

Transcriptome analysis shows activation of circulating CD8⁺ T cells in patients with severe asthma

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Background: Although previous studies have implicated tissue CD4⁺ T cells in the development and maintenance of the inflammatory response in asthmatic patients, little is known about the role of CD8⁺ T cells. There is now accumulating evidence that microRNAs and other noncoding RNAs are important regulators of T-cell function.

Objectives: We sought to use transcriptomics to determine the activation state of circulating CD4⁺ and CD8⁺ T cells in patients with nonsevere and severe asthma.

Methods: mRNA and noncoding RNA expression in circulating T cells was measured by means of microarray, quantitative real-time PCR, or both.

Results: Comparison of mRNA expression showed widespread changes in the circulating CD8⁺ but not CD4⁺ T cells from patients with severe asthma. No changes were observed in the CD4⁺ and CD8⁺ T cells in patients with nonsevere asthma versus those in healthy control subjects. Bioinformatics analysis showed that the changes in CD8⁺ T-cell mRNA expression were associated with multiple pathways involved in T-cell activation. As with mRNAs, we also observed widespread changes in expression of noncoding RNA species, including natural antisense, pseudogenes, intronic long noncoding RNAs (lncRNAs), and intergenic lncRNAs in CD8⁺ T cells from

patients with severe asthma. Measurement of the microRNA expression profile showed selective downregulation of miR-28-5p in CD8⁺ T cells and reduction of miR-146a and miR-146b in both CD4⁺ and CD8⁺ T cells.

Conclusions: Severe asthma is associated with the activation of circulating CD8⁺ T cells but not CD4⁺ T cells. This response is correlated with the downregulation of miR-146a/b and miR-28-5p, as well as changes in the expression of multiple species of lncRNA that might regulate CD8⁺ T-cell function. (*J Allergy Clin Immunol* 2011;■■■■:■■■■-■■■■.)

Key words: Severe asthma, CD8⁺ T cells, transcriptome, long non-coding RNA, microRNA

Asthma is characterized by reversible airway obstruction, remodeling, and inflammation and is one of the most common chronic allergic diseases, affecting 300 million persons worldwide and causing approximately 180,000 deaths per year.¹ Most patients with asthma respond well to anti-inflammatory treatment with inhaled corticosteroid therapy. However, 5% to 10% of patients experience ongoing symptoms of asthma despite taking corticosteroid treatments, often in combination with long-acting β -agonists and other controller therapies. These patients have been labeled as having severe or therapy-resistant asthma and form a heterogeneous group of patients that can be divided into distinct phenotypes.^{2,3}

Although the underlying mechanisms that drive asthma are still an area of contention, T cells have been proposed to contribute to the induction and maintenance of the inflammatory response. After their early identification in bronchoalveolar lavage fluid, much work has focused on the importance of the T_H2 CD4⁺ T cells.⁴ These cells are activated after the interaction between the T-cell receptor and peptide antigen presented in the context of MHC class II molecules expressed by airway dendritic/epithelial cells. In turn, this stimulates the release of T_H2 cytokines, including IL-4, IL-5, IL-9, and IL-13, which contribute to the development of many of the features of asthma, including airways hyperresponsiveness, mucus hypersecretion, airway eosinophilia, and B-cell activation.^{5,6} Significantly, recent studies have also implicated roles for other subtypes of CD4⁺ T cells, including regulatory T (Treg), T_H9, T_H17, T_H22, $\gamma\delta$ T, and natural killer T cells, although the majority of these studies have been performed in animal models and their role in human asthma remains to be confirmed.^{5,6}

Unlike CD4⁺ T cells, little is known about the role of CD8⁺ T cells, which are normally associated with the recognition and

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Abbreviations used

FDR: False discovery rate
 LCN: Lipocalin 2
 lncRNA: Long noncoding RNA
 miRNA: MicroRNA
 qRT-PCR: Quantitative RT-PCR
 Treg: Regulatory T

destruction of cells that are infected with viruses and other intracellular pathogens. Previous studies in patients have shown the presence of CD8⁺ cells in the bronchoalveolar lavage fluid and sputum of asthmatic patients, whereas increased CD8⁺ T-cell numbers were noted in patients with status asthmaticus.⁷⁻⁹ In addition, animal studies have shown that CD8⁺ T cells contribute to the development of allergen-induced airway hyperresponsiveness and pulmonary inflammation.¹⁰

In this report we have adopted a transcriptomics-based approach to investigate the activation state of circulating CD4⁺ and CD8⁺ T cells in patients with severe asthma and to identify potential targets/pathways that might provide novel therapeutic targets. In addition, we have investigated whether any changes in mRNA expression are regulated at the posttranscriptional level by microRNA expression and whether there are changes in the expression of other noncoding RNAs, including antisense, pseudogenes, long intronic noncoding RNA, and long intergenic noncoding RNAs. Significantly, measurement of the mRNA expression profile has shown activation of the circulating CD8⁺ but not CD4⁺ T-cell populations in patients with severe asthma. No significant changes in mRNA expression were observed in the CD4⁺ and CD8⁺ T cells obtained from patients with nonsevere asthma. As with the mRNAs, we have shown widespread changes in the expression of natural antisense, pseudogenes, long intronic noncoding RNAs, and long intergenic noncoding RNAs within the CD8⁺ population from patients with severe asthma. In addition, we observed reduced expression of miR-28-5p in CD8⁺ T cells and downregulated expression of miR-146a and miR-146b in both CD4⁺ and CD8⁺ T cells. Overall, these observations have revealed that activation of CD8⁺ T cells might have an important role in the pathogenesis of severe asthma and that this activation might be regulated through changes in expression of multiple classes of noncoding RNAs.

METHODS**T-cell isolation**

Human CD4⁺ and CD8⁺ T cells were isolated by means of negative selection with Miltenyi beads and a MACS Vario magnetic cell separation apparatus (Miltenyi Biotec, Bergisch Gladbach, Germany). T-cell purity was assessed by means of FACS with anti-CD4–fluorescein isothiocyanate and anti-CD8–phycoerythrin by using CellQuest software.

Microarray analysis of mRNA and noncoding RNA expression

Expression profiling was performed with the Affymetrix U133 Plus 2.0 microarrays (Affymetrix, Santa Clara, Calif) at AROS, Denmark. Total RNA was extracted with the *mirVana* miRNA isolation kit (Ambion Europe, Huntingdon, United Kingdom) eluted in 50 μ L of RNase-free water (Promega UK, Southampton, United Kingdom), and purity was measured with a BioTek PowerWave XS (SSI Robotics, Shoreview, Minn) spectrophotometer and

Agilent Bioanalyser (Agilent, Santa Clara, Calif), which showed RNA integrity number values of greater than 9.5. Raw data were analyzed with Gene Spring GX 10 software (Agilent) and then R/Bioconductor. Array data were then normalized by using a robust multiarray and batch effects corrected with the ComBat R package.¹¹ Data were filtered to remove probes that had low expression in all arrays; those with a robust multiarray signal value of less than 4 were removed. Differential gene regulation was determined by taking the output from a shrinkage *t* test¹² and then computing the *P* values and *q* values (false discovery rate [FDR]) with “fdrtool.”¹³ The cutoff used for significance is an FDR of less than 0.05, which is a 5% FDR. The *P* values are shown to allow readers to discern different rankings of genes in which the FDR value is less than 0.05.

Changes in antisense, pseudogenes, long intronic noncoding RNA, and long intergenic noncoding RNAs expression were determined by mapping nonannotated probes onto the human genome (GRCh37) with Ensembl (www.ensembl.org/index.html). Microarray data has been deposited at GEO (GSE31773).

Quantitative PCR measurement of micro RNA and mRNA expression

MicroRNA (miRNA) expression profiling was undertaken with the 2-step Applied Biosystems TaqMan RT-PCR protocol (Applied Biosystems, Foster City, Calif) and normalized to *RNU44*, as previously described.¹⁴ mRNA expression was determined by using TaqMan RT-PCR with Assays on Demand (Applied Biosystems). Alternatively, mRNA levels were determined by using the Power SYBR Green PCR mix (Applied Biosystems) with the listed probes (MWG-Biotech, Ebersberg, Germany). The separate-well 2^{-($\Delta\Delta C_t$)} method was used to determine relative quantitative levels of individual miRNAs and mRNAs.

The following primer sequences were used for the mRNA RT-PCR: lipocalin 2 (*LCN*) forward, CACCCTCTACGGGAGAACCAAGGA; *LCN* reverse, ACCTGTGCACTCAGCCGTCGATA; *S100A8* forward, CACATTCCTGTGCTATTGAGGGGTTA; *S100A8* reverse, GGGTGTCAAAATGCCCTTCCG; *S100P* forward, CAGGCTTCTCTGCAGAGTGGAAGA; *S100P* reverse, GCAGACGTGATTGCAGCCACGA; cathelicidin antimicrobial peptide (*CAMP*) forward, AGACACGCCAAAGCCTGTGAGCTT; *CAMP* reverse, TTCACGAGCCGTCCTTCTTGAAGT; bactericidal/permeability-increasing protein (*BPI*) forward, TGCAGAAGGAGCTGAAGAGGATCAA; *BPI* reverse, ACGGATGTCCATGCTGTAGAAGCTA; leukocyte elastase (*ELA*) forward, ACTGCGTGGCGAATGTAAACGT; *ELANE* reverse, CCGTTTTCGAAGATGCGCTGCA; eukaryotic translation initiation factor 2, subunit 3 (*EIF2S3*) forward, TGCTCCAGGCGGTCTTATGGAGTT; *EIF2S3* reverse, TCAGGTAAAGCTCCGACTGCACCA; annexin A3 (*ANAX3*) forward, TCGCTCGCAGTTTGTTCGCAGT; and *ANAX3* reverse, TGTTCCTCGGTGTCCAACCCAGAT.

Data and statistical analysis

The RT-PCR results are presented as means and SDs of at least 3 independent experiments. Differences in the expression of mRNA and miRNA were determined by using a 2-tailed nonparametric Mann-Whitney *U* test within GraphPad Prism 5 software (www.graphpad.com). *P* values of less than .05 were considered significant.

RESULTS**Patient groups**

Transcriptomics studies were performed in the circulating CD4⁺ and CD8⁺ T cells obtained from 12 patients with severe asthma, 4 patients with nonsevere asthma, and 8 healthy control subjects (Table I), which had been sanctioned by the Royal Brompton and Harefield NHS Trust Ethics committee. Severe asthma was defined according to the American Thoracic Society major criteria of needing either continuous or near-continuous oral corticosteroids, high-dose inhaled corticosteroids (≥ 2000 μ g/d

TABLE I. Subjects' data

Subject no.	Age (y)	Sex (M/F)	FEV ₁ (% predicted)	FVC (% predicted)	PC ₂₀ (mg/mL)	Medication
Control subjects						
1	39	M	78	76	>16	None
2	25	F	103	107	>16	None
3	26	F	105	115	>16	None
4	46	M	92	105	>16	None
5	33	M	78	77	>16	None
6	33	F	116	115	>16	None
7	31	F	85	96	15.67	None
8	56	M	111	111	>32	None
Mean ± SEM	36 ± 4	4/4	96 ± 5	100 ± 6		
Patients with nonsevere asthma						
1	23	M	82	98	Not done	Beclomethasone (Becotide), albuterol (Ventolin)
2	44	F	87	92	0.52	Fl
3	35	M	77	89	6.27	Beclomethasone (Becotide), albuterol (Ventolin)
4	23	F	84	81	0.17	Salbutamol
Mean ± SEM	31 ± 5	2/2	83 ± 2	90 ± 2		
Patients with severe asthma						
1	55	F	82	105	1.09	Fl, formoterol (Oxis), loratadine, beclomethasone dipropionate (Beconase)
2	21	F	105	101	Not done	Pr, Se, Si, aminophylline (Phyllocontin), albuterol (Ventolin)
3	32	M	98	113	0.01	Pr, Se, montelukast, albuterol (Ventolin), aminophylline (Phyllocontin), Fl
4	55	F	65	81	0.10	Pr, Sp, Se, methotrexate, alendronate (Fosamax), folic acid, co-codamol
5	54	F	44	61	Not done	Pr, Sy, montelukast, omeprazole, theophyllin (Uniphyllin), terbutaline (Bricanyl)
6	54	F	55	65	Not done	Pr, Co, Ce, terbutaline, Bu, aminophylline SR, formoterol
7	45	F	71	105	Not done	Pr, Se, Fl, metformin, esomeprazole (Nexium), tramadol and acetaminophen (Tramacet), valsartan, amitriptyline
8	42	F	33	94	Not done	Pr, Se, aminophylline, doxycycline, tiotropium
9	47	M	63	81	Not done	Se, theophyllin (Uniphyllin), alendronate (Fosamax), albuterol (Ventolin), ipratropium inhalation (Atrovent)
10	39	M	63	73	Not done	Sy, loratadine, beclomethasone dipropionate (Beconase), theophyllin (Uniphyllin)
11	61	M	57	98	Not done	Pr, Sy, Fl, omeprazole, atorvastatin
12	52	M	83	97	Not done	Sy, Betnasal
Mean ± SEM	46 ± 3	5/7	68 ± 6	90 ± 5		

Bu, Budesonide; Ce, cetirizine; Co, albuterol and ipratropium (Combivent); F, female; Fl, Flixotide; M, male; Pr, prednisolone; Se, salmeterol and fluticasone (Seretide); Si, montelukast (Singulair); Sp, tiotropium inhalation (Spiriva); Sy, budesonide and formoterol inhalation (Symbicort).

beclomethasone equivalent), or both to achieve a level of mild-to-moderate persistent asthma and by 2 or more minor criteria of asthma control.¹⁵ Patients with severe asthma underwent the Royal Brompton severe asthma protocol to confirm the diagnosis and maximize treatments. Patients with nonsevere asthma were those who did not fall into the severe asthma category and who used 0 to 1000 µg/d inhaled beclomethasone or an equivalent dosage with perfect control of their asthma. All patients showed either an improvement in baseline FEV₁ of 12% or greater over baseline values after inhalation of 400 µg of salbutamol aerosol or the presence of bronchial hyperresponsiveness defined by a methacholine PC₂₀ value of less than 4 mg/mL. Current and ex-smokers of greater than 5 pack-years were excluded. Healthy control subjects with no diagnosis of asthma and a negative PC₂₀ value (>16 mg/mL) using no medications and never smokers were also recruited as the comparative group.

Characterization of T-cell populations

Circulating blood (50 mL) was obtained from nonasthmatic control subjects or patients with nonsevere and severe asthma. Before negative selection, measurement of the total number of PBMCs in control subjects, patients with nonsevere asthma, and patients with severe asthma showed no significant difference at 97.6×10^6 cells (SD, 7.7×10^6 cells), 120.5×10^6 cells (SD, 10.2×10^6 cells), and 127.0×10^6 cells (SD, 30.0×10^6 cells), respectively. FACS analysis showed that antibody-based negative selection resulted in the production of highly purified (>98%) CD4⁺ and CD8⁺ T cells. After their isolation, we once again observed no significant difference in the number of CD4⁺ T cells at 11.2×10^6 cells (SD, 4.8×10^6 cells), 11.0×10^6 cells (SD, 8.4×10^6 cells), and 11.9×10^6 cells (SD, 2.6×10^6 cells) or CD8⁺ T cells at 5.5×10^6 cells (SD, 1.6×10^6 cells), 5.8×10^6 cells (SD, 5.0×10^6 cells), and 4.7×10^6 cells (SD, 1.6×10^6 cells).

TABLE II. Common mRNA expression changes in both CD4⁺ and CD8⁺ T cells in patients with severe asthma

Gene symbol	Gene name	CD4 ⁺ T cells		CD8 ⁺ T cells	
		Microarray (FC)	qRT-PCR (FC)	Microarray (FC)	qRT-PCR (FC)
<i>BPI</i>	Bactericidal/permeability-increasing protein	2.6 (<.001)	21.7 (.019)	2.8 (<.001)	29.1 (.001)
<i>CAMP</i>	Cathelicidin antimicrobial peptide	3.3 (<.001)	12.4 (.065)	2.1 (.017)	5.9 (.016)
<i>CASK</i>	Peripheral plasma membrane protein CASK	1.7 (<.001)	—	1.8 (<.001)	—
<i>CEACAM6</i>	Carcinoembryonic antigen-related cell adhesion molecule 6	2.4 (<.001)	29.1 (.012)	2.9 (<.001)	4.6 (.012)
<i>CEACAM8</i>	Carcinoembryonic antigen-related cell adhesion molecule 8	5.6 (<.001)	20.2 (.023)	4.4 (<.001)	3.7 (.023)
<i>CFLAR</i>	FLAME-1	1.6 (<.001)	—	2.4 (.019)	—
<i>GCA</i>	Grancalcin, EF-hand calcium-binding protein	2.6 (<.001)	—	2.6 (.002)	—
<i>LCN2</i>	Lipocalin 2	4.7 (<.001)	17.0 (.015)	2.4 (<.001)	14.8 (.01)
<i>LOC23117</i>	KIAA0220-like protein	2.4 (<.001)	—	3.3 (.007)	—
<i>LOC728358</i>	Defensin, α 1	6.2 (<.001)	—	2.3 (.004)	—
<i>LTF</i>	Lactotransferrin	5.0 (<.001)	21.1 (.012)	2.1 (<.001)	2.5 (.154)
<i>POLR2J2</i>	DNA-directed RNA polymerase II polypeptide J-related	1.9 (<.001)	—	3.8 (.001)	—
<i>RETN</i>	Resistin	1.7 (<.001)	—	2.3 (.005)	—
<i>RNASE3</i>	Ribonuclease, RNase A family, 3 (eosinophil cationic protein)	2.0 (<.001)	—	1.8 (.005)	—
<i>S100A12</i>	S100 calcium-binding protein A12	4.7 (<.001)	27.1 (.0078)	2.4 (.051)	6.5 (.031)
<i>S100A8</i>	S100 calcium-binding protein A8	4.8 (<.001)	15.3 (.015)	2.0 (<.001)	28.3 (.006)
<i>S100A9</i>	S100 calcium-binding protein A9	5.0 (<.001)	19.5 (.019)	1.9 (.011)	2.7 (.070)
<i>S100P</i>	S100 calcium-binding protein P	3.3 (<.001)	21.2 (.008)	2.1 (.008)	18.4 (.001)
<i>SLC38A2</i>	Amino acid transporter system A2 (ATA2)	1.6 (<.001)	—	1.6 (<.001)	—
<i>SPATA13</i>	Spermatogenesis-associated 13	1.7 (<.001)	—	1.5 (.001)	—
<i>SRGN</i>	Serglycin	2.0 (<.001)	—	1.8 (.016)	—
<i>TNIP1</i>	TNFAIP3-interacting protein 1	1.6 (<.001)	—	1.7 (<.001)	—
<i>UGCG</i>	UDP-glucose ceramide glucosyltransferase (UGCG)	2.0 (<.001)	—	2.5 (<.001)	—

This table lists those mRNAs whose expression increased in both the CD4⁺ and CD8⁺ T cells from patients with severe asthma when compared with that seen in nonasthmatic subjects. Microarray data are denoted as the fold change against nonasthmatic control subjects and is the mean of 8 individual patients. These values are derived from [Tables E1 and E2](#) and are for those genes that demonstrated an FDR of less than 0.05; associated *P* values are shown in parentheses. Changes in mRNA expression were confirmed by means of qRT-PCR, which was normalized against 18S and expressed as fold changes over nonasthmatic control subjects. In the case of the qRT-PCR data, statistical significance against nonasthmatic control subjects was determined by using a 2-tailed nonparametric Mann-Whitney *U* test. These values are the mean of 12 individual patient samples, and *P* values are indicated in parentheses.

FC, Fold change.

in control subjects, patients with nonsevere asthma, and patients with severe asthma, respectively.

Severe asthma is associated with changes in mRNA expression in CD4⁺ and CD8⁺ T cells

Measurement of the profile of mRNA expression with Affymetrix microarrays showed that it differed between healthy control subjects and patients with severe asthma but not patients with nonsevere asthma. These initial studies were performed by using the 8 control subjects, 4 patients with nonsevere asthma, and 8 patients with severe asthma ([Table I](#), patients 1-8). In the case of the CD4⁺ T cells, we identified a relatively small number of transcripts (*n* = 40) that were upregulated (see [Table E1](#) in this article's Online Repository at www.jacionline.org) and none that were downregulated by greater than 1.5-fold in patients with severe asthma versus control subjects. In contrast, with CD8⁺ T cells, we observed 1359 upregulated genes (see [Table E2](#) in this article's Online Repository at www.jacionline.org) and 207 downregulated genes (see [Table E3](#) in this article's Online Repository at www.jacionline.org) in the samples obtained from patients with severe asthma.

The general validity of these microarray observations in the samples from patients with severe asthma was assessed by using quantitative RT-PCR (qRT-PCR). Because the initial microarray studies were performed with a group of patients with severe asthma that had a female/male ratio of 7:1, we included an

additional 4 samples obtained from male patients with severe asthma to account for potential sex bias. These studies were able to confirm the increase in expression of 9 of 10 mRNAs examined in CD4⁺ T cells and in 13 of 16 mRNAs in CD8⁺ T cells ([Tables II and III](#)). These included *BPI*, carcinoembryonic antigen-related cell adhesion molecule 6/8 (*CEACAM6/8*), *LCN*, lactotransferrin (*LTF*), S100 calcium binding protein P/A8/A9/A12, chitinase 3-like 1 (*CHI3L1*), leukocyte elastase (*ELA2*), and arginase (*ARG*).

As might be predicted from the microarray data, we observed only a small number of mRNAs whose expression was changed in patients with nonsevere asthma versus nonasthmatic control subjects as measured using qRT-PCR (see [Table E4](#) in this article's Online Repository at www.jacionline.org). This included 2 of 10 mRNAs examined in CD4⁺ T cells and 5 of 15 mRNAs in CD8⁺ T cells (see [Table E4](#)).

Pathway analysis shows activation of CD8⁺ T cells but not CD4⁺ T cells in patients with severe asthma

To identify the pathways that were either activated or inhibited in the CD4⁺ and CD8⁺ T cells from patients with severe asthma, we analyzed the changes in profile of mRNA expression using the Ingenuity Systems software (<http://ingenuity.com/>; [Table IV](#)). Significantly, this showed an upregulation in the expression of those transcripts involved in multiple inflammatory and other signaling pathways in CD8⁺ T cells from patients with severe

TABLE III. mRNA expression showing the highest fold change in CD4⁺ and CD8⁺ T cells in patients with severe asthma

Gene symbol	Gene name	Array (FC)	RT-PCR (FC)
CD4 ⁺ T cells			
<i>LOC728358</i>	Defensin, α 1	6.6 (<.001)	—
<i>CEACAM8</i>	Carcinoembryonic antigen-related cell adhesion molecule 8	5.6 (<.001)	20.2 (.023)
<i>LTF</i>	Lactotransferrin	5.0 (<.001)	21.1 (.012)
<i>S100A9</i>	S100 calcium-binding protein A9	5.0 (<.001)	19.5 (.019)
<i>S100A8</i>	S100 calcium-binding protein A8	4.8 (<.001)	15.3 (.015)
<i>LCN2</i>	Lipocalin 2	4.7 (<.001)	17.0 (.015)
<i>S100A12</i>	S100 calcium-binding protein A12	4.7 (<.001)	27.1 (.008)
<i>DEFA4</i>	Defensin, α 4, corticostatin	4.3 (<.001)	—
<i>MMP8</i>	MMP-8 (collagenase 2)	3.6 (<.001)	—
<i>S100P</i>	S100 calcium-binding protein P	3.3 (<.001)	21.2 (.008)
<i>CAMP</i>	Cathelicidin antimicrobial peptide	3.3 (<.001)	12.4 (.645)
<i>MNDA</i>	Myeloid cell nuclear differentiation antigen	2.7 (<.001)	—
<i>BPI</i>	Bactericidal/permeability-increasing protein	2.6 (<.001)	21.7 (.019)
<i>GCA</i>	Grancalcin, EF-hand calcium-binding protein	2.6 (<.001)	—
<i>ZNF749</i>	Zinc finger protein 749	2.5 (<.001)	—
<i>CEACAM6</i>	Carcinoembryonic antigen-related cell adhesion molecule 6	2.4 (<.001)	29.1 (.012)
<i>LOC23117</i>	KIAA0220-like protein	2.4 (<.001)	—
<i>STOM</i>	Stomatin	2.3 (<.001)	—
<i>ALOX5</i>	Arachidonate 5-lipoxygenase	2.2 (<.001)	—
<i>UGCG</i>	UDP-glucose ceramide glucosyltransferase	2.0 (<.001)	—
CD8 ⁺ T cells			
<i>CEACAM8</i>	Carcinoembryonic antigen-related cell adhesion molecule 8	4.4 (<.001)	3.7 (.023)
<i>CHI3L1</i>	Chitinase 3-like 1 (cartilage glycoprotein-39)	3.9 (<.001)	4.8 (.019)
<i>LTF</i>	Lactotransferrin	3.8 (<.001)	2.5 (.154)
<i>ELA2</i>	Leukocyte elastase (neutrophil elastase)	3.7 (.002)	12.7 (.013)
<i>CD24</i>	CD24 molecule	3.6 (<.001)	3.5 (.015)
<i>LCN2</i>	Lipocalin 2	3.3 (<.001)	14.8 (.01)
<i>EIF2S3</i>	Eukaryotic translation initiation factor 2, subunit 3 γ , 52 kd	3.3 (.045)	1.5 (.232)
<i>ANXA3</i>	Annexin A3	3.2 (.001)	9.6 (.008)
<i>ARG1</i>	Arginase, liver	3.2 (.001)	4.0 (.04)
<i>SEPT6</i>	Septin 6	3.1 (.010)	—
<i>HSP90AB1</i>	Heat shock protein 90 kd α (cytosolic), class B member 1	3.1 (.013)	—
<i>CEACAM6</i>	Carcinoembryonic antigen-related cell adhesion molecule 6 (nonspecific cross-reacting antigen)	2.9 (<.001)	4.6 (.030)
<i>AZU1</i>	Azurocidin	2.9 (.003)	—
<i>LEF1</i>	Lymphoid enhancer-binding factor 1	2.9 (.026)	—
<i>BPI</i>	Bactericidal/permeability-increasing protein	2.8 (<.001)	29.1 (.001)
<i>SF1</i>	Splicing factor 1	2.8 (.030)	—
<i>SAMHD1</i>	SAM domain and HD domain 1	2.7 (.006)	—
<i>S100B</i>	S100 calcium-binding protein B	2.7 (.046)	—
<i>TCN1</i>	Transcobalamin I (vitamin B12-binding protein, R binder family)	2.7 (.002)	—

This table lists those mRNAs whose expression showed the largest fold change increase in either CD4⁺ and CD8⁺ T cells when compared with nonasthmatic control subjects. Microarray data are denoted as the fold change against nonasthmatic control subjects and are the mean of 8 individual patients. These values are derived from Tables E1 and E2 and are for those genes that demonstrated an FDR of less than 0.05; associated *P* values are shown in parentheses. Changes in mRNA expression were confirmed by means of qRT-PCR, which was normalized against 18S and expressed as the fold change over nonasthmatic control subjects. In the case of the qRT-PCR data, statistical significance against nonasthmatic control subjects was determined by using a 2-tailed nonparametric Mann-Whitney *U* test. These values are the mean of 12 individual patient samples, and the *P* values are indicated in parentheses.

FC, Fold change.

asthma. These included those involved in T-cell activation (eg, T-cell receptor signaling; nuclear factor of activated T cells transcription factor pathway, inducible costimulator-inducible costimulator ligand, and CD28 costimulation; and the pathways involved in the regulation of IL-2 expression in activated and anergic T lymphocytes) and inflammation (LPS-stimulated mitogen-activated protein kinase, Janus kinase/signal transducer and activator of transcription, and nuclear factor κ B signaling pathways). A number of pathways that are commonly associated with asthma, including IL-3, IL-4, and IL-9, were also activated, as was glucocorticoid signaling. In contrast, only the vitamin D pathway was upregulated in CD4⁺ T cells, whereas no pathways were shown to be downregulated in either CD4⁺ or CD8⁺ T cells.

Subsequent analysis aimed to identify mRNAs that were associated with differences in the T-cell phenotype from patients with severe asthma compared with nonasthmatic control subjects. Comparison of the 2 datasets showed that 23 genes were upregulated in both the CD4⁺ and CD8⁺ T-cell populations, but no genes were downregulated (Table II). Prominent among the upregulated mRNAs were members of the S100 calcium-binding and CEACAM family. Examination of those mRNAs that had the largest fold changes also highlighted the potential importance of the S100 and CEACAM families in both CD4⁺ or CD8⁺ T cells (Table III). Interestingly, levels of 2 proteins that have recently been linked to asthma, arginase 1¹⁶ and chitinase 3 protein 1,¹⁷ were also shown to be increased in CD8⁺ T cells (Tables II and III).

TABLE IV. Pathway analysis in CD4⁺ and CD8⁺ T cells in patients with severe asthma

Gene set name	P value
CD4 ⁺ T cells	
VDR/RXR activation	4.80E-04
CD8 ⁺ T cells	
Role of NFAT in regulation of the immune response	7.80E-06
Molecular mechanisms of cancer	3.20E-05
Death receptor signaling	3.70E-05
T-cell receptor signaling	3.90E-05
Glucocorticoid receptor signaling	4.20E-05
LPS-stimulated MAPK signaling	7.20E-05
Apoptosis signaling	8.80E-05
Acute myeloid leukemia signaling	1.00E-04
B-cell receptor signaling	1.10E-04
CD40 signaling	1.30E-04
JAK/Stat signaling	1.30E-04
IL-8 signaling	1.30E-04
PPARα/RXRα activation	1.50E-04
NF-κB signaling	1.90E-04
ICOS-ICOSL signaling in T _H cells	2.80E-04
IL-9 signaling	2.80E-04
Angiopoietin signaling	3.80E-04
EIF2 signaling	4.10E-04
IL-3 signaling	4.50E-04
PDGF signaling	4.50E-04
Prolactin signaling	4.50E-04
GM-CSF signaling	4.50E-04
IL-4 signaling	7.30E-04
Regulation of IL-2 expression in activated and anergic T lymphocytes	7.60E-04
CD28 signaling in T _H cells	9.50E-04

Circulating CD4⁺ and CD8⁺ T cells were isolated from 8 nonasthmatic control subjects and 8 patients with severe asthma, and mRNA expression was determined by using U133 Affymetrix microarrays. The mRNAs that showed significant change were analyzed with Ingenuity Systems software (<http://ingenuity.com/>), and those pathways showing a significant increase are listed.

ICOS-ICOSL, Inducible costimulator–inducible costimulator ligand; *JAK/Stat*, Janus kinase/signal transducer and activator of transcription; *MAPK*, mitogen-activated protein kinase; *NFAT*, nuclear factor of activated T cells; *NF-κB*, nuclear factor κB; *PDGF*, platelet-derived growth factor; *PPARα/RXRα*, peroxisome proliferator–activated receptor α/retinoid X receptor α; *VDR/RXR*, vitamin D receptor/retinoic acid X receptor.

Severe asthma is associated with reduced miRNA expression in CD4⁺ and CD8⁺ T cells

Previous investigations have shown that a number of miRNAs, including miR-21, miR-132, miR-146a, miR-150, miR-155, and miR-326, are important regulators of the differentiation, proliferation, and activation of immune cells.^{18–20} To determine whether miRNAs might regulate the changes in mRNA expression, we measured the expression of these immune-related miRNAs (Table V). These studies showed that the expression of miR-146a and miR-146b was reduced by 50% to 80% and that this occurred in both CD4⁺ and CD8⁺ T cells from patients with severe asthma (Table V). Significantly, we also observed a selective reduction of approximately 30% in the expression of miR-18a-5p in CD8⁺ T cells.

Because it might be expected that a reduction in miRNA expression would lead to increased expression of their predicted mRNA targets, we examined this possibility using the miRNA target prediction program TargetScan (<http://www.targetscan.org/>). Bioinformatics analysis showed an enrichment of miR-28a-5p ($P = .026$) and miR-146 ($P = .13$) targets in those mRNAs

that were downregulated in CD8⁺ T cells. As a result of the small number of differentially regulated mRNAs in CD4⁺ T cells, it was not possible to determine whether targets for miR-146 were enriched.

Severe asthma is associated with widespread changes in the expression of long noncoding RNAs in CD8⁺ T cells

In addition to the miRNA family of short noncoding RNAs (<200 nucleotides), there is now accumulating evidence that long noncoding RNAs (lncRNAs; >200 nucleotides) regulate multiple biological responses and that changes in their expression are related to the development of disease.^{21,22} At the present time, lncRNAs can be broadly divided into 4 families based on their sequence and relative position to the exonic regions of protein-coding sequences and include pseudogenes, natural antisense (to exonic regions), intronic lncRNAs, and intergenic lncRNAs. To identify novel lncRNAs, we used Emsembl (www.ensembl.org/index.html) to determine the genomic position of those probe sets from the Affymetrix U133 Plus 2.0 microarray, which (1) did not match known protein-coding genes and (2) were significantly changed in the CD4⁺ and CD8⁺ T cells in patients with severe asthma. Of relevance, a recent publication has indicated that these probe sets are able to detect up to 43% of the 5446 predicted lncRNAs in the human genome.²³ This approach identified a small number of lncRNAs that were downregulated in CD4⁺ T cells (see Table E5 in this article's Online Repository at www.jacionline.org), including 4 intergenic lncRNAs and 2 intronic lncRNAs. Of these lncRNAs, only a single transcript entitled AC090517.2 has been manually annotated by the Havana project team at the Wellcome Trust Sanger Institute (see Table E5). In the case of CD8⁺ T cells, we identified 167 potential lncRNAs that we significantly changed in patients with severe asthma (see Table E6 in this article's Online Repository at www.jacionline.org). These included 5 antisense sequences (4 upregulated and 1 downregulated), 4 pseudogenes (3 upregulated and 1 downregulated), 44 intergenic lncRNAs (28 upregulated and 16 downregulated), and 114 intronic lncRNAs (106 upregulated and 8 downregulated). In this case 19 of these transcripts have been manually annotated and include 1 antisense sequence, all 4 pseudogenes and 14 intergenic lncRNAs (Table VI). Overall, this bioinformatics analysis has shown for the first time that changes in lncRNA expression are associated with the severe asthma in circulating T cells, although their function remains to be determined.

DISCUSSION

In this report we have compared the profile of mRNA expression in circulating CD4⁺ and CD8⁺ T cells from healthy subjects, patients with nonsevere asthma, and patients with severe asthma by using microarray and qRT-PCR analyses. In contrast to previous studies that have implicated T_H2 CD4⁺ cells in driving the inflammatory response in asthmatic patients, we showed no major changes in the profile of mRNA expression in patients with nonsevere asthma and only a relatively small number of mRNAs that were upregulated in CD4⁺ T cells from patients with severe asthma. Pathway analysis indicated that these circulating CD4⁺ T cells from patients with severe asthma were nonactivated. In contrast, there were widespread changes in the expression of mRNAs in

TABLE V. miRNA expression levels in circulating CD4⁺ and CD8⁺ T cells from nonasthmatic control subjects and patients with severe asthma

miRNA	CD4 ⁺ T cells				CD8 ⁺ T cells			
	Baseline expression (ΔCT)	Fold change from control (%)			Baseline expression (ΔCT)	Fold change from control (%)		
	Control subjects	Control subjects	Asthmatic patient	P value	Control subjects	Control subjects	Asthmatic patients	P value
miR-16	−1.2 (0.5)	106.2 (32.3)	84.3 (43.7)	.302	−1.5 (0.5)	108.1 (51.1)	73.4 (19.9)	.069
miR-17-5p	4.1 (0.5)	107.0 (40.0)	105.5 (32.1)	.980	3.7 (0.8)	118.4 (73.4)	70.0 (20.9)	.243
miR-21	−0.8 (0.5)	108.0 (39.8)	125.3 (42.7)	.428	0.1 (0.5)	108.7 (53.0)	100.0 (35.0)	.739
miR-26a	0.1 (0.5)	105.7 (33.4)	84.8 (30.7)	.242	0.2 (0.8)	122.4 (98.6)	72.0 (70.2)	.149
miR-28-5p	5.1 (0.3)	97.4 (11.9)	106.8 (23.6)	.637	4.4 (0.5)	104.9 (32.3)	68.7 (32.1)	.046
miR-29a	−0.2 (0.5)	104.5 (29.7)	105.0 (38.7)	.926	−0.2 (0.5)	105.3 (33.4)	89.8 (33.9)	.360
miR-106a	3.9 (0.5)	104.3 (33.9)	109.4 (43.7)	.982	3.5 (1.1)	124.4 (94.6)	70.4 (25.4)	.159
miR-126	6.1 (2.1)	276.9 (351.9)	59.4 (57.0)	.784	6.0 (5.0)	190.0 (187.1)	30.9 (23.9)	.687
miR-142-3p	−4.0 (0.3)	101.2 (17.0)	103.3 (30.5)	.949	−3.8 (0.5)	104.6 (31.3)	90.7 (27.0)	.392
miR-142-5p	0.0 (0.3)	100.9 (13.5)	104.8 (22.3)	.952	0.0 (0.5)	103.9 (32.6)	95.0 (32.1)	.515
miR-146a	3.2 (0.3)	109.2 (35.8)	59.4 (29.7)	.021	4.9 (3.7)	135.2 (93.3)	47.5 (31.8)	.049
miR-146b	2.1 (0.3)	111.3 (38.4)	53.0 (32.9)	.010	4.1 (3.2)	122.4 (73.4)	51.3 (23.3)	.049
miR-150	−4.8 (0.3)	100.3 (8.0)	102.9 (23.9)	.641	−4.4 (0.3)	102.9 (23.6)	93.6 (17.2)	.491
miR-155	2.8 (0.5)	104.7 (31.0)	129.0 (23.1)	.105	2.2 (0.5)	105.2 (33.4)	76.3 (35.8)	.099
miR-155*	13.0 (1.1)	120.4 (66.8)	148.7 (82.7)	.601	12.4 (1.1)	129.3 (94.3)	114.7 (74.2)	.784
miR-181a	3.1 (0.3)	101.1 (15.1)	86.9 (22.3)	.159	2.7 (0.8)	117.8 (70.2)	77.3 (26.0)	.239
miR-196a	11.6 (1.1)	120.8 (71.6)	136.5 (41.9)	.351	8.5 (0.5)	108.2 (39.5)	145.8 (15.1)	.608
miR-223	2.4 (1.3)	150.3 (130.4)	170.8 (93.8)	.364	0.4 (1.3)	171.9 (230.8)	95.6 (45.3)	.673

Circulating CD4⁺ and CD8⁺ T cells were isolated from 8 nonasthmatic control subjects and 8 patients with severe asthma, and the levels of individual miRNAs were determined by means of TaqMan qRT-PCR. Baseline expression levels are expressed as Δ cycle threshold (ΔCT) and have been normalized against *RNU44*. Differences between control subjects and patients with severe asthma are expressed as the fold change from control values. All values are means (SDs) of 8 individual patient samples, and statistical differences were evaluated by using the Mann-Whitney *U* test.

TABLE VI. Changes in the expression of lncRNAs in CD8⁺ T cells from patients with severe asthma

Probe set	Class of lncRNA	Ensembl gene ID	Transcript	Name	P value	Fold change
220459_at	Antisense	ENSG00000215424	MCM3APAS	Minichromosome maintenance complex component 3-associated protein antisense RNA	.00014	1.73
1557008_at	Intergenic	ENSG00000248908	RP11-730N24.1		.00015	−1.80
1556453_at	Intergenic	ENSG00000229727	AC013460.1		.00107	−1.59
210794_s_at	Intergenic	ENSG00000214548	MEG3	Maternally expressed 3 (non-protein coding)	.00002	−1.54
215011_at	Intergenic	ENST00000413987	SNHG3	Small nucleolar RNA host gene 3 (non-protein coding)	.00148	1.53
1553449_at	Intergenic	ENSG00000180422	C16orf81	Chromosome 16 open reading frame 81	.00000	−1.86
228913_at	Intergenic	ENSG00000170919	XXyac-R12DG2.2-010		.00000	1.56
1554447_at	Intergenic	ENSG00000225470	NCRNA00183	Non-protein-coding RNA 183	.00152	2.27
223725_at	Intergenic	ENSG00000240288	GHRLOS	Ghrelin opposite strand (non-protein coding)	.00030	1.53
243656_at	Intergenic	ENSG00000215447	BX322557.10		.00021	−1.60
1563088_a_at	Intergenic	ENSG00000215458	AP001053.11		.00005	−1.53
1557505_a_at	Intergenic	ENSG00000251153	AL049776.2		.00000	1.67
241270_at	Intergenic	ENSG00000250233	AC015802.2		.00002	−1.62
1564211_at	Intergenic	ENSG00000246223	C14orf64		.00115	1.67
1564426_x_at	Intergenic	ENSG00000247397	AC130352.1		.00151	−1.95
237544_at	Pseudogene	ENSG00000107679	PLEKHA1	Pleckstrin homology domain containing, family A pseudogene	.00003	1.81
1562250_at	Pseudogene	ENSG00000176993	AL590233.1	Known pseudogene	.00080	1.99
1555890_at	Pseudogene	ENSG00000170356	OR2A20P	Olfactory receptor, family 2, subfamily A, member 20 pseudogene	.00095	1.70
244071_at	Pseudogene	ENSG00000188573	FBLL1	Fibrillarin-like 1	.00141	−1.74

This table lists those lncRNAs that have been manually annotated in the Ensembl database and the expression of which was significantly changed in circulating CD8⁺ T cells obtained from patients with severe asthma compared with those from healthy control subjects. Data are denoted as the fold change against nonasthmatic control subjects and are the mean of 8 individual patients. These values are derived from Table E6 and have in addition been annotated in Ensembl. Statistically, these lncRNAs demonstrated an FDR of less than 0.05; associated *P* values are shown.

circulating CD8⁺ T cells obtained from patients with severe asthma, including the pathways associated with T-cell activation and the allergic inflammatory response. Because these changes were not observed in patients with nonsevere asthma, this indicated

that the changes in mRNA expression in circulating CD8⁺ T cells are related to the severity of the asthma and not asthma *per se*.

Examination of the individual mRNAs showed that 23 mRNAs were upregulated in both CD4⁺ and CD8⁺ T cells from patients

with severe asthma, including members of the S100 calcium-binding and CEACAM families. The calgranulin family of S100 calcium-binding proteins is composed of 3 members, S100A8, S100A9, and S100A12, which were all shown to be upregulated in CD4⁺ and CD8⁺ T cells. Interestingly, increased calgranulin expression is linked to the development of rheumatoid arthritis, inflammatory bowel disease, and atherosclerosis^{24,25}; is released from neutrophils/macrophages; and is thought to induce an inflammatory response through activation of Toll-like receptor 4 and receptor for advanced glycosylation.²⁶ Much less is known about the immunoglobulin-related glycoprotein CEACAM family, of which CEACAM-6 and CEACAM-8 are upregulated in both CD4⁺ and CD8⁺ T cells, although they have been implicated in T-cell proliferation.²⁷

Specific mRNAs upregulated in CD8⁺ T cells from patients with severe asthma included chitinase 3 protein 1 and arginase. Chitinase 3 protein 1, which is also known as YKL-40 in human subjects and BRP-39 in mice, is upregulated in many inflammatory diseases, including osteoarthritis, rheumatoid arthritis, sarcoidosis, inflammatory bowel disease, idiopathic pulmonary fibrosis, and solid malignancy.^{17,28,29} Significantly, its levels are also increased in serum and lung macrophages/epithelium of patients with asthma, in whom expression correlates positively with the severity of the disease.^{30,31} Furthermore, murine studies have shown that YKL-40 is crucial in the initiation and effector stage of T_H2-driven inflammation and airway remodeling in models of allergen-induced asthma and in IL-13 transgenic mice.³² As with human subjects, the animal studies showed that the increase in chitinase 3 protein 1 expression was predominately associated with lung epithelium and macrophages.³² Arginase is a key metalloenzyme of the urea cycle that converts L-arginine into L-ornithine and urea. Levels of this metalloenzyme are known to be increased in asthmatic patients, as well as in various animal models of asthma.¹⁶ It has been speculated that increased arginase levels deprive nitric oxide synthase of arginine and thereby prevent the production of nitric oxide (NO), a key bronchodilator and anti-inflammatory agent in asthmatic patients.

Having demonstrated changes in the mRNA expression, we investigated whether this might be mediated by changes in miRNA expression. There is now accumulating evidence that miRNAs regulate the proliferation, differentiation, and activation of T cells. Thus studies involving DICER knockout (an RNase III enzyme that is crucial to the production of mature miRNAs) in T-cell development showed that miRNAs are important in T-cell proliferation and the generation of the T_H2 phenotype and forkhead box protein 3–positive CD4⁺ Treg cells.^{18–20} Subsequent investigations aimed at characterizing the role of individual miRNAs have implicated the miR-17~92 cluster in early T-cell proliferation, miR-155 in the development of the T_H2 and Treg phenotype, and miR-181a as an inhibitor of CD8⁺ T-cell function.^{18–20} Both miR-155 and miR-181a have also been implicated in the regulation of the acute response in activated CD4⁺ T cells, including the release of IL-2 and IFN- γ . In addition, recent animal studies have shown increased expression of miR-21 in the lungs during ovalbumin-, fungus-, and IL-13–induced allergic airway inflammation³³ and increased miR-126 expression in a house dust mite–induced model of asthma.³⁴

Our comparison of the expression of these immune-related miRNAs showed no changes in the expression of members of the miR-17~92 cluster, miR-155, miR-181a, miR-21, or miR-126 in CD4⁺ and CD8⁺ T cells from patients with severe asthma,

although we observed significant downregulation in the expression of miR-146a and miR-146b. Interestingly, there are an increasing number of publications that demonstrate a central role for miR-146a in the regulation of immune responses^{35–40} and in the development of inflammatory diseases, such as rheumatoid arthritis, osteoarthritis, systemic lupus erythematosus, and chronic obstructive pulmonary disease.^{41,42} However, although we observed significant downregulation of miR-146a and miR-146b in both CD4⁺ and CD8⁺ T cells, only CD8⁺ T cells showed widespread changes in the profile of mRNA expression. This would imply that miR-146a and miR-146b alone are not responsible for changes in the mRNA profile in CD8⁺ T cells, a conclusion that is supported by the fact that there was only a bias toward targets of miR-146a and miR-146b ($P = .13$). Significantly, we also observed selective downregulation of miR-28-5p in CD8⁺ T cells, and bioinformatics analysis showed an enrichment of miR-28a-5p ($P = .026$) targets in those mRNAs that were upregulated in CD8⁺ T cells. At the present time, virtually nothing is known regarding the biological function of miR-28-5p, and subsequent studies will need to investigate its function in CD8⁺ T cells.

Having shown altered expression of mRNAs and miRNAs, we proceeded to investigate whether severe asthma was also associated with changes in expression of lncRNAs. lncRNAs can be broadly divided into natural antisense, pseudogenes, long intronic noncoding RNAs, and long intergenic noncoding RNAs. Despite their widespread expression, their importance in the regulation of multiple physiological and pathological response is only now emerging.^{21,22} Thus recent reports have demonstrated that expression of long intergenic noncoding RNA is cell and developmentally specific and mediated through activation of common transcription factors, including p53, nuclear factor κ B, Sox2, Oct4, and Nanog.⁴³ Our initial analysis of the profile of lncRNA expression showed that severe asthma was also correlated with widespread changes in the expression of natural antisense, pseudogenes, long intronic noncoding RNAs, and long intragenic noncoding RNAs in circulating CD8⁺ T cells but not CD4⁺ T cells. This observation indicates for the first time that the changes in mRNA expression and subsequent T-cell function might be regulated by lncRNAs.

In summary, using a transcriptomics-based approach, we have demonstrated that circulating CD8⁺ T cells from patients with severe asthma but not patients with nonsevere asthma have a transcript expression profile associated with an activated phenotype and have shown that these changes might, in part, be mediated through alterations in noncoding RNA expression. In contrast, there was little evidence to suggest activation of circulating CD4⁺ T cells in patients with either nonsevere or severe disease, despite the fact that tissue CD4⁺ T_H2 cells have previously been linked to the asthmatics phenotype. Clearly, because antigen-specific T cells compartmentalize to local or regional lymph nodes, these observations could be the result of circulating (spilled over) factors presumably from the lungs and might not reflect *in situ* activation and effector function for these cells. It will therefore be important to confirm these observations by using lung-derived T cells. In addition, it will also be important to examine whether the activation of CD8⁺ T cells observed at the transcriptional level is reflected by changes in effector function, the effect of therapy, or both. However, given the increasing evidence to suggest that viral infection is important to both asthma development in early life and subsequent exacerbations^{44,45} and the

role of CD8⁺ T cells in the immune response to viral infection, it might therefore be speculated that chronic activation of CD8⁺ T cells might contribute to the symptoms observed in patients with severe asthma. If confirmed, this would suggest that targeting either the activation of CD8⁺ T cells or their effector functions might provide a novel therapeutic approach to the treatment of severe asthma.

Clinical implications: Severe asthma is associated with the activation of circulating CD8⁺ T cells, and targeting this response might provide a novel therapeutic approach.

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