Affymetrix GeneChips. This confirmation gave us confidence that the gene-expression data derived from the gene arrays were reliable.

We focused on circulating CD4⁺ lymphocytes, a highly relevant cell type in asthma, and isolated extremely pure cell populations for our studies. Substantial experimental evidence supports the notion that circulating lymphocytes are abnormal in asthma in several respects (eg, resistance to apoptosis, heightened activation, enhanced gene expression) and that the analysis of circulating cells provides a window into the pulmonary immune response in subjects with asthma.^{55–58} Although we acknowledge the possibility of compartmentalization in the pulmonary immune response and understand that our study of peripheral-blood cells may not accurately reflect lymphocytes recruited to the lung, we believe that analyses based on samples that are not drawn at the main sites of inflammation in the lung have the potential to yield much insight. Furthermore, successful management of asthma requires integrated approaches targeting both local and systemic pathways. For the development of such approaches, we need to focus research efforts on both the elucidation of systemic and local mechanisms of disease.

To date, the data presented in this study represent the most comprehensive analysis of gene expression patterns in peripheral-blood lymphocytes in subjects with asthma of varying disease severity and contributes globally to our understanding of CD4⁺T-cell involvement in airway inflammation. We have demonstrated a method by which CD4⁺ lymphocytes can be successfully extracted from peripheral blood for microarray analysis and have shown that T-lymphocytes from the peripheral blood of patients with severe and mild asthma differ significantly in their gene-expression profiles. Differential transcriptional regulation may thereby influence the development or severity of disease. Subjects with severe asthma demonstrated increased expression of genes involved in T-cell activation, proliferation, cytoskeletal changes, and adhesion, supporting the notion that lymphocyte recirculate to the lung or areas of inflammation. T-cells also exhibited increased gene expression of genes that may be involved in airway function and remodeling. Comparing and contrasting transcriptional programs and gene expression may help identify markers for those at increased risk of severe asthma and molecular targets for preventive and therapeutic options.

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