

Corticosteroid-resistant asthma is associated with classical antimicrobial activation of airway macrophages

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Background: The cause of corticosteroid-resistant (CR) asthma is unknown.

Objective: We sought to perform gene microarray analyses by using bronchoalveolar lavage (BAL) cells from well-characterized subjects with CR asthma and subject with corticosteroid-sensitive (CS) asthma to elucidate the differential expression of genes that contribute to the development of corticosteroid resistance.

Methods: The patients were characterized as having CR or CS asthma based on FEV₁ percent predicted improvement after a 1-week course of oral prednisone. Expression of selected gene targets was verified by means of real-time PCR and ELISA.

Results: Microarray analyses demonstrated significantly higher levels (>3-fold increase, $P < .05$) of transcripts for TNF- α , IL-1 α , IL-1 β , IL-6, CXCL1, CXCL2, CXCL3, CXCL8 (IL-8), CCL3, CCL4, and CCL20 in BAL cells of subjects with CR asthma. These findings, confirmed by means of RT-PCR in additional BAL samples, were consistent with classical macrophage activation by bacterial products. In contrast, markers of alternatively activated macrophages, arginase I and CCL24, were decreased. Genes associated with activation of the LPS signaling pathway (early growth response 1, dual-specificity phosphatase 2, molecule possessing ankyrin repeats induced by LPS, and TNF- α -induced protein 3) were significantly increased in BAL samples from subjects with CR asthma ($P < .05$). These patients had significantly higher amounts (1444.0 ± 457.3 pg/mg total protein) of LPS in BAL fluid than seen in subjects with CS asthma (270.5 ± 216.0 pg, $P < .05$), as detected by using the LAL assay and confirmed by means of gas chromatographic/mass spectrometric analysis. Prolonged exposure to LPS induced functional steroid resistance to dexamethasone in normal human monocytes, as

demonstrated by persistently increased IL-6 levels in the presence of dexamethasone.

Conclusions: Classical macrophage activation and induction of LPS signaling pathways along with high endotoxin levels detected in BAL fluid from subjects with CR asthma suggest that LPS exposure might contribute to CR asthma. (*J Allergy Clin Immunol* 2008;122:550-9.)

Key words: Corticosteroids, asthma, resistance, genes, endotoxin

Corticosteroids are highly effective in the treatment of inflammation and are currently recommended for management of persistent asthma as the preferred therapy for control of airway inflammation.¹ However, a subset of asthmatic subjects does not respond to steroid therapy²⁻⁶ and is therefore at risk for escalation of disease severity. Corticosteroid resistance presents a major management problem and accounts for a disproportionate amount of health care costs in this group of patients.^{7,8} The studies suggest that there is a substantial need for further improvement in asthma therapy because a large group of asthmatic patients remain symptomatic despite optimal therapy.

The data collected to date suggest that, in most cases, corticosteroid-resistant (CR) asthma is an acquired condition.³ However, the environmental factors that contribute to CR asthma are unknown. Use of new technologies, such as gene microarray analyses, which can examine the complete gene expression profile of inflammatory airway cells from subjects with CR asthma versus those from subjects with corticosteroid-sensitive (CS) asthma, have the potential to identify new hypotheses concerning the cause of this increasingly recognized challenge in the treatment of asthma. The gene microarray approach is a comprehensive technique that is not limited by a preconceived hypothesis. The present study was undertaken to identify genes that are differentially expressed in bronchoalveolar lavage (BAL) cells from subjects with CR asthma and subjects with CS asthma to determine what might contribute to CR asthma and the potential role that endotoxin (LPS) might have in this condition.

METHODS

Subjects

Patients with a diagnosis of asthma according to American Thoracic Society criteria⁹ were selected for evaluation. Asthmatic subjects had a baseline FEV₁ percent predicted of 55% to 85% of predicted value, a β_2 -adrenergic response of 12% or greater of baseline FEV₁ percent predicted, and/or a methacholine PC₂₀ value of 8 mg/mL or less. Asthmatic patients were further subdivided into those with CR or CS asthma based on their response to steroids. The definition was based on change in FEV₁ percent predicted over a 1-week course of 40 mg/d oral prednisone. Asthmatic patients were defined as having CS asthma if they had an increase in FEV₁ percent predicted of 15% or greater after a 1-week course of prednisone and as having CR asthma if a 12% or less change in FEV₁ percent predicted was observed. None of the asthmatic

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Data deposition footnote: The microarray data reported in this article have been deposited in the Gene Expression Omnibus database at www.ncbi.nlm.nih.gov/geo (accession no. GSE7368).

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Abbreviations used

BAL: Bronchoalveolar lavage
CR: Corticosteroid resistant
CS: Corticosteroid sensitive
LAL: Limulus amoebocyte lysate
TLR: Toll-like receptor

subjects recruited for this study had evidence of other types of lung diseases. None of the subjects had received systemic corticosteroid therapy for at least 1 month before bronchoscopy. Normal healthy adult donors with no history of chronic respiratory tract disorders and normal spirometric results were recruited for the study as well. All subjects provided written informed consent to participate in the study. The study was approved by the Institutional Review Board at National Jewish Health, Denver, Colorado.

Ten subjects with CR asthma and 8 subjects with CS asthma were recruited for the study and underwent bronchoscopy with collection of BAL fluid. Gene array studies of BAL macrophages were performed in 3 subjects with CR asthma and 3 subjects with CS asthma. Real-time PCR was performed with BAL samples from an additional 7 subjects with CR asthma and 5 subjects with CS asthma to confirm gene array results. Two subjects with CS asthma underwent bronchoscopy twice: the first time the RNA from cells was used for the gene array study, and the second time the RNA from BAL cells was used for real-time PCR. No difference in BAL differential counts between 2 bronchoscopies was found for these 2 subjects with CS asthma (data not shown). Blood monocytes for evaluation of steroid response after preincubation with LPS were obtained from 6 healthy control subjects.

Preparation of BAL cells and fluids

Fiberoptic bronchoscopies with BAL were performed according to the guidelines of the American Thoracic Society.¹⁰ BAL cells were filtered through a 70- μ m nylon cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ), spun at 200g for 10 minutes, washed 2 times, and resuspended in HBSS. After filtration of BAL cells, BAL fluid was placed in aliquots and stored at -80°C . BAL differentials were obtained on cytospin preparations by using Diff-Quick (Scientific Products, McGraw Park, Ill) stain, counting a minimum of 500 cells. RNA from freshly isolated 3×10^6 BAL cells was preserved in the RLT buffer and stored at -80°C for further RNA extraction.

Gene expression profile analysis

The gene microarray studies were performed by the Gene Microarray Core Laboratory at the University of Colorado at Denver and Health Sciences Center, Denver, Colorado, as described by us earlier (for details, see this article's [Methods](#) section in the Online Repository at www.jacionline.org).^{11,12}

Real-time PCR was used to confirm the expression of selected genes in BAL cells. RNA was transcribed into cDNA and analyzed by means of real-time PCR with the dual-labeled fluorogenic probe method on an ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, Calif), as previously described.^{11,13} All primers and probes for the target genes and housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase [GAPDH] and 18sRNA) were purchased from Applied Biosystems. Standard curves for all targets were generated with the fluorescent data from 10-fold serial dilutions of total cDNA of the highest expression sample. Quantities of target genes in test samples were normalized to the corresponding housekeeping gene levels in each sample. A blinded investigator who was unaware of the clinical status of the patients performed the assays.

Multiplex ELISA assay

A Human 17-plex panel ELISA (171-A11127; Bio-Rad, Hercules, Calif) was performed to analyze human cytokine/chemokine expression in BAL fluid samples from 6 subjects with CR asthma and 4 subjects with CS asthma. The

data were normalized to the total protein amounts in the samples, as detected by using the Bradford assay (Bio-Rad), to account for the variability in protein content in the BAL fluid caused by differences in the recovered BAL fluid volume.

Preparation of PBMCs and culture

Human PBMCs were isolated by means of Ficoll-Hypaque density gradient centrifugation of heparinized venous blood. Monocytes were isolated from PBMCs by using magnetic bead negative selection (Monocyte isolation kit II; Miltenyi Biotec, Auburn, Calif) and resuspended in RPMI 1640 (BioWhittaker, Los Angeles, Calif) containing 10% charcoal-filtered, heat-inactivated, steroid-free FCS (Gemini Bio Products, Calabasas, Calif); 40 $\mu\text{mol/L}$ L-glutamine; 100 U/mL penicillin; 100 U/mL streptomycin; and 20 mmol/L HEPES (GIBCO BRL Life Technologies, Carlsbad, Calif) at a concentration of $5 \times 10^5/\text{mL}$. Monocytes were pretreated with 10 ng/mL LPS for 1 or 24 hours and then cultured for an additional 24 hours in the absence or presence of 10^{-9} to 10^{-6} mol/L dexamethasone. Culture supernatants were collected and stored at -20°C until analysis for IL-6 and TNF- α production by means of ELISA with a human IL-6 ELISA kit or human TNF- α ELISA kit (R&D Systems, Inc, Minneapolis, Minn), according to the manufacturer's instructions.

Measurement of LPS in BAL fluid

The content of LPS in BAL fluid was analyzed by using the chromogenic limulus amoebocyte lysate (LAL) test (Cambrex Bio Science Walkersville, Inc, Walkersville, Md), according to the manufacturer's instructions and normalized to the total protein level in each sample.

Gas chromatographic/electron ionization-mass spectrometric analysis of BAL fluid

This analysis was performed by the Analytical Toxicology Laboratory, Department of Environmental and Radiological Health Sciences, Colorado State University, Fort Collins, Colorado, based on quantification of 3-hydroxy fatty acids as a chemical marker of LPS (for details, see this article's [Methods](#) section in the Online Repository).

Statistical methods

Patient clinical variables were analyzed by using descriptive statistics, including proportions or means with ranges when appropriate. For details on gene microarray analysis, see this article's [Methods](#) section in the Online Repository.

Analysis for statistically significant differences for the data gathered by means of ELISA and real-time PCR was performed with the unpaired 2-tailed *t* test and the nonparametric Mann-Whitney test, respectively. The Pearson correlation analysis was performed with log-transformed data that assumed Gaussian distribution. Graph Pad Prism, version 4.01 (San Diego, Calif), was used for all statistical calculations. *P* values of .05 or less were considered significant.

RESULTS

Patient characteristics

Eighteen asthmatic subjects (10 with CR asthma and 8 with CS asthma) were recruited for this study and underwent bronchoscopy with collection of BAL fluid. Patients were divided into CR and CS groups based on FEV₁ percent predicted responses after a 1-week course of oral prednisone. As shown in [Table E1](#) (available in this article's Online Repository at www.jacionline.org), patients in the CR group did not show any improvement in pre-bronchodilator FEV₁ percent predicted after exposure to prednisone (mean $\pm$ SEM: 67.0% $\pm$ 2.7% and 65.8% $\pm$ 2.5% before vs after steroid burst, respectively; *P* = .54). In contrast, patients

in the CS group showed significant improvement in their lung function after the steroid burst ($68.6\% \pm 2.7\%$ and $83.1\% \pm 2.8\%$ before vs after the steroid burst, respectively; $P < .0001$, compared with FEV₁ percent predicted before the steroid burst). During the study, the patients continued to use inhaled steroids but were asked to withhold them for 24 hours before bronchoscopy. Patients who had respiratory tract infections, used antibiotics, or both within 4 weeks of scheduled bronchoscopy were excluded from the study. There was no evidence of active infection during the time of bronchoscopy in either of the asthma groups studied.

The number of total white cells in BAL samples (see Table E2 in this article's Online Repository at www.jacionline.org) collected was significantly lower in samples from the CR group compared with those from the CS group ($P = .002$) and also varied between patients (mean $\pm$ SEM total white cell counts for the CR [$n = 10$] and CS [$n = 8$] groups were $9.3 \pm 1.7 \times 10^6$ and $17.3 \pm 1.2 \times 10^6$, respectively). This difference is due to the differences in the volume of BAL fluid (percentage of BAL fluid return) recovered after bronchoscopy (BAL fluid percentage return was $41.3\% \pm 5.7\%$ vs $62.8\% \pm 2.6\%$ in the CR and CS asthma groups, respectively; $P = .008$). However, the percentages of macrophages, lymphocytes, neutrophils, and eosinophils did not differ between the 2 groups. Equal amounts of BAL cells were processed from each patient for RNA isolation. Macrophages were the major cell population in BAL samples: macrophages composed a mean percentage of $91.3\% \pm 3.9\%$ and $93.2\% \pm 1.3\%$ BAL samples from the CR ($n = 10$) and CS ($n = 8$) asthma study groups, respectively.

Gene microarray analysis of BAL samples reveals classical macrophage activation in samples from subjects with CR asthma

BAL samples collected from 3 white subjects with CR asthma and 3 white subjects with CS asthma were initially analyzed to screen for potential gene candidates with gene microarray. None of the patients selected were current or former smokers. In terms of asthma severity, both groups were equivalent: baseline prebronchodilator FEV₁ percent predicted was $66.6\% \pm 3.9\%$ in the CR group and $69.4\% \pm 5.9\%$ in the CS group ($P = .71$). Postbronchodilator FEV₁ percent predicted was $80.0\% \pm 8.5\%$ and $85.0\% \pm 8.1\%$, respectively, in the CR versus CS groups ($P = .69$).

By using the unpaired *t* test with the Welch correction, we found significant ($P < .05$) upregulation (>3 -fold) of 38 probe sets representing 30 genes in samples from subjects with CR asthma compared with samples from subjects with CS asthma. Of these 30 genes, 8 were transcribed sequences and hypothetical proteins of unknown function. Three genes were significantly downregulated (>3 -fold) in samples from subjects with CR asthma compared with those from subjects with CS asthma.

It was found that BAL cells from subjects with CR asthma are remarkably more activated than those from subjects with CS asthma. As shown in Table I, a number of proinflammatory genes demonstrated significantly greater upregulation in subjects with CR asthma compared with that seen in subjects with CS asthma; this pattern of activation was confirmed with MAS 5.0 and Robust Multichip Average algorithms of data analysis in GeneSpring software (Agilent Technologies, Santa Clara, Calif).

Because BAL samples were mostly composed of macrophages (see Table E2 in this article's Online Repository at www.jacionline.org), a majority of genes identified by means of gene microarray

were related to macrophage function and activation. Macrophages are known to express different functional programs in response to microenvironmental signals.^{14,15} It is known that cytokines and microbial products profoundly affect the function of these cells. Recently, the concept of classical (activation by microbial products and proinflammatory cytokines) and alternative activation patterns of macrophages (T_H2) has been proposed.^{14,15} These stimuli induce distinct cell-activation programs that differ with regard to receptor expression and effector function and produce a distinct pattern of cytokines and chemokines.¹⁴

Interestingly, our microarray analysis of BAL cells obtained from subjects with CR asthma and subjects with CS asthma suggest that macrophages from subjects with CR asthma have undergone classical activation, expressing significantly higher levels of TNF- α , IL-6, IL-1 α , and IL-1 β mRNA compared with those from subjects with CS asthma (Table I). In addition, a distinct set of chemokines (CXCL1, CXCL2, CXCL3, CXCL8 [IL-8], CCL3, CCL4, and CCL20) was found to be significantly activated in BAL cells from subjects with CR asthma. LPS is an important stimulus for triggering classical macrophage activation.¹⁴ Along with cytokines and chemokines, several other genes linked to the LPS signaling pathway were found to be significantly upregulated in BAL samples from subjects with CR asthma: early growth response 1,¹⁶⁻¹⁸ molecule possessing ankyrin repeats induced by LPS,^{19,20} dual-specificity phosphatase 2, and TNF- α -induced protein 3²¹ (Table I). This indicates the potential exposure of macrophages to LPS in the airways of subjects with CR asthma. In contrast, markers of alternatively activated macrophages (arginase I and CCL24) that are reported to be upregulated by T_H2 cytokines in allergic asthma^{15,22,23} were significantly downregulated in macrophages from subjects with CR asthma (Table I). Analysis of gene array data revealed no difference in the expression of T_H2 cytokines and their receptors in BAL samples from subjects with CR asthma and subjects with CS asthma (data not shown). These findings suggest that macrophages from subjects with CR asthma exhibit a proinflammatory pattern of gene expression and have undergone classical macrophage activation in contrast to the macrophages from subjects with CS asthma that typically exhibit a pattern of gene expression characteristic of T_H2 exposure and alternative activation.

Confirmation of gene microarray results by means of real-time PCR and ELISA

To rule out the possibility of false-positive results when running single microarrays on a sample, we confirmed the gene array data by using real-time PCR and ELISA with 14 additional BAL samples (7 from subjects with CR asthma and 7 from subjects with CS asthma; 2 of the subjects with CS asthma were repeat bronchoscopies involving subjects with CS asthma studied in gene microarrays). As shown in Fig 1, real-time PCR amplification of TNF- α , IL-8, IL-1 β , and CCL20 (macrophage inflammatory protein 3 α) mRNA confirmed our gene array findings that airway BAL cells from subjects with CR asthma are associated with significantly greater proinflammatory cytokine and chemokine gene expression than found in subjects with CS asthma ($P \leq .01$). As for markers of alternative macrophage activation, significantly higher ($P < .05$) levels of CCL24 mRNA expression were found in BAL samples from subjects with CS asthma, but no differences in CCL22 and suppressor of cytokine signaling 1

TABLE I. Gene profiling of BAL cells from subjects with CR asthma versus subjects with CS asthma

Classification	Affymetrix U133 + 2.0	Gene accession no.	Mean CR asthma $\pm$ SE*	Mean CS asthma $\pm$ SE*	Fold induction in CR/CS asthma (MAS 5.0)	Fold induction in CR/CS asthma (Robust Multichip Average)
Molecules associated with the classical pattern of macrophage activation						
IL-6	205207_at	NM_000600	8199 $\pm$ 2183	231 $\pm$ 52	35.54†	34.22†
TNF- α	207113_s_at	NM_000594	4640 $\pm$ 593	431 $\pm$ 114	10.76†	8.9†
IL-1 β	205067_at	NM_000576	20,205 $\pm$ 6204	1947 $\pm$ 351	10.38†	7.38†
IL-1 α	210118_s_at	M15329	8921 $\pm$ 4015	1053 $\pm$ 327	8.47†	6.5†
CCL20	205476_at	NM_004591	15,591 $\pm$ 3047	580 $\pm$ 56	26.90†	21.7†
CXCL8 (IL-8)	202859_x_at	NM_000584	30,651 $\pm$ 1958	2014 $\pm$ 837	15.22†	24.05†
CCL3	205114_s_at	NM_002983	6374 $\pm$ 2466	752 $\pm$ 184	8.48†	11.09†
CXCL3	207850_at	NM_002090	20,093 $\pm$ 5258	2590 $\pm$ 569	7.72†	4.92†
CCL4	204103_at	NM_002984	9648 $\pm$ 747	1372 $\pm$ 277	7.03†	5.89†
CXCL2	209774_x_at	M57731	22,556 $\pm$ 2913	3738 $\pm$ 1174	6.03†	7.35†
CXCL1	204470_at	NM_001511	663 $\pm$ 161	117 $\pm$ 19	5.66†	7.34†
Molecules associated with an alternative pattern of macrophage activation						
CCL24	221463_at	NM_00299	330 $\pm$ 58	2388 $\pm$ 290	0.14†	0.16†
Arginase 1	231662_at	AV652232	9 $\pm$ 10	37 $\pm$ 18	Absent	Absent
LPS/TNF-related signaling molecules						
Early growth response 1	227404_s_at	AI459194	39,963 $\pm$ 13,583	2696 $\pm$ 299	14.82†	12.45†
Molecule possessing ankyrin repeats induced by LPS	223217_s_at	BE646573	23,852 $\pm$ 4436	3040 $\pm$ 1170	7.85†	7.15†
Dual-specificity phosphatase 2	204794_at	NM_004418	1643 $\pm$ 83	212 $\pm$ 18	7.73†	10.52†
TNF- α -induced protein 3	202643_s_at	AI738896	11,911 $\pm$ 4214	1849 $\pm$ 389	6.44†	4.92†

*Mean signal intensity $\pm$ SEM of 3 BAL samples from subjects with CR asthma and 3 BAL samples from subjects with CS asthma.

† $P < .05$ comparing data sets from CR asthma versus CS asthma groups analyzed with the 2-tailed unpaired t test.

expression were observed (Fig 2). No mannose receptor C type 1, another gene associated with alternative activation of macrophages,¹⁴ was detected in either group of asthmatic subjects. The summarized data concerning additional targets associated with classical macrophage activation analyzed by means of real-time PCR are presented in Table II.

A subset of samples (6 from subjects with CR asthma and 4 from subjects with CS asthma) was screened by using a 17-plex ELISA assay to determine whether these differences in cytokine production between the groups can also be seen in BAL fluid. Clinical features of the subset of patients selected for the BAL fluid cytokine analysis were similar to those described in the patient characteristics table (see Table E1). No difference in the total protein concentration was observed between BAL fluids from the 2 groups of patients studied. Significantly higher levels of TNF- α , IL-1 β , and IL-8 were found in BAL fluid from subjects with CR asthma compared with that from subjects with CS asthma (Fig 3). No difference in T_H2 cytokine levels in BAL fluid was found (Fig 3).

Presence of LPS in the BAL fluid of subjects with CR asthma

Because LPS signaling genes were activated on gene microarray, we evaluated the levels of LPS in BAL fluid. The chromogenic LAL test was performed, and the data obtained were normalized to total protein in BAL fluid. It was found that

BAL fluid from 9 of 10 subjects with CR asthma tested contained substantial amounts of LPS. In contrast, only trace amounts of LPS could be detected in BAL fluid of subjects with CS asthma (Fig 4, A). LPS, 1444.0 $\pm$ 457.3 pg/mg total protein, was detected in BAL fluid from subjects with CR asthma compared with 270.5 $\pm$ 216.0 pg/mg total protein found in BAL fluid of subjects with CS asthma ($P \leq .05$; Fig 4, A).

To rule out false-positive readings of the LAL assay caused by the potential effect of other biologically active components in BAL fluid samples, additional experiments were performed with 4 selected BAL fluid samples in which serial dilutions of the BAL fluid samples were tested by using the LAL assay or the samples were boiled, spiked with known amounts of LPS, or measured by using the LAL assay in the presence of the proteinase inhibitor (to rule out confounding results of colorimetric end point of the LAL assay caused by the potential presence of active proteases in BAL fluid samples). No significant effect on LPS detection was noted (data not shown).

We confirmed the presence of LPS in a set of several BAL fluid samples by means of LPS gas chromatographic/mass spectrometric analysis based on quantification of 3-hydroxy fatty acids as a chemical marker of LPS (Table III).^{24,25} A significant correlation between the amounts of LPS detected by means of LAL assay and the LPS mass spectrometric assay was noted ($R = 0.9949$, $P = .0051$). In addition, the BALF3 and BALF4 specimens were split in half (samples A and B) to assess variability of endotoxin detection by means of mass spectrometry, and based

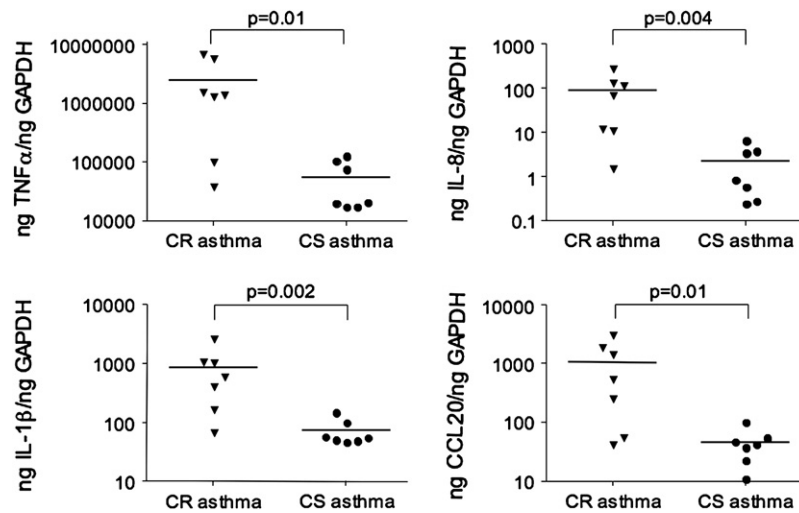


FIG 1. Significant increase in expression of markers associated with classical macrophage activation in BAL cells from subjects with CR asthma compared with subjects with CS asthma, as shown by means of real-time PCR. *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase.

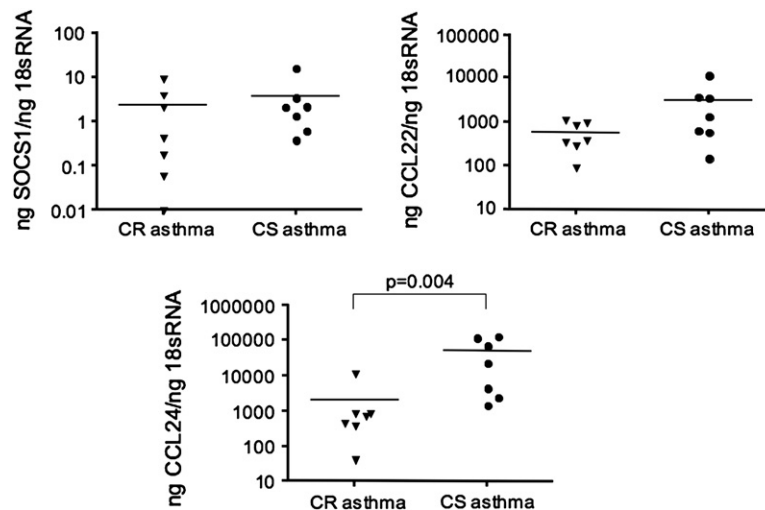


FIG 2. Expression of markers of alternative macrophage activation in BAL cells from subjects with CR asthma compared with those from subjects with CS asthma, as shown by means of real-time PCR. *SOCS1*, Suppressor of cytokine signaling 1.

on the data (Table III), the reproducibility of the results was satisfactory.

The biologic significance of these observations was supported by evidence for a significant correlation between the percentage of neutrophils in BAL samples and the amount of IL-8 mRNA expression in BAL cells ($R = 0.60$, $P = .02$), as well as between the amount of LPS in BAL fluid and IL-8 mRNA expression by BAL cells ($R = 0.65$, $P = .02$; Fig 4, B and C).

Prolonged exposure to LPS alters normal steroid responses of human monocytes *in vitro*

To assess the effects of LPS on cellular steroid responses, normal human monocytes ($n = 6$) isolated from PBMCs by using magnetic bead negative selection were stimulated with LPS alone for 1 or 24 hours, and then various concentrations of dexamethasone were added and the cells were cultured for an additional 24

hours in the continued presence of LPS. Cell supernatants were collected and analyzed for IL-6 by means of ELISA. It was found that the duration of LPS exposure determined the steroid responsiveness of the cells. IL-6 production by human monocytes was significantly suppressed by steroids when the cells were pretreated with LPS for 1 hour and then cultured with dexamethasone in the continued presence of LPS (Fig 5, A). Pretreatment of cells with LPS for 24 hours resulted in loss of the ability of dexamethasone to suppress IL-6 production by human monocytes (Fig 5, B). Similar effects had been observed when TNF- α levels were measured in cell-culture supernatants from monocytes treated with LPS (Fig 5, C and D). Thus LPS re-exposure induced functional steroid resistance.

DISCUSSION

The current study determined gene expression profiles of BAL cells collected from subjects with CR asthma and subjects with CS

TABLE II. Gene profiling of BAL cells from subjects with CR asthma versus subjects with CS asthma by means of real-time PCR

Classification	Mean CR asthma $\pm$ SE*	Mean CS asthma $\pm$ SE*	Fold induction in CR/CS asthma	P value
IL-6	503 $\pm$ 172	7.2 $\pm$ 1.9	69.9	.0007
CXCL8 (IL-8)	2740 $\pm$ 847	10.4 $\pm$ 1.6	263.5	.0006
CCL3	25.9 $\pm$ 9.1	4.2 $\pm$ 1.7	6.2	.0379
CXCL3	14.2 $\pm$ 3.3	3.8 $\pm$ 1.3	3.74	.0175
CCL4	13.2 $\pm$ 5.8	3.3 $\pm$ 1.3	4.0	.0379
CXCL2	13.2 $\pm$ 3.5	4.1 $\pm$ 1.2	3.22	.0530
CXCL1	8.4 $\pm$ 4.0	2.5 $\pm$ 1.3	3.36	.1282
Early growth response 1	14.8 $\pm$ 3.9	2.2 $\pm$ 0.6	6.73	.0006
Molecule possessing ankyrin repeats induced by LPS	10.5 $\pm$ 3.5	2.8 $\pm$ 0.8	3.75	.0262
Dual-specificity phosphatase 2	34.3 $\pm$ 17.0	0.8 $\pm$ 0.4	42.88	.0041
TNF- α -induced protein 3	11,911 $\pm$ 4214	1849 $\pm$ 389	2.37	.0379

*Mean signal intensity $\pm$ SEM (in nanograms) of the target mRNA/nanogram of 18sRNA of 7 additional BAL samples from subjects with CR asthma and 7 additional BAL samples from subjects with CS asthma.

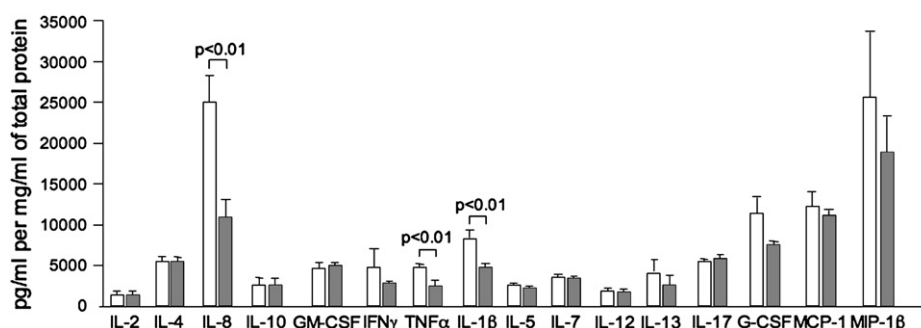


FIG 3. Increased expression of proinflammatory cytokines in the BAL fluid samples from subjects with CR asthma compared with those from subjects with CS asthma, as detected by using the 17-plex panel ELISA ($n = 6$ for the CR asthma group [open bars], $n = 4$ for the CS asthma group [solid bars]). Expression of 17 different targets was analyzed in 100 μ L of BAL fluid and normalized to the total protein amount in each sample. MCP-1, Monocyte chemoattractant protein 1; MIP-1 β , macrophage inflammatory protein 1 β .

asthma. As demonstrated by activation of multiple proinflammatory cytokines and chemokines accompanied by activation of LPS signaling pathways, BAL macrophages were remarkably more skewed toward a proinflammatory, antimicrobial, classically activated phenotype in subjects with CR asthma compared with subjects with CS asthma. These data gathered by means of gene microarray were further confirmed in a separate group of subjects with CR asthma and subjects with CS asthma by means of real-time PCR and ELISA. The significance of the gene expression profiles presented in Table I is supported not only by the large observed between-group differences but also by their interrelationships (ie, a related set of genes was activated). A set of related proinflammatory cytokine and chemokine genes (TNF- α , IL-6, IL-1 β , IL-1 α , CXCL1, CXCL2, CXCL3, CXCL8 [IL-8], CCL3, CCL4, and CCL20) with strong observed differences reflects a more convincing trend than a strong difference from just 1 gene and supports a programmed pattern of cell activation.

Macrophages are known to express different functional programs in response to unique microenvironmental signals.¹⁴ It has been recognized that macrophages respond to microbial products, such as LPS,²⁶ by undergoing classical macrophage activation. This response is associated with increased expression of multiple genes, including proinflammatory chemokines (CXCL8), cytokines (TNF- α , IL-1 β , and IL-6), and regulators of host defense.²⁷ Early studies have suggested that classical macrophage activation is inhibited by T_H2 molecules, including IL-4, IL-13, and IL-10.

More recently, it has been recognized that these molecules do more than simply downregulate classically activated macrophages but that they promote a distinct response, termed alternative macrophage activation. Gene profiling studies have shown that alternatively activated macrophages are characterized by a different pattern of gene expression than classically activated macrophages^{12,13,15} and include genes involved in phagocytosis (mannose receptor, dectin-1, and scavenger receptor A), tissue remodeling (arginase I and insulinlike growth factor 1), and the termination of inflammation (CCL24 and CCL16).

There had been only 2 studies that attempted to identify novel pathways involved in the pathogenesis of steroid responses in asthma.^{28,29} One study included gene expression profiling in inflammatory cells of subjects with CR asthma and subjects with CS asthma, and this was done with PBMCs in search of genes that accurately predict responders and nonresponders to inhaled steroids.²⁸ Their analysis focused on genes that were upregulated or downregulated by means of *in vitro* IL-1 β /TNF- α stimulation and reversed with steroid treatment in the CS asthma group. Through analysis of the most extreme phenotypes of patients, 15 genes were selected that predicted steroid responses of subjects with CR asthma and subjects with CS asthma with 84% accuracy.²⁸ Our current study was performed with inflammatory cells from airways, thereby allowing us, for the first time, to investigate corticosteroid resistance at the target organ level.

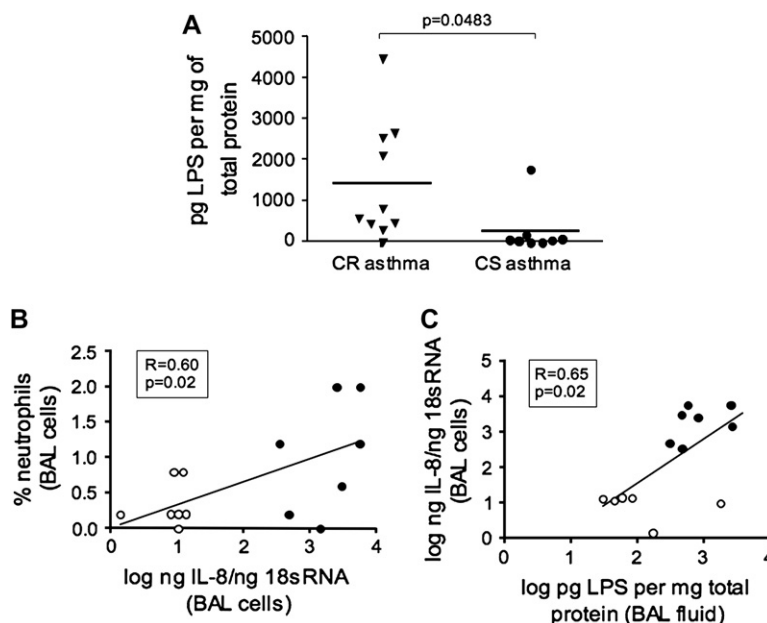


FIG 4. Presence of high amounts of LPS in BAL fluid from subjects with CR asthma. **A**, The content of LPS in BAL fluid was analyzed by using the chromogenic LAL test and normalized to the total protein level in each sample. IL-8 mRNA expression by BAL cells from subjects with CR asthma and subjects with CS asthma positively correlates with the percentage of neutrophils in BAL samples (**B**) and amounts of LPS in BAL fluid (**C**; solid circles, subjects with CR asthma; open circles, subjects with CS asthma).

TABLE III. Endotoxin analysis for C8-C17 3-hydroxy fatty acids in BAL fluid samples

Sample ID	3-Hydroxy fatty acids (ng/mL)	LAL assay results (ng/mL)
BALF1	1.33	2.67
BALF2	1.85	4.48
BALF3 (A)	34.76	92.25
BALF3 (B)	25.02	92.25
BALF4 (A)	18.05	39.02
BALF4 (B)	14.17	39.02

Another recent study²⁹ compared gene expression in epithelial brushings from subjects with asthma before and after treatment with either an inhaled corticosteroid (fluticasone) or placebo in a double-blind, randomized controlled trial. Also, gene expression in epithelial brushings from 2 control groups (healthy nonsmokers and habitual smokers) was analyzed to identify epithelial abnormalities that are asthma specific. The patients in this study demonstrated mild airway obstruction with FEV₁ percent predicted values of $87\% \pm 12\%$, and no patients with asthma were using corticosteroids or long-acting β -agonists before enrollment. All patients enrolled into our study had FEV₁ percent predicted values of less than 80%, and many patients were taking inhaled corticosteroids. All 3 studies (including ours) yielded nonoverlapping datasets of genes that were differentially expressed, likely because of the differences in tissue and cell components analyzed, disease severity, and differences in sample acquisition. Nevertheless, we believe that these studies provided useful information and insights into the contribution of various cell types in the pathogenesis of steroid resistance in asthma.

In the current study we demonstrated a significant increase in TNF- α mRNA and protein production by BAL cells of subjects

with CR asthma. This observation is consistent with 2 recent studies that also demonstrated evidence of upregulation of the TNF- α axis in refractory asthma.^{30,31} It has been shown that these patients had increased expression of membrane-bound TNF- α , TNF- α receptor 1, and TNF- α -converting enzyme by peripheral blood monocytes.³⁰ In addition, significant increases of TNF- α levels were reported in BAL fluid, with a significant change in the number of TNF- α -producing cells in endobronchial biopsy specimens of subjects with severe asthma who were refractory to corticosteroids.³¹ Two clinical trials confirmed that treatment of this group of patients with etanercept (soluble TNF- α receptor IgG1 Fc fusion protein) resulted in significant improvement in asthma symptoms, lung function, and bronchial hyperresponsiveness,^{30,31} suggesting that TNF- α is a potential factor that requires intervention in CR asthma. There is mounting evidence that corticosteroid insensitivity is associated but not limited to severe asthma, and given the heterogeneity of asthma, treatment resistance might not represent a distinct phenotype in children and adults.³²⁻³⁴ Thus it is difficult to directly extrapolate the results of the studies that demonstrate association of TNF- α with refractory asthma because these studies defined their study population by severity of disease but not by degree of reversibility after steroid use.

Nothing is known about the phenotypic characteristics of airway macrophages of subjects with CS asthma versus those of subjects with CR asthma. Studies in the allergen-driven models of airway hyperresponsiveness suggest that the T_H2 environment of the sensitized and antigen-challenged lung promotes the expression of genes associated with alternative macrophage activation, including arginase.²³ In addition, increased expression of arginase has been detected in BAL cells from asthmatic subjects, suggesting that macrophages might undergo alternative macrophage activation in asthma. However, our current studies involving microarray analyses of macrophages obtained from subjects

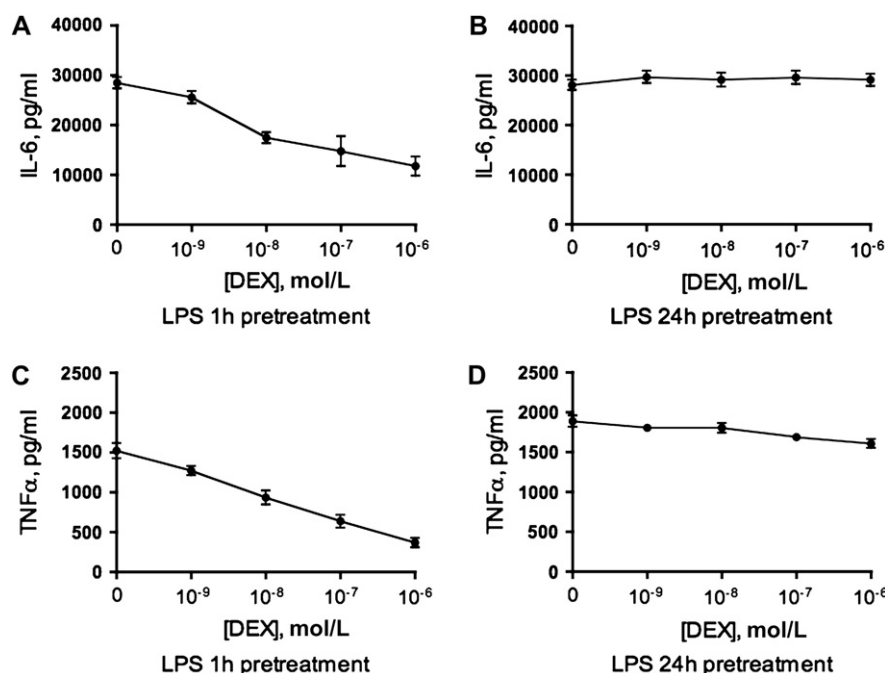


FIG 5. LPS induces functional steroid resistance in normal human monocytes *in vitro*. Monocytes were cultured with 10 ng/mL LPS for 1 or 24 hours before addition of dexamethasone for an additional 24 hours. IL-6 (**A and B**) and TNF- α (**C and D**) levels were measured in cell-culture supernatants by means of ELISA (n = 6).

with CS asthma and subjects with CR asthma suggest that macrophages from subjects with CR asthma exhibit a proinflammatory pattern of gene expression and might have deviated toward a gene expression profile similar to that seen during classical microbial macrophage activation. This contrasts with macrophages from subjects with CS asthma, found in most asthmatic subjects, which typically exhibit a pattern of gene expression characteristic of alternative macrophage activation. The genes activated, including cytokines and chemokines and molecules in the LPS/nuclear factor κ B signaling pathway (early growth response 1,^{11,13} molecule possessing ankyrin repeats induced by LPS,^{14,15} dual-specificity phosphatase 2, and TNF- α -induced protein 3¹⁶) indicate the potential exposure of inflammatory cells to LPS in the airways of subjects with CR asthma. Of interest, no arginase and mannose receptor C type 1, genes associated with alternative activation of macrophages, were detected in either group of asthmatic subjects. A recent article detected arginase activity/protein only in lung lysates and positive cells (macrophages) located deep in the tissue, suggesting that alternatively activated macrophages are not well lavagable.²⁸

Our current study detected the presence of high endotoxin levels in BAL fluid samples of subjects with CR asthma. The presence of LPS in BAL fluid was confirmed by 2 independent methods: the LAL assay and gas chromatography/mass spectrometry. Importantly, a significant correlation between the percentage of neutrophils in BAL samples and the amount of IL-8 mRNA expression by BAL cells, as well as between the amount of LPS in BAL fluid and IL-8 mRNA expression by BAL cells, was found, suggesting a functional relationship between LPS exposure and production of IL-8 by BAL macrophages and neutrophil infiltration.

Interestingly, the total number of white cells in BAL samples from subjects with CR asthma was significantly lower than in

subjects with CS asthma. There might be fewer macrophages in the CR group because the cells are more activated and adhesive and therefore resistant to lavage caused by LPS because endotoxin is known to stimulate monocyte adhesion.³⁵

Endotoxin, an LPS that comprises most of the outer layer of the bacterial membrane of gram-negative bacteria, is a potent immunomodulator capable of inducing activation of numerous immune cells and cytokine secretion. Exposure to environmental endotoxin is a leading cause of occupational asthma.³⁶ Higher exposure to house dust endotoxin has also been associated with increased asthma severity in several studies.³⁷⁻³⁹ The National Survey of Endotoxin in US Housing demonstrated significant relationships between household endotoxin and diagnosed asthma, recent asthma symptoms, current use of asthma medications, and wheezing.⁴⁰

Cellular responses to endotoxin are dependent on its membrane receptor, Toll-like receptor (TLR) 4, and are enhanced by CD14.⁴¹ Intracellular signaling mediated by endotoxin shares a common MyD88-dependent pathway with other TLRs and also has an MyD88-independent pathway that is particular to TLR4 and TLR4-mediated activation. Our *in vitro* experiments indicate that exposure of monocytes to LPS induces cellular steroid resistance. Further studies are needed to delineate the exact activation pathways involved in LPS-induced steroid resistance.

The cause of increased LPS in the airways of subjects with CR asthma is currently unknown. Bacterial colonization in the airways^{42,43} and environmental and occupational exposures^{44,45} should be further explored to assess their contribution to high endotoxin presence in the airways of subjects with CR asthma. A recent study by Bisgaard et al⁴² reported that neonates colonized in the hypopharyngeal region with *Streptococcus pneumoniae*, *Haemophilus influenzae*, or *Moraxella catarrhalis* or with a combination of these organisms are at risk for recurrent wheeze and

asthma early in life, suggesting that microbial colonization of the airways early in life might predispose to more severe asthma. In addition, several studies demonstrated anti-inflammatory and steroid-sparing effects of macrolides as add-on therapy for patients with refractory asthma.^{43,46-48} They had been shown to improve pulmonary function and asthma quality-of-life score and reduce noneosinophilic airway inflammation, particularly neutrophil inflammation, in subjects with asthma.^{47,48} Recently, Platts-Mills et al⁴⁴ showed that living with a dog, as compared with living with a cat, significantly increases exposure to airborne endotoxin, thus creating an environment for worsening asthma. Because dog ownership was reported to be associated with increased endotoxin levels in house dust,^{44,45} we have administered a questionnaire to subjects with CR asthma and subjects with CS asthma on dog ownership and allergic sensitivity to dogs. Using an established cohort of subjects with CR asthma versus subjects with CS asthma,^{49,50} we gathered preliminary data demonstrating a significantly increased prevalence of dog ownership among subjects with CR asthma.⁵¹ In this regard we found that 12 (66.6%) of 18 subjects with CR asthma lived with at least 1 dog at home compared with 6 (37.5%) of 16 subjects with CS asthma ($P \leq .05$). In addition, it was noted that dog allergy is more prevalent among subjects with CR asthma. To date, 13 of 18 subjects with CR asthma studied were sensitized to dog allergen versus 5 of 16 subjects with CS asthma ($P \leq .01$). No differences in cat exposures between the 2 groups were noted.

Thus understanding what conditions will lead to the presence of LPS in the airways will help to develop alternative approaches in the treatment of subjects with CR asthma.

We thank Maureen Sandoval for her help in preparing this manuscript. We also thank Brian Cranmer and Stephen Reynolds (Analytical Toxicology Laboratory, Department of Environmental and Radiological Health Sciences, Colorado State University, Fort Collins, Colo) for their assistance in the LPS detection in BAL fluid by means of gas chromatographic/electron ionization-mass spectrometric analysis.

Clinical implications: Classical macrophage activation and induction of LPS signaling pathways in BAL cells, along with high endotoxin levels in BAL fluid, were found in subjects with CR asthma, suggesting contribution of endotoxin exposure to corticosteroid resistance.

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METHODS

Gene expression profile analysis

In brief, total RNA (5 μ g) was converted into double-stranded cDNA by using the Superscript Choice System (Life Technologies Inc). An oligo-dT primer containing a T7 RNA polymerase promoter (Genset Corp, Paris, France) was used. After the second-strand synthesis, the reaction mixture was extracted with phenol-chloroform-isoamyl alcohol, and the double-stranded cDNA was recovered by means of ethanol precipitation. *In vitro* transcription was performed to generate biotin-labeled cRNA by using an RNA Transcript Labeling Kit (Enzo, Farmingdale, NY) and 3.3 μ L of the double-stranded cDNA template in the presence of a mixture of biotin-labeled ribonucleotides. Biotin-labeled cRNA was purified with an RNeasy affinity column (Qiagen, Valencia, Calif). The cRNA was fragmented into fragments between 35 and 200 bases in length by incubating the cRNA at 94°C for 35 minutes in a fragmentation buffer to ensure optimal hybridization to the oligonucleotide array. The samples were then added to a hybridization solution containing 100 mmol/L 2-[N-morpholino] ethanesulfonic acid, 1 mol/L NaCl, and 20 mmol/L EDTA in the presence of 0.01% Tween 20. The final concentration of the fragmented cRNA was 0.05 μ g/ μ L. The quality of total RNA and labeled cRNA was assessed with Agilent's Lab-on-a-Chip total RNA nano-biosizing assay (Agilent Technologies, Palo Alto, Calif).

Hybridization was performed by incubating 200 μ L of each sample to the Affymetrix GeneChip human arrays. The GeneChip Human Genome U133 Plus 2.0 (Affymetrix, Inc, Santa Clara, Calif) was used. Hybridization was carried at 45°C for 16 hours by using a GeneChip Hybridization Oven 640 (Affymetrix). After incubation, hybridization solutions were removed, and arrays were washed and stained with streptavidin-phycoerythrin by using a GeneChip Fluidics Station 400 (Affymetrix). The chips were scanned with the Affymetrix GeneChip Scanner 3000 (Affymetrix). The image data on each individual microarray chip was scaled to 500 target intensity by using the Microarray Suite software (Affymetrix).

To capture the differences between BAL samples from subjects with CR asthma versus subjects with CS asthma, we have carried out gene expression profiling studies by using Affymetrix U133+2.0 gene microarray analysis, which examined 54,675 targets, 24,568 of which were unique gene probe sets and 9018 of which were expressed sequence tags. 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, the degree of differential expression representing biologically important changes in asthma is unknown. Therefore as a means for reducing the false-positive rate, we selected genes with fold changes greater than 3 between subjects with CR asthma and subjects with CS asthma as candidates for further study.

The GeneChip Suite version 5.0 program (Affymetrix) was used to determine the intensity of expression for all genes on the microarray and provide comparison between chips. Genes called present or marginal in the majority of patients in each group were sorted into a preliminary list and then sorted into genes that are significantly different between the CR and CS asthma groups with a *P* value of less than .05 and then sorted by means of fold change. The difference between CR asthma and CS asthma samples were defined as significant if there was at least a 3-fold difference in average signal intensity with a *P* value of less than .05 by using a 2-tailed unpaired *t* test.

Gene array data were analyzed with Robust Multichip Average and MAS 5.0 processing algorithms in GeneSpring 3.2f to identify genes that are differentially expressed and to perform cluster analysis. MAS 5.0 uses the original Affymetrix method, which compares perfect-match data with mismatch data values to assign a present, absent, or marginal call to each gene, as well as a normalized signal value. Robust Multichip Average uses only the perfect-

match information to background correction and normalizes and summarizes it in a set of expression measures. Robust Multichip Average is comprised of 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 are 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.

Gas chromatographic/electron ionization–mass spectrometric analysis of BAL fluid

BAL fluid samples and external 3-hydroxy fatty acids standards of 8 to 18 carbon chain lengths (except C13:0, method surrogate) were digested and methylated with 0.5 mL of 3 mol/L methanolic HCl (2.5 mL of acetyl chloride added to 11 mL of methanol) for 16 to 18 hours at 80°C and cooled to room temperature. Samples and standards were amended with 10 μ L of pentadecanol (100 μ L/mL in heptane) as a keeper solvent and diluted with 1 mL of deionized water for solid-phase extraction cleanup. For solid-phase extraction cleanup, samples were applied to a 60 mg/3 mL Strata-X polymeric reversed phase cartridge. Cartridges were conditioned with 1 mL of diethyl ether and 1 mL of deionized water before sample loading. After 20 minutes of aspiration, 3-hydroxy fatty acid methyl esters were eluted with 2 mL of diethyl ether and evaporated to dryness with a nitrogen stream. Samples were converted to trimethylsilyl analogs for gas chromatographic/electron ionization–mass spectrometric analysis by adding 50 μ L N,O-bis-trimethylsilyl-trifluoroacetamide and 5 μ L of pyridine and heating for 20 minutes at 80°C. Derivatized samples and standards were diluted with 50 μ L of heptane, and a 2- μ L aliquot of each was analyzed by means of gas chromatography/electron ionization–mass spectrometry with an HP 5890 Series II Plus GC equipped with HP-5MS column (0.25 mm $\times$ 30 m, 0.25- μ m film thickness; Hewlett-Packard, Palo Alto, Calif) with split/splitless inlet, electronic pressure control, 7673 automatic liquid sampler, and an HP 5972 Mass Selective Detector. Selected ion monitoring for individual 3-hydroxy fatty acids was used for endotoxins, and the concentration was calculated in picomoles. For each 3-hydroxyfatty acid, monitored ions were as follows: C8:0, *m/z* 175 and 231; C9:0, *m/z* 175 and 245; C10:0, *m/z* 175 and 259; C11:0, *m/z* 175 and 273; C12:0, *m/z* 175 and 287; C13:0, *m/z* 175 and 301; C14:0, *m/z* 175 and 315; C15:0, *m/z* 175 and 329; C16:0, *m/z* 175 and 343; C17:0, *m/z* 175 and 357; and C18:0, *m/z* 175 and 371. Selected ions represented the parent ion and *m/z* 175, the acid portion of the fatty acid cleaved between C3 and C4. Ion ratios were monitored to identify interference from the 2-hydroxy fatty acids, which have the same parent ion as the corresponding 3-hydroxy fatty acids but lack the *m/z* 175 fragment.

The 3-hydroxy fatty acids of C8:0 to C18:0, except C13:0, were processed identically to samples instead of introducing 3-hydroxy fatty acid methyl esters only at the final derivatization step. The 3-hydroxy fatty acid of C13:0 was added as surrogate to each sample before digestion and methylation. Spike levels of 2, 6, 20, 100, and 500 ng of individual 3-hydroxy fatty acids (in ethanol, stored at -20°C) were used for creating the calibration curve to account for a wide range of endotoxin concentration levels.

Limit of detection and limit of quantitation were determined on the basis of signal-to-noise ratios based on the chromatograms of controls and 0.5-ng spikes (signal-to-noise ratio >2 for limit of detection and >10 for limit of quantitation). Coefficient of variation of C13:0 3-hydroxy fatty acid surrogate peaks on the chromatogram were calculated to test the accuracy and reproducibility of the modified method.

TABLE E1. Characteristics of BAL samples for asthmatic patients used for GeneArray studies

Parameter	Asthma	
	CR	CS
No. of subjects	10	8
Age, y (mean $\pm$ SD)	41.3 $\pm$ 3.0	36.1 $\pm$ 1.6
Sex (M/F)	3/7	5/3
Atopic subjects*	10	8
Active smokers	0	0
Prior smokers	1	1
Age of asthma onset, y (median [range])	18.5 (6-42)	9.0 (2-30)

Spirometry	FEV ₁ % predicted (mean $\pm$ SEM)	Δ FEV ₁ % predicted compared with baseline (median [range])	FEV ₁ % predicted (mean $\pm$ SEM)	Δ FEV ₁ % predicted compared with baseline (median [range])	P value	P value¶
Baseline	67.0 $\pm$ 2.7	NA	68.6 $\pm$ 2.7	NA	.68	
Baseline postbronchodilator (albuterol) response	74.0 $\pm$ 3.5	7.4 (4.2-29.1)	83.7 $\pm$ 5.1†	21.9 (8.8-44.3)	.108	.02
Response after 1 wk of oral prednisone treatment	65.8 $\pm$ 2.5	-0.6 (-16.3 to 11.4)	83.1 $\pm$ 2.8‡	21.8 (14.9-35.4)	.0002	.000001
Postbronchodilator (albuterol) response after 1 wk of oral prednisone treatment	72.6 $\pm$ 3.6	7.4 (-14.4 to 28.7)	89.4 $\pm$ 2.9§	31.7 (9.2-47.1)	.003	.003

Medications	Patients on medication	Dose per day#	Patients on medication	Dose per day#
ICS	4/10	Flovent, 352 μ g (1), 880 μ g (2); Q-var, 320 μ g (1)	3/8	Flovent, 352 μ g (1), 880 μ g (1); Q-var, 320 μ g (1)
ICS+LABA	3/10	Q-var, 640 μ g/Serevent, 100 μ g (1); Advair, 500 μ g/100 μ g (2)	1/8	Advair, 1000 μ g/100 μ g (1)
Systemic CS	0		0	

ICS, Inhaled corticosteroids; LABA, long-acting β -agonists; CS, corticosteroids.

*Patients were defined as atopic based on the degree of skin prick responses to the set of common allergens.

† $P < .05$; ‡ $P < .01$; and § $P < .0001$ compared with baseline FEV₁ percent predicted.

||FEV₁ percent predicted comparison between the CR and CS asthma groups.

¶ Δ FEV₁ percent predicted comparison between the CR and CS asthma groups.

#Number of patients using corresponding dose of medication is indicated in parentheses.

TABLE E2. BAL cell differentials for the samples from subjects with CR asthma and subjects with CS asthma used for the gene array study

Cell type	Asthma		P value
	CR group (n = 10)	CS group (n = 8)	
Total white blood cells, mean $\pm$ SEM	9.3 $\pm$ 1.7 $\times 10^6$	17.3 $\pm$ 1.2 $\times 10^6$	.002
Macrophages/monocytes (%), mean $\pm$ SEM	91.3 $\pm$ 3.9	93.2 $\pm$ 1.3	.68
Polymorphonuclear cells (%), mean $\pm$ SEM	3.7 $\pm$ 3.0	0.4 $\pm$ 0.1	.34
Lymphocytes (%), mean $\pm$ SEM	4.2 $\pm$ 1.4	5.2 $\pm$ 1.3	.60
Eosinophils (%), mean $\pm$ SEM	0.8 $\pm$ 0.2	2.0 $\pm$ 1.0	.17