

Subsets of airway myeloid-derived regulatory cells distinguish mild asthma from chronic obstructive pulmonary disease

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Background: Subsets of myeloid-derived regulatory cells (MDRCs), which are phenotypically similar to the myeloid-derived suppressor cells found in patients with cancer, have recently been appreciated as critical regulators of airway inflammation in mouse models of asthma.

Objective: We test the hypothesis that subsets of airway MDRCs contribute differentially to the inflammatory milieu in human asthma and chronic obstructive pulmonary disease (COPD).

Methods: We used bronchoalveolar lavage to identify and characterize human airway MDRCs from 10 healthy subjects, 9 patients with mild asthma, and 8 patients with COPD, none of whom were treated with inhaled or systemic corticosteroids. We defined subsets of airway MDRCs using flow cytometry, the molecular mediators they produce, and their abilities to regulate proliferation of polyclonally activated autologous T lymphocytes.

Results: We found substantial differences in the functional potential of MDRC subsets in healthy subjects, patients with asthma, and patients with COPD, with these differences regulated by the nitrosative and oxidative free radicals and cytokines they produced. Nitric oxide-producing MDRCs suppressed and superoxide-producing MDRCs enhanced proliferation of polyclonally activated autologous CD4 T cells. HLA-DR⁺CD11⁺CD11c⁺CD163⁻ superoxide-producing

MDRCs, which stimulated proliferation of autologous T cells, comprised a high fraction of MDRCs in the airways of patients with mild asthma or COPD but not those of healthy control subjects. CD11b⁺CD14⁺CD16⁻HLA-DR⁻ nitric oxide-producing MDRCs, which suppressed T-cell proliferation, were present in high numbers in airways of patients with mild asthma but not patients with COPD or healthy control subjects.

Conclusion: Subsets of airway MDRCs conclusively discriminate patients with mild asthma, patients with COPD, and healthy subjects from each other. The distinctive activities of these MDRCs in patients with asthma or COPD might provide novel targets for new therapeutics for these common disorders. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: Myeloid cell, macrophage, nitric oxide, superoxide, regulatory T cell

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of immature myeloid cells that inhibit lymphocyte function through a range of mechanisms. These include production of reactive oxygen species (ROS) and reactive

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Abbreviations used

BAL:	Bronchoalveolar lavage
BMI:	Body mass index
COPD:	Chronic obstructive pulmonary disease
DAF-FM-DA:	4-Amino-5-methylamino-2',7'-difluorofluorescein diacetate
DHE:	Dihydroxyethidium
DPI:	Diphenylethiodonium
FACS:	Fluorescence-activated cell sorting
iNOS:	Inducible nitric oxide synthase
MDRC:	Myeloid-derived regulatory cell
MDSC:	Myeloid-derived suppressor cell
NADPH:	Nicotinamide adenine dinucleotide phosphate
NK:	Natural killer
NO:	Nitric oxide
nor-NOHA:	N(omega)-hydroxy-nor-L-arginine
PCA:	Principal component analysis
PMA:	Phorbol 12-myristate 13-acetate
RNS:	Reactive nitrogen species
ROS:	Reactive oxygen species
SOD:	Superoxide dismutase
UAB:	University of Alabama at Birmingham

nitrogen species (RNS), which are generated by the inducible nitric oxide synthase (iNOS) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzymes, and depletion of key nutrients required for normal function of T cells, especially arginine through activation of arginase and tryptophan and cysteine through sequestration in tumor-specific T cells.¹⁻⁶ Additionally, activation of T cells can be impaired by nitration of their antigen or chemokine receptors⁷ or suppressed by induction of regulatory T cells through TGF- β produced by MDSCs.⁸

We and others have shown that the iNOS, NADPH oxidase, and arginase pathways are critical for the ability of these myeloid lineage cells to control T-cell responses.^{2,6,9-14} MDSCs are significant sources of nitric oxide (NO) and ROS in patients with cancer, as well as in those with other conditions characterized by chronic inflammation.^{2-4,9,10} In a mouse model of allergic airway inflammation, we demonstrated that distinct subsets of NO-producing anti-inflammatory MDSCs and O₂⁻-producing proinflammatory myeloid cells are major sources of free radicals and critical regulators of the inflammatory response.¹⁰ NO-producing myeloid cells suppressed airway hyperresponsiveness in mice through iNOS-derived NO, arguing for a protective function of NO in attenuation of the inflammatory response in asthmatic patients.¹⁰ Superoxide generated by a subpopulation of cells with phenotypic characteristics of MDSCs contributed to increased T-cell inflammatory responses and increased airway hyperresponsiveness in an NADPH oxidase-dependent fashion.¹⁰ We referred to these NO- and O₂⁻-producing cell subsets as myeloid-derived regulatory cells (MDRCs) because of their broad functions as both upregulators and downregulators of the inflammatory response. An imbalance in the ratio of these anti-inflammatory and proinflammatory myeloid cell subsets might contribute to many chronic airway inflammatory disorders.

Increased levels of RNS, including NO and its metabolites, and ROS, especially O₂⁻, are prevalent in human subjects with inflammatory disorders of the lung.¹⁵⁻¹⁸ In asthmatic patients

levels of NO produced by iNOS and levels of urea produced by arginase are correlated with the degree of inflammation and with clinical exacerbations.¹⁹⁻²² The NO synthase/arginase ratio can also contribute to bronchial tone in patients with chronic obstructive pulmonary disease (COPD).^{23,24} Although levels of exhaled NO are much lower in patients with stable COPD than in asthmatic patients, cross-talk between ROS and RNS and the role of RNS, particularly peroxynitrite, in the inflammatory mechanisms underlying COPD are well appreciated.^{22,25,26} Despite the fact that there might be differences in the inflammatory patterns and contributions of nitrosative and oxidative stress between patients with bronchial asthma and those with COPD, the iNOS, NADPH oxidase, and arginase pathways are likely to contribute to the inflammatory milieu in both of these common airway diseases.

We and others have shown that increased concentrations of the metabolites of iNOS are localized to the smaller distal airway in human asthmatic patients.^{15,27} This suggests that in these patients the primary cellular sources of iNOS-derived NO might be localized in the bronchiolar and/or alveolar compartments. In contrast, we found that ROS were present in both the proximal and distal airway compartments.¹⁵ We hypothesize that MDRC subsets contribute importantly to the inflammatory milieu in the airways of patients with asthma and those with COPD. We hypothesize further that the production of RNS and ROS by individual MDRC subsets contributes to their abilities to regulate the inflammatory and immune responses. Although peripheral blood MDSC subsets have recently been shown to be increased in asthmatic patients,^{28,29} the phenotypic and functional relationships of these cells in the airways of these patients have not been investigated.

In this study we have analyzed free radical-producing myeloid cells recovered by means of bronchoalveolar lavage (BAL) from 10 healthy subjects, 9 patients with mild asthma, and 8 patients with moderate COPD. We identified distinct subsets of NO- and O₂⁻-producing MDRCs in the airways of patients with asthma and those with COPD and showed that these cells can modulate T-cell responses through free radical-dependent mechanisms. Importantly, we found that the proportions of HLA-DR⁺CD11c⁺CD163⁻ O₂⁻-producing MDRCs were high in the airways of patients with mild asthma or COPD but low in healthy subjects and that proportions of CD11b⁺CD14⁺CD16⁻HLA-DR⁻ NO-producing MDRCs were high only in patients with mild asthma but not in patients with COPD or healthy control subjects. Although additional airway MDRC subsets with distinct free radical profiles and T cell-modulating functions were identified and showed some degree of association with the 3 clinical phenotypes studied here, the MDRC subsets described above contributed prominently to the ability to completely discriminate the asthmatic, COPD, and healthy populations we analyzed. The tight association of MDRC phenotypes with the discrete inflammatory phenotypes that characterize asthma and COPD suggests that MDRCs and their associated regulatory free radical and immunoregulatory cytokine mechanisms are central participants in these disorders. An improved understanding of the nature of these MDRC populations and their mechanisms of action might lead to identification of new therapeutic targets that focus on unique disease features in patients with asthma and those with COPD.

TABLE I. Characteristics of study subjects

Parameters	Group			P value*
	Healthy subjects (n = 10)	Asthmatic patients (n = 9)	Patients with COPD (n = 8)	
Age (y)	52.1 ± 9.4	51 ± 10.4	56.3 ± 7.9	.3752
Male/female sex	3/7	1/8§	7:1§	.0031
White/African American subjects	3/7	2/7	1/1	.5258
Never smoker	9:1†	6:3§	0:8‡§	.0002
Atopic/nonatopic	2/8†	7/2§	1/7§	.0079
Prebronchodilator FEV ₁ (% predicted)	89 ± 13‡	83 ± 26§	55 ± 15‡§	.0006
Postbronchodilator FEV ₁ (% predicted)	92 ± 12‡	89 ± 11§	56 ± 14‡§	.0007

Age and prebronchodilator and postbronchodilator FEV₁ are represented as means ± SDs.

*For continuous variables, *P* values were determined by using the Kruskal-Wallis test to assess whether the distributions differ among the study groups. For categorical variables, *P* values were determined by using an exact Pearson χ^2 test.

†Distributions differ between the healthy and asthmatic groups by using a Bonferroni-adjusted type I error rate of 0.0167.

‡Distributions differ between the healthy and COPD groups by using a Bonferroni-adjusted type I error rate of 0.0167.

§Distributions differ between the asthmatic and COPD groups by using a Bonferroni-adjusted type I error rate of 0.0167.

METHODS

Study design and patient recruitment

BAL fluid collected from asthmatic patients (n = 9), patients with COPD (n = 8), and healthy control subjects (n = 10) aged 25 to 65 years (Table I) were used for identification and purification of MDRC subpopulations, and venous blood was used for purification of PBMCs. In a previous publication¹⁵ we provided a preliminary characterization of a subgroup of 8 each of these healthy and asthmatic subjects, comparing the levels of metabolites of the NO and arginase pathways that were recovered in small-volume lavage specimens obtained from the large proximal airways with larger-volume specimens that sampled the more distal airway compartments. All subjects, including healthy subjects and patients with asthma or COPD, were recruited through the University of Alabama at Birmingham (UAB) Lung Health Center according to the exclusion and inclusion criteria described previously.¹⁵ Treatment with inhaled or systemic corticosteroids within the 6 weeks before or during the study was an absolute exclusion criterion.

Control subjects had no history of lung disease and had normal spirometric results. Asthmatic patients carried a clinical diagnosis and had mild disease as defined by Global Initiative for Asthma guidelines (Global Strategy for Asthma Management and Prevention, <http://www.ginasthma.org/>). Seven had a clinical diagnosis of atopy, but none had peripheral blood hypereosinophilia. None required treatment with oral or inhaled corticosteroids to maintain symptom control. All patients with COPD had an established history of COPD, 6 of 8 with moderate airflow obstruction (50% ≤ postbronchodilator FEV₁ ≤ 80% of predicted value) and 2 with severe airflow obstruction (FEV₁ = 33% and 39% of predicted value). Patients with COPD were excluded if they had previous lung surgery, respiratory disorders other than COPD (including mixed asthma and COPD), or a COPD exacerbation (defined as worsening cough, sputum, or dyspnea that required treatment with systemic corticosteroids, antibiotics, or an emergency department visit or hospitalization) in the 6 weeks before the study. This study was approved by the Institutional Review Board at UAB (protocol no. F090116008), and written informed consent was obtained from all participants.

Statistical analysis

Analyses began with calculation of measures of central tendencies as assessed by sample means and medians and measures of dispersion as assessed by sample variances and interquartile ranges for the number and proportion of cells in each subset. To compare the demographics (sex and race) or clinical characteristics (atopic/nonatopic and smoking history) measured as categorical variables among groups, exact *P* values for the χ^2 analyses, instead of asymptotic approximations, were calculated to address small sample sizes. Because of the relatively small number of subjects in each of the healthy, asthmatic, and COPD groups, we used the Kruskal-Wallis procedure, which does not assume normality, to test whether the distributions of cell types differed by clinical disease group. The Kruskal-Wallis procedure was also used to compare distributions of continuous characteristics (age,

prebronchodilator/postbronchodilator FEV₁, and body mass index [BMI]) among groups. Pairwise comparisons were conducted by using the Wilcoxon rank sum procedure³⁰ with the Bonferroni correction. The principal components technique was used to calculate the first 2 principal components to develop 2-dimensional graphs based on numbers of cell types and proportions. By using these 2 principal components, scatter plots were developed to visualize the clustering of healthy subjects, asthmatic patients, and patients with COPD. The first 2 principal components were used in a discriminate analysis to determine to what degree the information could accurately predict the study groups. For analyses of all other parameters, including BAL volume, total cell numbers, cytokine profiles, and T-cell proliferation, the Kruskal-Wallis procedure was performed. All procedures were performed with SAS 9.3 (SAS Institute, Cary, NC) and GraphPad Prism (GraphPad Software, La Jolla, Calif) software.

RESULTS

Multiple subsets of airway MDRCs can be recovered from patients with asthma or COPD

The characteristics of the human subjects studied here are described in Table I. None of the study subjects were undergoing treatment with inhaled or systemic corticosteroids at the time of or at any time during the 6 months before their participation in this study. Statistically significant differences existed among the groups with regard to sex, history of remote smoking, atopic/nonatopic status, and prebronchodilator/postbronchodilator FEV₁. The patients with mild asthma were predominantly female (8/9), and the patients with COPD were predominantly male (7/8). The effect of sex on the biology of free radical-producing myeloid cells or MDRCs has not been studied systematically. Feng et al³¹ observed no sex-based differences in the numbers of MDSCs in 44 patients with renal carcinoma. Studies with much larger sample sizes will be required to evaluate whether sex affects the phenotype and function of MDRCs in patients with inflammatory diseases of the airway. As anticipated, all patients with COPD had a past history of cigarette smoking. Additionally, a self-reported history of atopy was more common in asthmatic patients compared with patients with COPD. Patients with COPD showed reduced levels of prebronchodilator and postbronchodilator FEV₁, differing from both healthy subjects and asthmatic patients. This is consistent with the patients with COPD carrying the designation of moderate-to-severe disease. The absence of significantly reduced prebronchodilator FEV₁ measurements in our asthmatic population in association with the absence of treatment with inhaled corticosteroids defines this group as having mild asthma. Obesity was prevalent in all 3 of the study

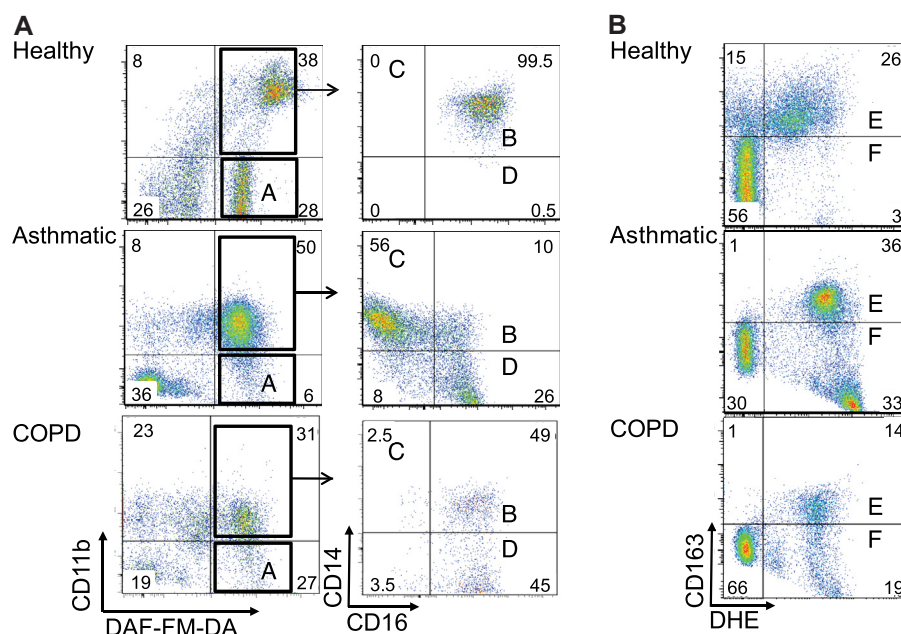


FIG 1. Different profiles of free radical-producing subsets of MDRcs are recovered from the airways of healthy subjects and patients with asthma or COPD. Cells obtained by means of BAL were stained with fluorescently labeled antibodies and with the free radical-reactive fluorescent indicator dyes DAF-FM-DA or DHE before analysis by means of flow cytometry. **A**, FACS plots of MDRcs recovered from BAL fluid demonstrating that the NO-producing CD11b⁺DAF-FM-DA⁺ population can be fractionated into 3 subsets based on differential expression of CD14 and CD16. These subsets are labeled **B** (CD11b⁺DAF-FM-DA⁺CD14⁺CD16⁺), **C** (CD11b⁺DAF-FM-DA⁺CD14⁺CD16⁻), and **D** (CD11b⁺DAF-FM-DA⁺CD14⁻CD16⁺). The CD11b⁻DAF-FM-DA⁺ population is designated **A**. **B**, ROS-producing MDRcs identified by staining with DHE can be fractionated into CD163⁺ subsets (labeled **E**) and CD163⁻ subsets (labeled **F**).

groups (data not shown). Although there was a trend toward higher BMI in asthmatic patients, the differences in BMI among the groups did not reach statistical significance. BMI was 32.3 ± 4.9 kg/m² for healthy subjects, 39.9 ± 12.9 kg/m² for patients with mild asthma, and 30.5 ± 12.1 kg/m² for patients with moderate-to-severe COPD ($P = .091$).

To investigate the characteristics of myeloid cells that might modulate inflammatory responses in the airways, we recovered airway leukocytes by means of BAL, analyzed them by using flow cytometry, purified selected subsets by means of cell sorting, and analyzed cellular functions with ELISA and assays of cell proliferation. First, we used the fluorescent probe 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM-DA) and a selection of cell-surface markers to identify NO-producing MDRcs in cells recovered by using BAL.¹⁰ When BAL leukocytes from healthy subjects were stained with the NO indicator dye DAF-FM-DA and the myeloid cell marker CD11b, two DAF-FM-DA⁺ populations were observed (Fig 1, A), one that was CD11b⁻ and one that was CD11b⁺. The CD11b⁻ population was also CD14⁻CD16⁻ (data not shown) and was designated population A. Both the proportions (Fig 1, A, and Table II) and absolute numbers of population A cells (Fig 2) differed between healthy subjects and patients with asthma or COPD. There were also substantial differences in the absolute numbers of CD11b⁺DAF-FM-DA⁺ cells among the 3 study groups (Table II).

The CD11b⁺DAF-FM-DA⁺ population could be further subdivided based on expression of CD14 and CD16. In BAL fluid from healthy subjects, there was a prominent population of CD11b⁺DAF-FM-DA⁺CD14⁺CD16⁺ cells (designated population B). Both populations A and B were CD11c⁻HLA-DR⁻ (see

Fig E1 in this article's [Online Repository](http://www.jacionline.org) at www.jacionline.org). Reductions in the percentages of population B cells were observed in BAL samples from asthmatic patients and patients with COPD compared with values in healthy control subjects (Fig 1, A, and Table II), whereas reductions in the absolute numbers of population B cells were observed only in patients with COPD (Fig 2).

CD11b⁺DAF-FM-DA⁺ cells that were CD14⁺CD16⁻ (Fig 1, A, and Table II) and CD11c⁻HLA-DR⁻ (see Fig E1) were designated population C, and their numbers were increased in asthmatic patients compared with healthy subjects (26-fold in proportions and 68-fold in absolute numbers) and decreased in patients with COPD (7.6-fold in proportions and 34-fold in total numbers compared with asthmatic patients). In asthmatic patients population C was the predominant DAF-FM-DA⁺ MDRc population (Figs 1, A, and 2 and Table II). The CD11b⁺DAF-FM-DA⁺CD14⁻CD16⁺ cells designated as population D (Fig 1, A, and Table II) were also CD11c⁻HLA-DR⁻ (see Fig E1). Numbers of population D cells were low in healthy subjects compared with those in asthmatic patients or patients with COPD (Figs 1, A, and 2 and Table II; 24- and 30-fold difference in percentages and absolute numbers, respectively, between healthy subjects and asthmatic patients and 23- and 16-fold differences in percentages and absolute numbers between healthy subjects and patients with COPD).

We next used the fluorescent dye dihydroxyethidium (DHE), as previously reported for murine cells,¹⁰ to identify superoxide-producing airway MDRcs. We detected 2 distinct subsets of CD11b⁺CD11c⁺DHE⁺ cells that could be distinguished by their expression of cell-surface CD163, a scavenger receptor for hemoglobin. The CD11b⁺CD11c⁺DHE⁺ cells that were

TABLE II. Proportions of MDRCs

Cell population	Group			P value*
	Healthy subjects (n = 10)	Asthmatic patients (n = 9)	Patients with COPD (n = 8)	
(A) CD11b ⁺ DAF-FM-DA ⁺	14.8 ± 12.5 [†]	3.1 ± 1.3 [†]	7.0 ± 5.8	.0267
CD11b ⁺ DAF-FM-DA ⁺	29.0 ± 13.9	42.1 ± 11.0 [§]	24.6 ± 11.9 [§]	.0211
(B) CD14 ⁺ CD16 ⁺	91.3 ± 6.0 ^{†‡}	29.8 ± 20.7 [†]	35.2 ± 25.4 [†]	.0001
(C) CD14 ⁺ CD16 ⁺	1.5 ± 0.8 ^{†‡}	40.0 ± 23.0 ^{†§}	5.2 ± 3.7 ^{†§}	.0001
(D) CD14 ⁺ CD16 ⁺	1.6 ± 1.7 ^{†‡}	22.6 ± 16.5 [†]	37.3 ± 15.5 [†]	.0001
(E) CD163 ⁺ DHE ⁺ CD11b ⁺ CD11c ⁺ HLA-DR ⁺	17.7 ± 6.8 [†]	31.9 ± 11.5 [†]	18.1 ± 12.5	.0243
(F) CD163 ⁺ DHE ⁺ CD11b ⁺ CD11c ⁺ HLA-DR ⁺	3.9 ± 3.5 ^{†‡}	22.4 ± 14.4 [†]	19.2 ± 15.6 [†]	.0008

Populations A to F are subsets described in Figs 1, A and B, and 2. Populations B to D are proportions of CD11b⁺DAF-FM-DA⁺ cells. Values shown represent proportions of the individual cell populations in the total leukocytes recovered by means of BAL (± SD).

*P values were determined by using the Kruskal-Wallis test to assess whether the distributions differ among the study groups.

[†]Distributions differ between the healthy and asthmatic groups by using a Bonferroni-adjusted type I error rate of 0.0167.

[‡]Distributions differ between the healthy and COPD groups by using a Bonferroni-adjusted type I error rate of 0.0167.

[§]Distributions differ between the asthmatic and COPD groups by using a Bonferroni-adjusted type I error rate of 0.0167.

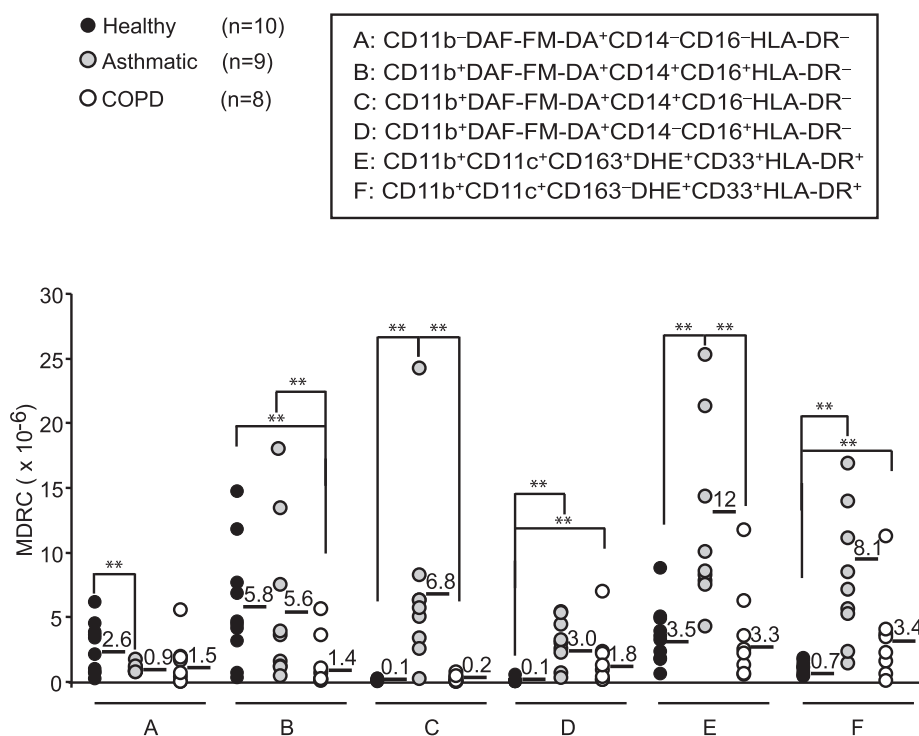


FIG 2. Absolute numbers of the different MDRC subsets recovered from the airways of patients with asthma or COPD compared with healthy control subjects. BAL cells were stained and analyzed as in Fig 1. MDRC populations were identified as A through F, as described in Fig 1, A and B. The absolute numbers of each of the MDRC subsets present in the total BAL fluids were calculated for each study subject as the product of total BAL cell numbers and the proportions of each. Open symbols, Patients with COPD; gray symbols, asthmatic patients; black symbols, healthy subjects. Numeric data represent pooled means. Population A: **P = .046 comparing healthy subjects versus asthmatic patients. Population B: **P = .024 comparing patients with COPD versus healthy subjects and asthmatic patients. Population C: **P < .001 comparing asthmatic patients versus patients with COPD and healthy subjects. Population D: **P < .001 comparing healthy subjects versus asthmatic patients and patients with COPD. Population E: **P = .0023 comparing asthmatic patients versus healthy subjects and patients with COPD. Population F: **P < .001 comparing asthmatic patients and patients with COPD versus healthy subjects.

CD163⁺ were designated population E, and those that were CD163⁺ were designated population F (Fig 1, B, and Table II). Both of these ROS-producing subsets were also CD33⁺ (data not shown) and HLA-DR⁺ (see Fig E2 in this article's Online

Repository at www.jacionline.org). In asthmatic patients population E showed a 1.8-fold increase in proportions and a 3.4-fold increase in absolute numbers compared with those in healthy subjects and patients with COPD (Figs 1, B, and 2 and

Table II). The proportions of population F cells were 5.7-fold greater in asthmatic patients and approximately 5-fold greater in patients with COPD compared with those seen in healthy subjects, whereas the absolute numbers were increased 11-fold in asthmatic and 4.7-fold in COPD samples.

To determine the nature of the cytokines and other mediators released by myeloid cells in each of these populations, we sorted the cells using fluorescence-activated cell sorting (FACS) with the same sets of fluorescently labeled antibodies, as well as DAF-FM-DA and DHE. We first assessed the morphology of cells in the sorted populations that were present in sufficient numbers to permit analysis by using Diff-Quik staining of cytospin preparations (see Fig E3 in this article's [Online Repository](#) at www.jacionline.org). Cells in populations A, B, C, and D showed variable cell sizes with varying degrees of nuclear condensation but no unique characteristics either between the populations in an individual study group or between the different study groups. Cells in populations E and F demonstrated more extensive vacuolization, which was perhaps even more extensive between the healthy subjects (who had lower amounts of vacuolization) and patients with asthma or COPD (with higher levels of vacuolization). Using sorted purified population B cells, we tested whether production of NO is iNOS dependent in these cells. After incubation with 1400W (N-(3-(Aminomethyl)benzyl)acetamide; Cayman Chemical, Ann Arbor, Mich), population B cells from a representative asthmatic patient showed ablation of DAF-FM-DA staining (see Fig E4 in this article's [Online Repository](#) at www.jacionline.org). Similar results were obtained with population B cells from all study cohorts (not shown). Population C cells from asthmatic patients produced the highest levels of nitrate and nitrite in supernatants from cultures of purified MDRCs compared with population A, B, and D cells (see Fig E5 in this article's [Online Repository](#) at www.jacionline.org). High levels of nitrate and nitrite were also detected in culture supernatants from the CD163⁺ population E, which produces ROS through NADPH oxidase, suggesting that a portion of the cells within population E could produce both NO and ROS. Stimulation of purified CD163⁺CD11b⁺CD11c⁺CD33⁺HLA-DR⁺ cells with phorbol 12-myristate 13-acetate (PMA) resulted in increased fluorescence with DHE (see Fig E6, A, in this article's [Online Repository](#) at www.jacionline.org), which was ablated by treatment with diphenyleneiodonium (DPI; an inhibitor of NADPH oxidase). The time-dependent increase in DPI-sensitive 2',7'-dichlorodihydrofluorescein diacetate cellular fluorescence (see Fig E6, B) confirmed that population E cells produced O₂⁻ through the NADPH oxidase complex. The supernatants from cultured BAL MDRC populations B and E from patients with COPD had reduced levels of nitrate compared with those in asthmatic patients or healthy subjects (see Fig E5). Production of nitrite by these populations was reduced only for MDRC population E. In contrast, the CD163⁻ ROS-producing MDRC population F secreted substantial levels of nitrate only in patients with COPD.

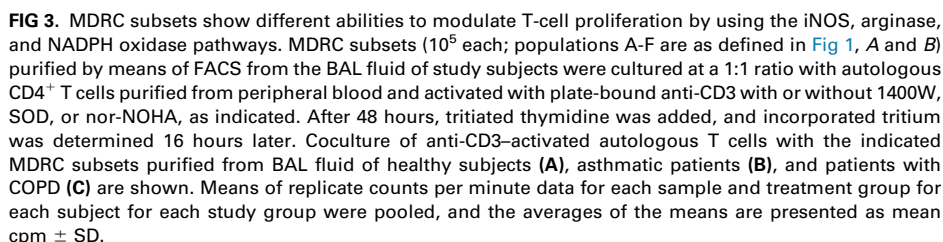
In the lung microenvironment, when L-arginine, which is required for T-cell receptor signaling, is depleted by activation of arginase, levels of O₂⁻ and other ROS can be increased by the uncoupling of NO synthase enzymes, including iNOS.³²⁻³⁴ We measured urea concentrations in culture supernatants of purified MDRC populations to assess arginase activity in these cells, finding that population E cells from patients with COPD showed a greater than 20-fold increase in urea concentrations

compared with the control group and an approximately 2-fold increase compared with population F from the same study group (see Fig E5). Both populations E and F purified from BAL leukocytes of asthmatic patients produced urea, indicating that these cells also expressed arginase. Altogether, these data showed that human airway MDRC populations purified from BAL leukocytes of healthy subjects, asthmatic patients, and patients with COPD possess unique signatures for activation of the iNOS, NADPH oxidase, and arginase pathways.

Analysis of a panel of signature cytokines and chemokines showed substantial heterogeneity among the MDRC subpopulations and between study groups (see Fig E7 in this article's [Online Repository](#) at www.jacionline.org). The T_H2 mediators IL-4, IL-13, and eotaxin were detected at significantly higher concentrations in culture supernatants from the DHE⁺ populations E and F purified from BAL fluid of both asthmatic patients and patients with COPD compared with healthy subjects. IL-4 and IL-13 levels were also significantly higher in population F from asthmatic patients compared with patients with COPD. In contrast, there were no significant differences noted in the levels of eotaxin comparing asthmatic patients or patients with COPD. The regulatory cytokines IL-10 and TGF- β , which are implicated in MDRC-mediated immunosuppression and the induction of regulatory T cells by MDRCs, were differentially expressed by populations A to F. IL-10 was produced predominantly in CD14⁺ cells of populations A, B, and C. In contrast, TGF- β showed a more promiscuous pattern, with both the NO- and ROS-producing populations C, E, and F secreting this cytokine. Levels of TGF- β produced by the MDRC subsets E and F in asthmatic patients and patients with COPD were increased compared with those in healthy subjects. In asthmatic patients the NO-producing MDRC populations B and C produced approximately 400-fold more IL-6 than their counterparts purified from samples from patients with COPD or healthy subjects or compared with ROS-producing MDRCs. Levels of IL-1 β , a proinflammatory signature cytokine for MDRCs that has been implicated in the pathogenesis of asthma,³⁵ were increased in BAL MDRC culture supernatants from asthmatic patients compared with those seen in patients with COPD and healthy subjects. IL-17 was produced by the ROS-producing populations E and F in all study groups at increased levels compared with those seen in the NO-producing MDRCs. Additionally, IL-7, which can activate eosinophils and drive eosinophilic inflammation,³⁶ was greater than 60-fold higher in culture supernatants from CD163⁺ MDRCs (population E) purified from asthmatic patients or patients with COPD compared with those from healthy subjects, with significantly higher levels produced by cells from asthmatic patients compared with patients with COPD.

Free radical pathways regulate heterogeneity in function of MDRCs in patients with chronic airway inflammation

During inflammation, production of RNS and ROS and arginase-mediated depletion of L-arginine by MDRC subsets are critical factors that affect T-cell responses.⁴ Sorted MDRC populations were cocultured with autologous CD4⁺ T cells in the presence of activating anti-CD3 antibody to define the regulatory behavior of the heterogeneous MDRC subsets in modulating the T-cell response. Population A cells did not suppress proliferation of polyclonally activated T cells



In contrast, population D cells from asthmatic patients or patients with COPD did not suppress proliferation when cocultured with autologous T cells (Fig 3, B and C). However, addition of the iNOS inhibitor 1400W or the O_2^- scavenger SOD to cocultures of population D cells from asthmatic patients and autologous T lymphocytes suppressed T-cell proliferation by 75% ($P = .0025$). This inhibition was not due to the toxicity of 1400W or SOD because the inhibitors by themselves did not affect T-cell proliferation (data not shown). In contrast, population D cells purified from patients with COPD did not modulate proliferation of autologous T cells in the presence of 1400W or SOD. Adequate numbers of population D cells were not recovered from healthy subjects to allow coculture studies. In an effort to delineate the roles of the ROS and arginase pathways in the function of MDRCs, the purified O_2^- -producing populations E and F (from asthmatic patients or patients with COPD only) were cocultured with autologous T cells in the presence or absence of inhibitors of these free radical pathways. MDRC population E increased T-cell proliferation (2-fold) in cocultures from all 3 study groups (healthy subjects, $P = .018$;

TABLE III. Summary of phenotypic and functional characteristics of MDRC populations

MDRC populations	Proportions of MDRCs			Modulation of T-cell response			Free radical pathway		
	Healthy subjects	Asthmatic patients	Patients with COPD	Healthy subjects	Asthmatic patients	Patients with COPD	Healthy subjects	Asthmatic patients	Patients with COPD
A	High	Low	Low	None	ND	ND	NA	NA	NA
B	High	High	Low	↓	↓	↓	iNOS	iNOS	iNOS
C	Low	High	Low	ND	↓	ND	NA	iNOS ROS	NA
D	Low	High	High	ND	None	None	NA	NA	NA
E	Low	High	Low	↑	↑	↑	ROS Arg	iNOS ROS Arg	ROS Arg
F	Low	High	High	ND	↑	↑	NA	iNOS ROS Arg	iNOS ROS Arg

Populations A to F are as described in [Figs 1, A and B, and 2](#).

Arg, Arginase; NA, not applicable; ND, not determined.

asthmatic patients, $P = .023$; patients with COPD, $P = .0034$; [Fig 3](#)). In cocultures from healthy subjects ([Fig 3, A](#)), this enhancement of T-cell proliferation was inhibited in a O_2^- - and arginase-dependent but iNOS-independent manner because SOD or the arginase inhibitor nor-NOHA, but not 1400W, ablated the increase in proliferation ($P = .002$).

Of interest, although population E cells from asthmatic patients, similar to those from healthy subjects, enhanced proliferation of polyclonally activated autologous T cells in a O_2^- - and arginase-dependent fashion ($P = .024$), addition of 1400W to these cocultures further enhanced T-cell proliferation ($P = .016$), suggesting that iNOS expressed in this population reduced the enhancement of T-cell proliferation ([Fig 3, B and C](#)). Population E cells from patients with COPD also enhanced T-cell proliferation in a O_2^- - and arginase-dependent fashion ($P = .0021$), but addition of 1400W did not show iNOS-dependent regulatory activity of these cells ($P = .69$).

The CD163⁺ population F from asthmatic patients or patients with COPD also enhanced polyclonal autologous T-cell proliferation ([Fig 3, B and C](#)). Although an arginase- and superoxide-dependent mechanism modulated this response in either asthmatic patients or patients with COPD (asthmatic patients, $P = .0032$; patients with COPD, $P = .0095$), there was also modest involvement of iNOS-derived NO in COPD samples because 1400W significantly reduced the increase in T-cell proliferative response ($P = .046$). The numbers of population F cells recovered from BAL fluid of healthy subjects were too low for coculture experiments.

Altogether, as summarized in [Table III](#), these data show that MDRCs can regulate T-cell proliferative responses by using free radical pathways; however, the distinct MDRC populations we have defined by means of analysis with a panel of well-defined cell-surface markers and the free radical-reactive dyes DAF-FM-DA and DHE are differentially regulated by the iNOS, O_2^- , and arginase pathways. Furthermore, the immunomodulatory mechanisms used by these cell populations varied depending on the patient population.

Alterations in leukocyte numbers in BAL fluid from asthmatic patients and patients with COPD

MDRC numbers are increased in blood and tumor tissues in human subjects, in mouse models of cancer, and in peripheral

blood of patients with chronic inflammation.^{2,3,5,8,37-39} Recently, we identified in the lungs of mice with experimental asthma subsets of MDRCs whose numbers and function were regulated by the iNOS and NADPH oxidase pathways.¹⁰ Our data suggested that the quantities of RNS and ROS might modulate the infiltration of MDRC subsets during chronic allergic inflammation. In the current study the total volumes of BAL fluid recovered from study subjects were not significantly different when comparing healthy subjects with asthmatic patients but were significantly greater in asthmatic patients compared with those in patients with COPD (see [Fig E8, B](#)). Total cell numbers recovered from pooled BAL samples were similar when comparing samples from healthy subjects and patients with COPD but were significantly higher when comparing asthmatic patients with healthy subjects and patients with COPD (see [Fig E9](#) in this article's [Online Repository at www.jacionline.org](#)). The percentages of eosinophils in BAL fluid were increased in asthmatic patients compared with those in patients with COPD but not the percentages of monocyte/macrophages, polymorphonuclear leukocytes, or lymphocytes (see [Fig E10](#) in this article's [Online Repository at www.jacionline.org](#)). We compared the absolute numbers and percentages of BAL fluid lymphocytes, natural killer (NK) cells, and invariant NK T cells, which have previously been linked with asthmatic airway inflammation (see [Fig E11](#) and [Table E1](#) in this article's [Online Repository at www.jacionline.org](#)).⁴⁰ The numbers of invariant NK T cells ($CD3^+CD4^+CD16^+CD56^+$) were higher in both the asthmatic and COPD groups compared with those in healthy subjects. The numbers of $CD3^+CD4^+CD8^+$ cells were increased only in the COPD group, whereas the $CD3^-CD4^-CD16^+CD56^+$ cell numbers were increased only in asthmatic patients. Significant differences were not noted among the study groups for the proportions ($P = .058$) or absolute numbers ($P = .96$) of $CD4^+CD3^+CD8^-$ T cells. The proportions of B lymphocytes were higher in patients with COPD compared with those seen in healthy subjects ($P = .011$), whereas the total numbers of B lymphocytes were not significantly different among the study groups ($P = .64$).

Frequencies of MDRC subsets discriminate the study groups

We used multivariate discriminant analysis of the proportions of MDRC subsets defined based on cell-surface phenotypes and

patterns of free radical production (Table II) or of the absolute numbers of cells in each of the subsets (Fig 2) to investigate whether the different MDRC subsets could discriminate and predict whether subjects belonged to the COPD, asthma, or healthy groups. With this group of samples, the proportions of the different MDRC subsets discriminated the study groups 100%. As shown in Table II, population A discriminated healthy subjects from asthmatic patients but did not discriminate healthy subjects or asthmatic patients from patients with COPD. The proportions of population B discriminated healthy subjects from both asthmatic patients and patients with COPD but not asthmatic patients from patients with COPD. Interestingly, analysis of absolute numbers of cells with the population B phenotype (Fig 2) discriminated patients with COPD from healthy subjects and also from asthmatic patients. Analysis of proportions of cells in population C distinguished all 3 groups from each other (Table II); however, based on absolute numbers (Fig 2), population C discriminated asthmatic patients from both healthy subjects and patients with COPD but did not distinguish healthy subjects from patients with COPD. Population D discriminated both asthmatic patients and patients with COPD from healthy subjects based on analysis of either proportions or absolute numbers. Based on proportions, population E discriminated healthy subjects and asthmatic patients, whereas analysis of absolute numbers discriminated asthmatic patients from both healthy subjects and patients with COPD. Based on absolute numbers (Fig 2), population F discriminated healthy subjects from asthmatic patients and patients with COPD based on either proportions or absolute numbers but did not discriminate asthmatic patients from patients with COPD.

Principal component analysis (PCA) transforms possibly correlated variables into a smaller number of uncorrelated variables called principal components and displays the data in a way that facilitates appreciation of sample variance. This analysis helps not only to determine how one sample is different from another but also to identify which variables contribute most to this difference and whether those variables contribute in the same way (ie, are correlated) or independently from each other. It also enables assessment of whether any particular grouping distinguishes among the samples. PCA using the frequencies of the MDRC populations and subpopulations we have defined identified the dimensions that summarized the most variance among the study groups (Fig 4). Weightings for each of the first 2 principal components are provided in Table E2 in this article's [Online Repository](#) at www.jacionline.org. The first 2 principal components accounted for 74% and the first 3 components accounted for 85% of the variability. Importantly, both linear discriminant and PCA analyses demonstrated that MDRC subsets by themselves provided the best discrimination between the study groups and predicted group status more efficiently than the frequencies of NK cells or lymphocytes (not shown). Additionally, grouping NK cells or lymphocyte populations with the MDRC subsets did not improve the ability to discriminate or predict group status (not shown).

DISCUSSION

This study has established a consistent association between respiratory disease phenotypes in human subjects and subpopulations of airway MDRCs by combining cell-surface phenotype, profile of molecular mediators produced, and ability to regulate proliferation of polyclonally activated CD4⁺ T cells.

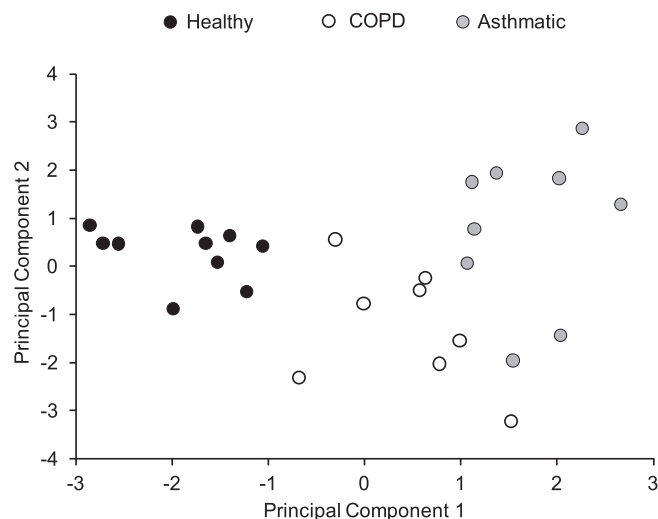


FIG 4. Multivariate analysis of MDRC subsets discriminates and predicts the group status of the healthy, asthmatic, and COPD study groups. A 2-dimensional PCA plot using the proportions of cells with surface phenotypes defined by means of flow cytometry, free radical production defined by means of DAF-FM-DA or DHE staining, and ability to regulate T-cell proliferation shows the variance in principal components 1 and 2 and separation of asthmatic patients (gray symbols) from patients with COPD (open symbols) and healthy subjects (black symbols).

To avoid the potentially confounding effects of treatment with either inhaled or systemic corticosteroids, we limited this study to patients with mild asthma who were receiving no corticosteroid medications and patients with moderately severe COPD who also had not received either inhaled or systemic steroids in the 6 weeks before entering our study. By comparing healthy subjects and patients with mild asthma or moderately severe COPD, we showed differences between the study groups in the cell-surface markers displayed on subsets of MDRCs^{3,37,41,42} and in the free radicals produced by them. Additional studies will be needed to test whether there are significant differences in the numbers and functional activities of MDRC subsets in the airways of patients with mild asthma compared with those with moderate and severe asthma and how treatment with inhaled or systemic corticosteroids might alter the MDRC repertoire.

Our results indicate that NO- and ROS-producing airway MDRC subsets recovered from lavage fluid from healthy subjects are distinctly different compared with those recovered from patients with asthma or COPD. We determined the proportions and absolute numbers of these subsets and established their production of free radicals by using fluorescent indicator dyes. The free radical-producing iNOS, arginase, and NADPH oxidase pathways, together with the regulatory cytokines IL-10 and TGF- β that govern important MDRC functions, determine the heterogeneity in immunoregulatory activities of the MDRC subsets. Our data indicate that phenotypic differences between MDRC subsets are able to predict with complete accuracy the clinical group status of the subjects in our study.

Differences in the profiles of cytokines produced by these MDRC subsets parallel the differences in ROS and RNS production. We observed a T_H2 and proinflammatory cytokine bias for the ROS-producing MDRCs and a regulatory cytokine profile for the NO-producing cells in both asthmatic patients and patients with COPD compared with healthy subjects. Interestingly, the production of IL-4 and IL-13 was more

increased in the O_2^- -producing HLA-DR⁺CD11c⁺CD163⁻ subset and the production of IL-7 was greater in the HLA-DR⁺CD11c⁺CD163⁺ subset from asthmatic patients than from patients with COPD, whereas similarly increased levels of eotaxin were produced by these cells from asthmatic patients and patients with COPD (see Fig E7). Thus there were important differences in the production of T_H2-associated cytokines in the O_2^- -producing cells from the different clinical groups.

These results are consistent with the observed differences in the percentage of eosinophils in BAL fluid from asthmatic patients compared with patients with COPD (see Fig E10). Although the numbers of eosinophils recovered in BAL fluid from asthmatic patients were significantly increased compared with those in fluid recovered from patients with COPD, increased numbers of peripheral blood eosinophils were detected in only a small fraction of subjects (1/10 healthy subjects, 0/9 asthmatic patients, and 2/8 patients with COPD). The absence of correlation between the numbers of peripheral blood eosinophils and airway eosinophils and the production of IL-7 by MDRCs underscores the likelihood that cytokines other than IL-7 and the products of other regulatory cells play important roles in determining airway and blood eosinophil numbers.

On the basis of experiments in which individual MDRC subsets were cocultured with polyclonally activated autologous CD4⁺ T cells, we observed that the CD163⁻ and CD163⁺ HLA-DR⁺CD11c⁺ O_2^- -producing subsets enhanced proliferation of T cells, with this proliferation-enhancing activity being dependent on the production of O_2^- by these MDRCs (Fig 2). These findings suggest that these MDRC subsets express proinflammatory or immune-enhancing activities. Thus it is significant that the numbers of both of these cell types are substantially increased in asthmatic patients (Fig 2). With these subsets, numbers of cells that were CD163⁻ were also increased in patients with COPD, whereas numbers of cells that were CD163⁺ were increased only in asthmatic patients. Perhaps significant in these differing associations is the production of high levels of IL-17 by the HLA-DR⁺CD11c⁺CD163⁻ subset (see Fig E7), likely contributing to the neutrophilic inflammatory response that is typically observed in the airways of patients with COPD. In contrast, the asthma-associated HLA-DR⁺CD11c⁺CD163⁺ subset produces low levels of IL-17 but high levels of IL-7, likely supporting the more T_H2-type inflammation that is often present in asthmatic patients.

Although O_2^- -producing MDRCs showed proinflammatory activities supporting enhanced proliferation of activated T cells, in almost all cases NO-producing MDRCs suppressed T-cell proliferation. NO-producing CD11b⁺CD14⁺CD16⁺ cells from all study groups suppressed T-cell proliferation in an NO-dependent fashion (Fig 3). The NO-producing CD11b⁺CD14⁺CD16⁻ subset that was present in substantial numbers only in the airways of asthmatic patients also suppressed T-cell proliferation in an NO-dependent fashion. These data have important implications. They establish clearly that NO produced by these subsets of MDRCs can downmodulate T-cell responses. This suggests that, in the context of inflammatory disorders, such as asthma and COPD, these cells are induced as part of the autoregulatory network to attempt to control the inflammatory response and restore homeostasis. In healthy subjects NO-producing MDRCs (primarily the CD11b⁺CD14⁺CD16⁺ and the CD11b⁻ subsets) might help establish the anti-inflammatory set point of the airways. Acknowledging that in

addition to myeloid cells other cell types contribute to total airway NO and specifically to exhaled NO levels, our data support a model in which the airway NO level is an indicator of an increased inflammatory state because the cells that produce the NO are elicited as part of the host response that aims to suppress the inflammation.

Interestingly, the CD11b⁺CD14⁻CD16⁺ subset that is present in almost undetectable numbers in the airways of healthy subjects when recovered from asthmatic patients suppressed T-cell proliferation but had no effect on T-cell proliferation when recovered from the airways of patients with COPD. Whereas the CD11b⁺CD14⁺CD16⁺ and CD11b⁺CD14⁺CD16⁻ populations produce substantial amounts of either IL-10, TGF- β , or both, the CD11b⁺CD14⁻CD16⁺ subset produces extremely low levels of these immunoregulatory cytokines and substantial amounts of IL-1 β , a cytokine with strong potential to enhance T-cell proliferation. These differences in production of immunoregulatory and proinflammatory cytokines might underlie the absence of T-cell suppressive activity when this subset is purified from patients with COPD. Additional currently unrecognized factors must contribute to the ability of this subset from asthmatic patients to suppress T-cell proliferation. It might also be possible that MDRC subsets that appear similar because they share cell-surface markers but that have distinct functional characteristics might be differentially activated forms of the same subset. Alternatively, they might be fundamentally different subsets differentiated along different pathways.

The highest capacity of MDRC subsets to discriminate between healthy subjects, asthmatic patients, and patients with COPD depended on consideration of all of the cell subsets that we identified; however, selected subsets were particularly useful in this regard. For example, the presence in BAL fluid of a high proportion of CD11b⁺CD14⁺CD16⁻ NO-producing cells was selectively and strongly associated with patients with mild asthma (Table II). In contrast, there was no single MDRC subset that was selectively associated with COPD; however, the presence of a high proportion of CD11b⁺CD14⁻CD16⁺ NO-producing cells together with a low proportion of airway CD11b⁺CD14⁺CD16⁻ NO-producing cells was highly associated with this disorder. Finally, the inflammatory subsets of HLA-DR⁺CD11b⁺CD11c⁺CD163⁻ and CD11b⁺CD14⁺CD16⁻ cells were both associated with either asthma or COPD, and when their proportions were low in BAL fluid, this was highly associated with the normal clinical phenotype.

Prior studies have demonstrated that other innate and adaptive immune cell types, including neutrophils, dendritic cells, and B and T lymphocytes, are associated with inflammatory diseases of the lung. Our analyses indicated that although the numbers and proportions of some of these cell types vary significantly in BAL fluid of asthmatic patients or patients with COPD, in PCA these cell types were not important predictors permitting classification of samples to the asthmatic, COPD, or healthy groups. This was in striking contrast to our observations that specific phenotypic and functional MDRC subsets were associated with these 2 common respiratory diseases. Consequently, we suggest that the phenotypic and functional differences in the MDRC subsets are integral to the ability of these populations to predict group status between healthy subjects and patients with asthma or COPD.

The discrete functional repertoires of the several MDRC phenotypes, together with the physical segregation of O_2^- -producing

and NO-producing MDRCs that we previously observed in proximal and distal airways of asthmatic patients,¹⁵ could indicate that diverse types of immune responses can occur at the same time in discrete tissue compartments, with locally active MDRC subsets providing regional immune regulation. Morphologic, phenotypic, and functional heterogeneity are accepted hallmarks of MDRCs, as is plasticity of the immunosuppressive myeloid compartment.⁴³ Notably, expression of myeloid cell-surface antigens, particularly CD11b, in selected MDRC subsets was less in asthmatic patients or patients with COPD compared with that seen in healthy subjects. It would be important to determine in future studies whether changes in the levels of expression of these myeloid differentiation and activation antigens correlate with changes in the functional properties of individual MDRC subsets or severity of respiratory inflammatory disease state. For the first time, our study describes that heterogeneous subsets of MDRCs differentiate 2 chronic inflammatory lung diseases. Our data are thus consistent with MDRCs contributing importantly to the differences in inflammatory reactions that characterize asthma and COPD.

Our studies also provide a conceptual framework for further study of the regional behavior of myeloid cells as they differentiate from immature cells in the bone marrow hematopoietic compartment into more mature cells in peripheral compartments with functions polarized into anti-inflammatory or proinflammatory regulatory populations. Investigating the mechanisms underlying the phenotypic differences of MDRCs will help elucidate whether the inflammatory milieu in the lung microenvironment is constantly modulating a pool of immature bone marrow cells to influence their maintenance and expansion during chronic inflammation, thereby supporting the formation of a central immunosuppressive core myeloid population plus discrete proinflammatory effector cells, or whether these different MDRC subpopulations originated from one major population of lung-resident myeloid cell precursors with common biological function, which was influenced by signals in the lung microenvironment to differentiate into phenotypically and functionally distinct subsets.

The fact that the proportions and numbers of MDRC subsets and their differing functional profiles discriminate patients with mild asthma from patients with COPD and both disease groups from healthy subjects suggests a critical role for these myeloid lineage cells in the pathogenesis of these inflammatory diseases of the lung. The data shown here lay the foundation for future studies to test the relationship between MDRC heterogeneity and the severity of chronic asthma and COPD. These results also provide strong evidence for the involvement of diverse MDRC subsets in regulation of the immune response. Furthermore, they underscore the need to evaluate MDRC subsets as biomarkers for monitoring the severity of airway inflammatory diseases and responses to anti-inflammatory therapeutics.

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Key messages

- Airway MDRCs can be recovered by BAL and characterized into distinct subsets, each with signature profiles based on cell-surface markers and secreted reactive free radicals and immunoregulatory cytokines.
- Superoxide-producing HLA-DR⁺CD11c⁺ MDRCs enhance the proliferation of polyclonally activated autologous T cells and express a proinflammatory signature of cytokines, whereas NO-producing CD11b⁺CD14⁺CD16⁺HLA-DR⁻ and CD11b⁺CD14⁺CD16⁻HLA-DR⁻ MDRCs suppress T-cell proliferation and express several anti-inflammatory cytokines.
- Airway HLA-DR⁺CD11c⁺CD163⁻ superoxide-producing cells were present as a small fraction of MDRCs in healthy subjects but as a substantial fraction in patients with mild asthma or COPD, whereas CD11b⁺CD14⁺CD16⁻HLA-DR⁻ NO-producing MDRCs were present in high numbers only in patients with mild asthma, permitting discrimination of the 3 clinical groups.

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METHODS

Collection of BAL samples and BAL cell differential

Airway samples were collected through fiberoptic bronchoscopy, as described previously.^{E1} The bronchoscope was wedged in a fourth- or fifth-generation airway, as is typical for traditional BAL. A low-volume bronchial wash was performed first before traditional BAL, as detailed in our earlier publication.^{E1} The cells recovered in the bronchial washes represented less than 3% of total cells obtained from pooled BAL samples. These were largely epithelial cells^{E1} and thus were not used in the current study. Then traditional BAL was performed by instilling 6 aliquots of 33 mL of warmed sterile saline, followed immediately by gentle aspiration of the lavage fluid. Aspirated airway cells and lavage fluids were separated by means of centrifugation. Cells from all the aliquots of BAL fluid from each subject were pooled for flow cytometric analysis, FACS, and BAL differential. Cell counts and BAL cell differentials were carried out, as described previously.^{E1}

Flow cytometric analysis and purification of MDRC subsets

Myeloid cells recovered from the airways by means of BAL were analyzed and sorted based on their expression of myeloid lineage-specific cell-surface antigens. The cell pellets obtained after centrifugation of pooled BAL cells were resuspended in RPMI-1640 medium containing 10% human AB Serum (negative for HBsAb, HIV1/2, and HCV; Mediatech, Herndon, Va) and supplemented with 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, 10 µg/mL penicillin-streptomycin, 25 µmol/L 2-β mercaptoethanol, and 0.1 mmol/L nonessential amino acids (Life Technologies, Gaithersburg, Md) and incubated for 20 minutes on ice. Then cells were stained with antibodies against CD11c (B-Ly6), CD33 (HIM3-4), HLA-DR (L243), CD11b (ICRF44), CD14 (M5E2), CD16 (3G8), CD56 (B159), CD4 (RPA-T4), CD3 (HIT3A), and CD8 (RPA-T8; all from BD Biosciences, San Jose, Calif), as well as CD163 (GHI/61; eBioscience, San Diego, Calif), for 30 minutes. Streptavidin-PE-Cy-7 (Southern Biotechnology, Birmingham, Ala) was used for the detection of cells stained with biotin-conjugated antibodies. Isotype control antibodies (BD Biosciences) and single-color compensation controls were used in all analyses. After staining with the indicated antibodies, cells were washed twice with PBS before analysis by using a Becton Dickinson LSR II with FACSDiva software (BD Biosciences). Data were further analyzed with FlowJo 7.6 software (Tree Star, Ashland, Ore). Cells were purified by sorting with a FACSARIA (Becton Dickinson). For identification and purification of MDRCs with the potential to produce NO, BAL cells were stained with DAF-FM-DA (fluorescent indicator for NO and its metabolites; Invitrogen, Carlsbad, Calif), as described previously,^{E1} together with the antibodies mentioned above. For identification of ROS⁺ cells, BAL cells were stained with DHE (a fluorescent indicator for ROS, Invitrogen), as described previously,^{E1} together with the antibodies mentioned above. Long-term viability of MDRCs was reduced after DHE staining. Therefore for purification of these subsets, cells stained with a cocktail of antibodies to CD11c, CD11b, HLA-DR, CD163, and CD33 were analyzed with and without DHE, and cell populations identified by means of comparison with DHE-stained samples were used to identify the ROS⁺ subsets. Once identified, the populations were aligned for forward- and side-scatter comparisons with DHE-stained samples for validation.

Measurement of total nitrite/nitrate production by MDRCs

BAL cells that were CD11b⁺DAF-FM-DA⁺ were analyzed further, and cell populations that were also either CD14⁺CD16⁺HLA-DR⁺CD11c⁺, CD14⁺CD16⁺HLA-DR⁺CD11c⁺, or CD14⁺CD16⁺HLA-DR⁺CD11c⁺ were purified by means of FACS, as described above. Purified MDRCs (10⁴) for selected subsets were cultured in serum-free RPMI 1640 media for 48 hours in a 96-well plate (control group; n = 7; asthmatic group, n = 8; COPD group, n = 6). Cell-culture supernatants were clarified by using

centrifugation to remove any cell debris. Total nitrate and nitrite levels were measured in these cell-culture supernatants in triplicate wells for each sample by using the Griess assay (Cayman Chemicals), according to the manufacturer's recommendations.

Detection of ROS in MDRCs

MDRCs were detected by means of flow cytometry after staining with myeloid cell-specific antibodies and incubation for 20 minutes at room temperature with DHE (10 µmol/L; Molecular Probes, Eugene, Ore).^{E2} The specificity of DHE for O₂⁻ was validated by inducing a respiratory burst in the sorted myeloid cells by means of incubation at 37°C for 15 minutes with PMA (1 µg/mL) or PMA plus the NADPH oxidase inhibitor DPI (1 µmol/L; Tocris Bioscience, Ellisville, Mo) added 15 minutes before PMA.^{E2} For detection of a time-dependent increase in ROS production of MDRCs, cells treated with PMA or PMA plus DPI were incubated with 2'-7'-dichlorodihydrofluorescein diacetate, and fluorescence intensity was used for ROS detection at 0, 15, 30, and 60 minutes after treatment by means of time-lapse confocal microscopy.^{E2} O₂⁻ production by MDRCs was determined based on O₂⁻-dependent reduction of cytochrome c (Sigma, St Louis, Mo).^{E2} Briefly, 4 × 10⁴ purified MDRCs were stimulated with PMA in RPMI 1640 plus 0.5% FBS, and the time course of reduction of cytochrome c (10 µmol/L) was monitored at a λ value of 550 nm and a M value of 21 mM⁻¹·cm⁻¹ with a spectrophotometer (UV-2501PC Shimadzu; Shimadzu, Kyoto, Japan). O₂⁻ produced by NADPH oxidase was determined based on the subtraction of the DPI (1 µmol/L)-sensitive rate of cytochrome c reduction from the rate determined in the absence of DPI.

Measurement of arginase activity

The concentration of urea, an end product of the arginase pathway, was used as an estimate of the arginase activity in the cell-culture supernatants harvested as described above from cultures of MDRC subsets (control group, n = 7; asthmatic group, n = 8; COPD group, n = 6). Detection of urea was carried out with the Quantichrom Urea Assay kit (DIUR-500; BioAssay Systems, Hayward, Calif). Fifty microliters of the cell-culture supernatant were analyzed in triplicate, and the concentration of urea was determined by comparison with a urea standard reference curve with absorbances at 520 nm as measured by using spectrophotometry.

Cytokine analysis

Purified BAL MDRC subsets (10⁴ cells each) from 7 control subjects, 6 asthmatic patients, and 6 patients with COPD were cultured in triplicate in serum-free RPMI media for 48 hours, and the culture supernatants harvested after centrifugation were used for cytokine analyses. For the detection of cytokines, duplicate measurements were made with the 42-plex MILLIPLEX MAP Human Cytokine/Chemokine Panel (Millipore, Billerica, Mass). Fluorescent signals were measured with a Luminex 100 laser (Bio-Rad Laboratories, Hercules, Calif) and compared with the internal standard curves established according to the manufacturer's recommendations.

Measurement of T cell-suppressive function by MDRCs

MDRC subsets were purified as described above by using FACS. Autologous CD4⁺ T cells were purified from 40 mL of peripheral blood with Human T Cell Enrichment Medium (Rosette Sep; STEMCELL Technologies, Vancouver, British Columbia, Canada) and density gradient centrifugation, with a yield of 25 × 10⁶ T cells with greater than 95% viability and greater than 94% purity. Subsets of purified MDRCs were then cocultured for 48 hours with naive CD4⁺ T cells in a 1:1 ratio in microtiter plates coated with activating anti-CD3 (OKT-3) mAb in complete R-10 medium (RPMI 1640 containing 2 mmol/L L-glutamine, 10 µg/mL penicillin-streptomycin,

25 $\mu\text{mol/L}$ 2-mercaptoethanol, 0.1 mmol/L nonessential amino acids; Life Technologies) and 10% AB serum, as described above. Tritiated thymidine (1 μCi per well) was added, and incorporation of tritium was determined 16 hours later. To determine the roles of iNOS, NADPH oxidase, O_2^- , and arginase as modulators of T-cell proliferation, 500 nmol/L 1400W, 1000 U/mL SOD (Sigma), or 20 $\mu\text{mol/L}$ nor-NOHA or 10 $\mu\text{mol/L}$ DPI was added at the start of the cultures.

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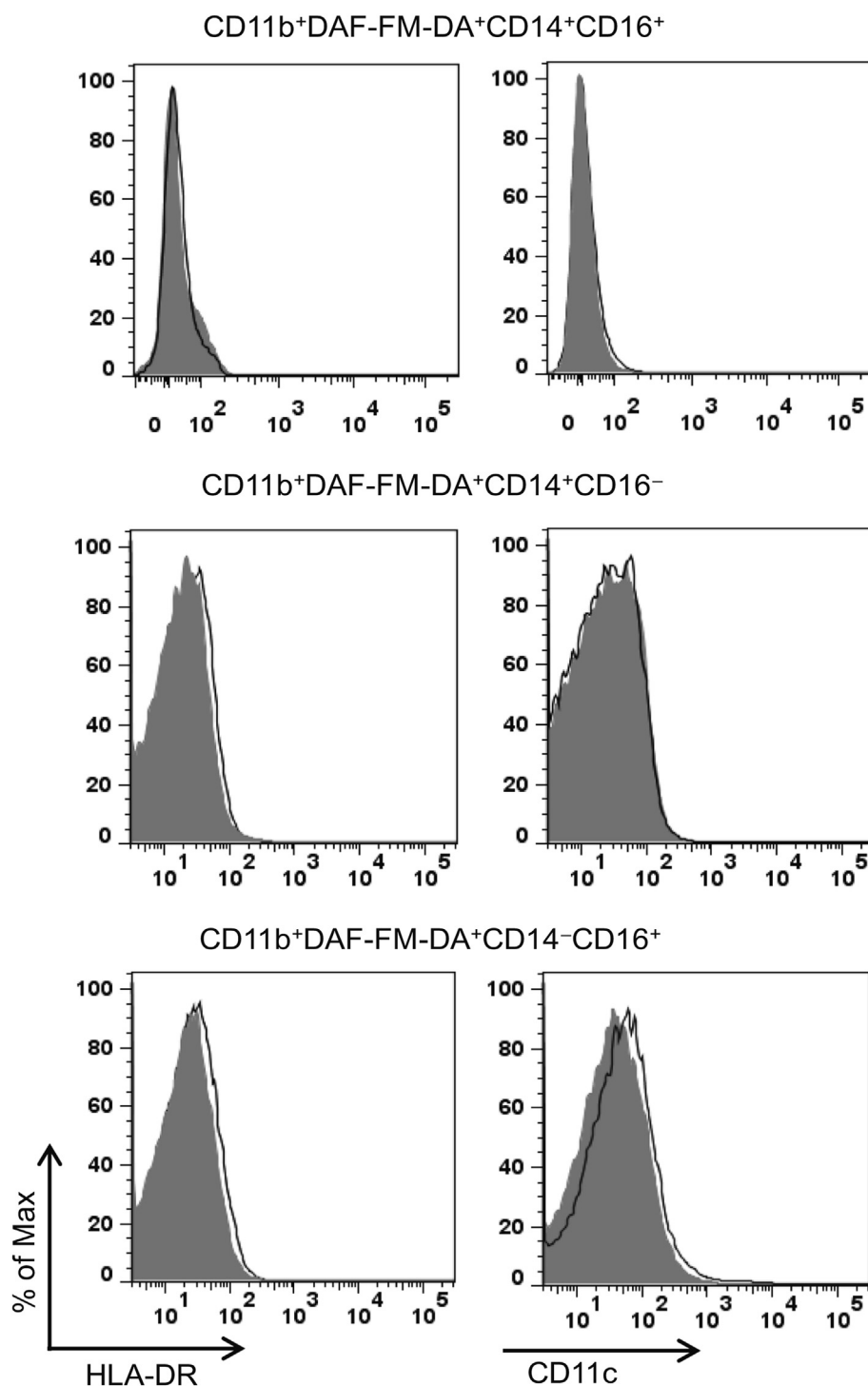


FIG E1. NO-producing airway MDRC subsets lack surface expression of CD11c and HLA-DR. Representative overlaid histogram plots of CD11b⁺DAF-FM-DA⁺ cells, which are CD14⁺CD16⁺ (*top panels*), CD14⁺CD16⁻ (*middle panels*), or CD14⁻CD16⁺ (*bottom panels*), isolated from BAL fluid from an asthmatic patient were stained with antibodies for HLA-DR and CD11c (*open tracings*) and compared with cells stained with isotype control antibodies (*shaded tracings*). Similar data were obtained for all subjects, including healthy subjects and patients with asthma or COPD.

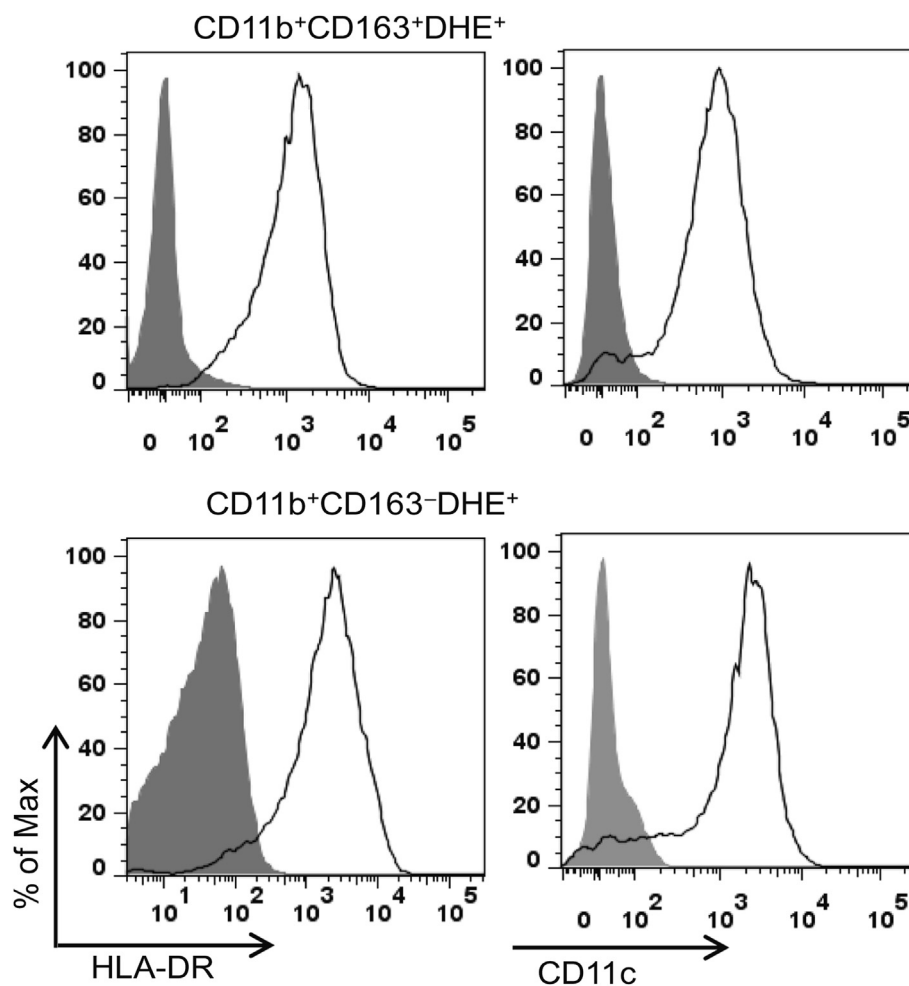


FIG E2. ROS-producing airway MDRC subsets express surface CD11c and HLA-DR. Representative overlaid histogram plots of CD11b⁺CD163⁺DHE⁺ and CD11b⁺CD163⁻DHE⁺ cells isolated from BAL fluid from an asthmatic patient and stained with antibodies for CD11c and HLA-DR (*open tracing*) compared with cells stained with isotype control antibodies (*shaded tracings*). Similar data were obtained for all patients with asthma or COPD. Numbers of CD11b⁺CD163⁻DHE⁺ cells were small in healthy subjects and were not analyzed further.

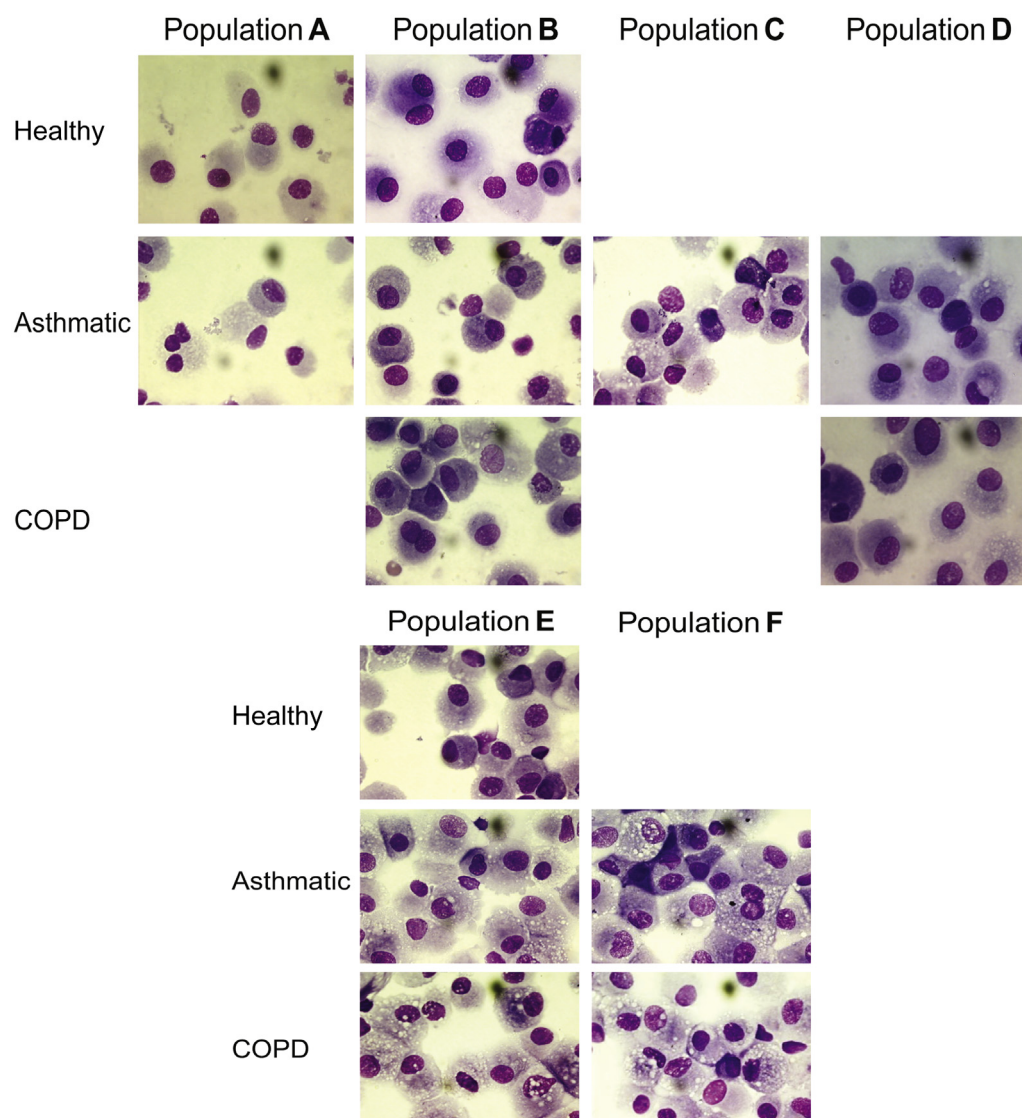
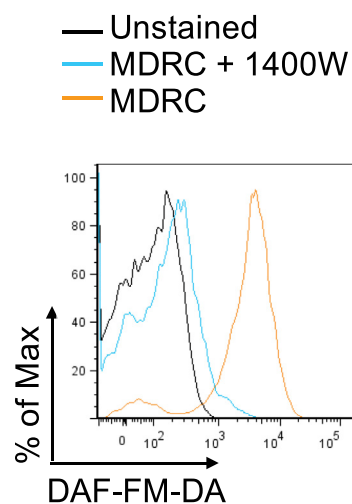


FIG E3. Cellular morphology of purified airway myeloid populations. BAL cells were sorted into distinct populations based on their expression of CD11b, CD11c, CD14, CD16, CD33, CD163, and HLA-DR and their staining with DAF-FM-DA or DHE, as specified in Fig 2. Cytospin preparations of individual populations were stained with Diff-Quik. Populations not shown were recovered in insufficient numbers for analysis. Data shown are representative of data from all study subjects.



CD11b⁺CD11c⁻CD14⁺CD16⁺CD33⁺HLA-DR⁻

FIG E4. Production of NO by MDRCs is dependent on iNOS. Representative overlaid histogram plots of CD11b⁺CD14⁺CD16⁺CD11c⁻HLA-DR⁻ cells purified by means of FACS from BAL fluid of an asthmatic patient and stained with DAF-FM-DA in the presence or absence of 500 nmol/L 1400W compared with an unstained control. Similar data were obtained by using the same MDRC subset purified from all healthy subjects, patients with COPD, and asthmatic patients.

■ Healthy ■ Asthmatic □ COPD

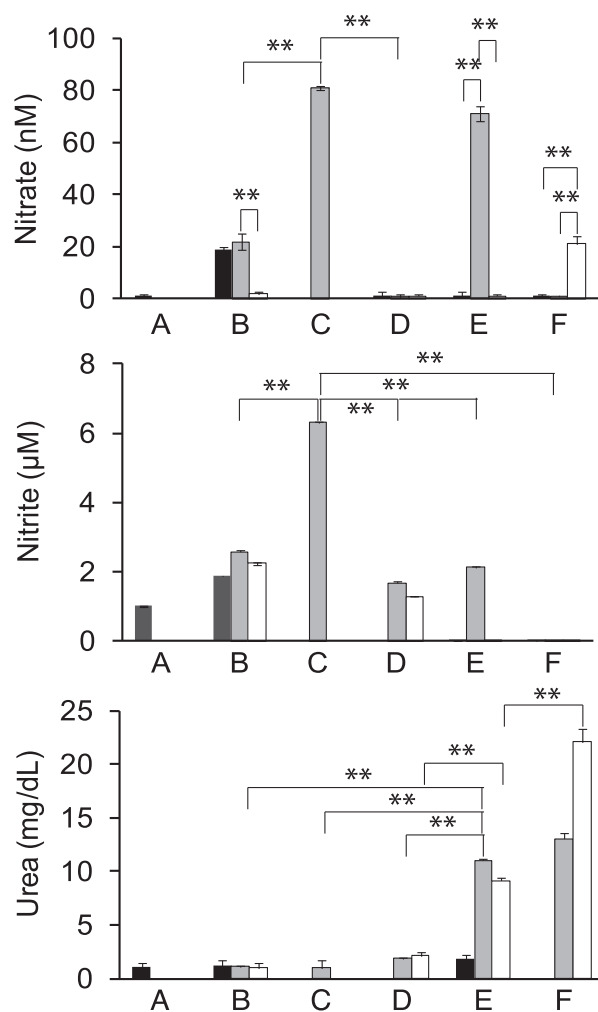


FIG E5. MDRC subsets show differential induction of iNOS and arginase activity. FACS-purified DAF-FM-DA⁺ (NO-producing) MDRC subsets A through D and DHE⁺ (ROS-producing) MDRC subsets E and F from healthy subjects and patients with asthma or COPD were cultured for 48 hours and analyzed for total levels of nitrate (*top panel*), nitrite (*middle panel*), and urea (*bottom panel*). ** $P < .001$.

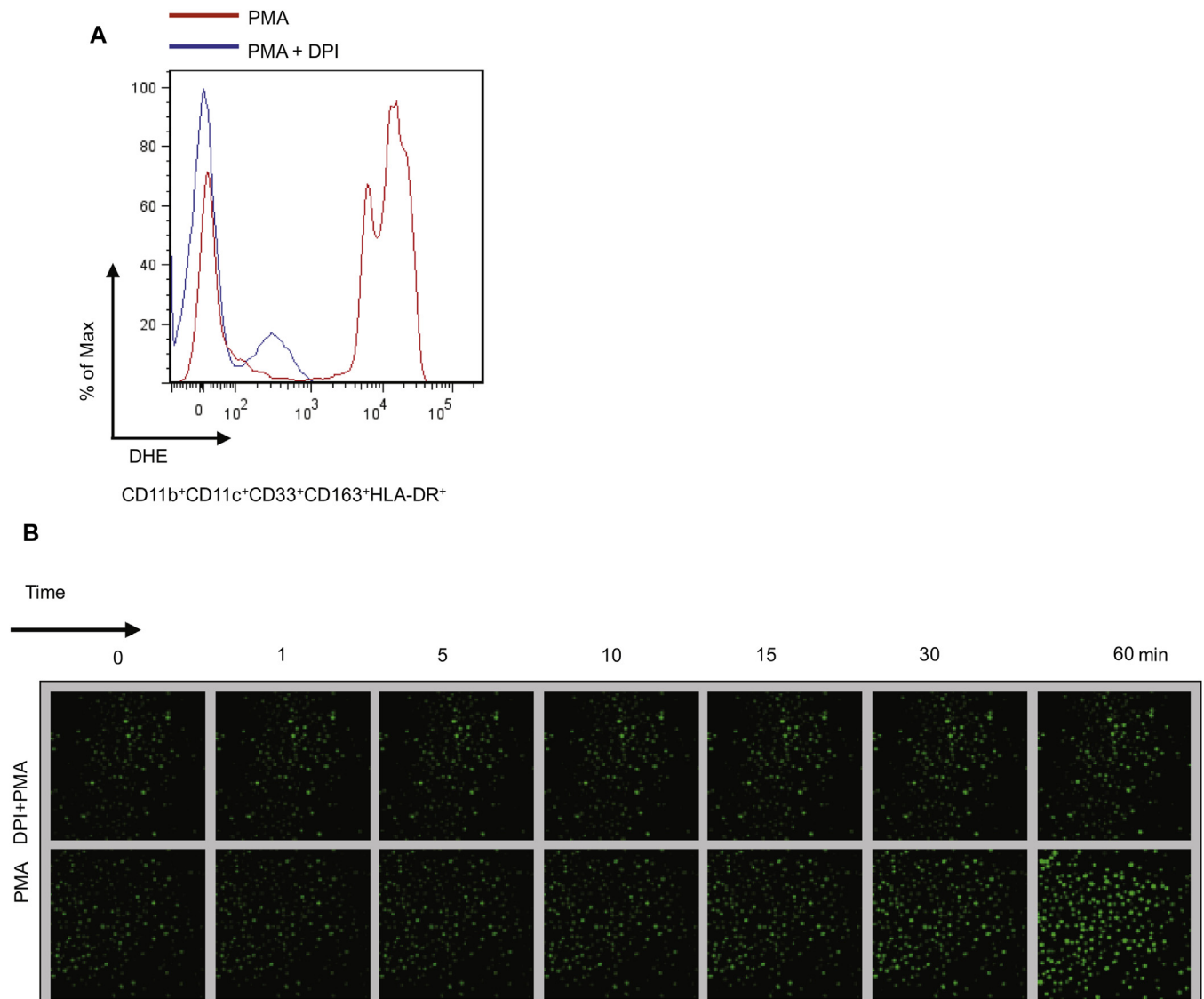


FIG E6. Production of ROS through NADPH oxidase in MDRC subsets. **A**, Representative overlaid histogram plots of CD11b⁺CD163⁺CD11c⁺HLA-DR⁺CD33⁺ cells purified by means of FACS from BAL fluid of an asthmatic patient, stained with DHE, and incubated at 37°C for 15 minutes with PMA (1 μ g/mL) or PMA plus the NADPH oxidase inhibitor DPI (1 μ mol/L). **B**, Representative time-lapse photomicrographs of CD11b⁺CD163⁺CD11c⁺HLA-DR⁺CD33⁺ cells purified by means of FACS from BAL fluid of an asthmatic patient, stained with DCF-DA, and incubated with PMA (1 μ g/mL) or PMA plus DPI (1 μ mol/L) for a period of 0 to 60 minutes before analysis by means of flow cytometry. Similar data were obtained with all healthy subjects, asthmatic patients, and patients with COPD.

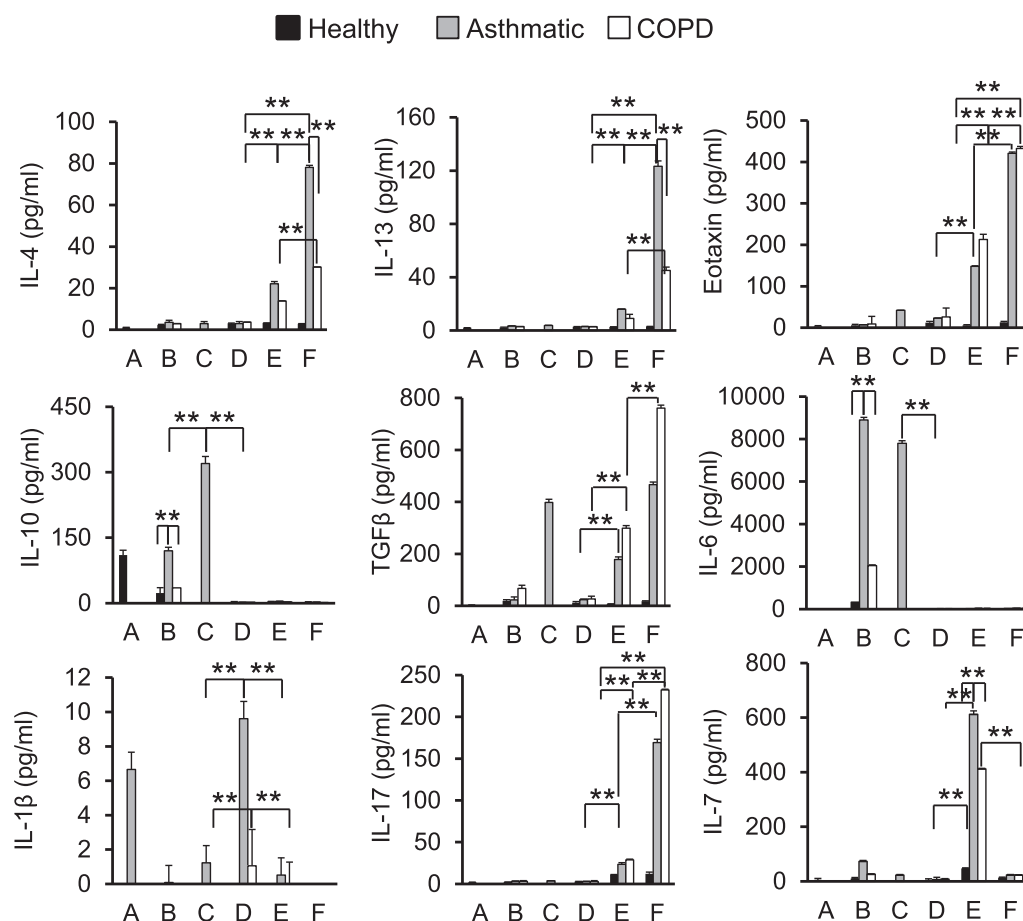


FIG E7. MDRC subsets show different multiplex cytokine profiles. FACS-purified NO-producing (subsets A-D) and ROS-producing (subsets E and F) MDRCs from healthy subjects and patients with asthma or COPD were cultured for 48 hours without additional stimulus, and then culture supernatants were evaluated by using multiplex analysis for total cytokine levels (in picograms per milliliter). Data shown are means from duplicate determinations. ** $P < .001$ for all comparisons.

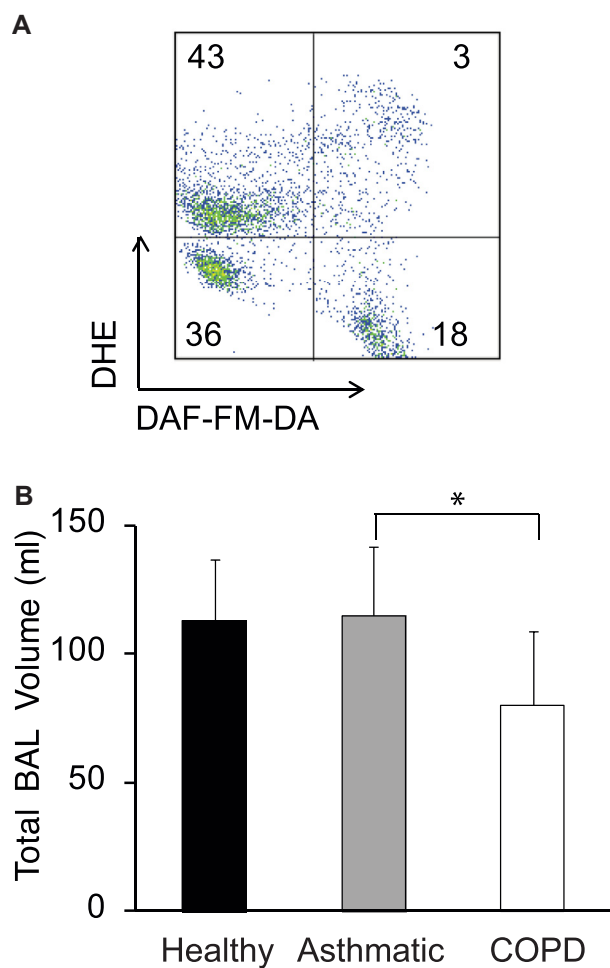


FIG E8. A, A portion of airway cells from asthmatic patients generate NO and ROS simultaneously. FACS plot of BAL cells from a study subject with asthma stained with DHE and DAF-FM-DA shows that 3% of cells are positive for both ROS and NO and its metabolites. Similar data were obtained for all other study subjects with asthma. **B,** Histograms showing comparisons of the total volume of BAL fluid recovered from healthy subjects and patients with asthma or COPD. Smaller quantities of BAL fluid were recovered from patients with COPD compared with those with asthma. * $P = .029$.

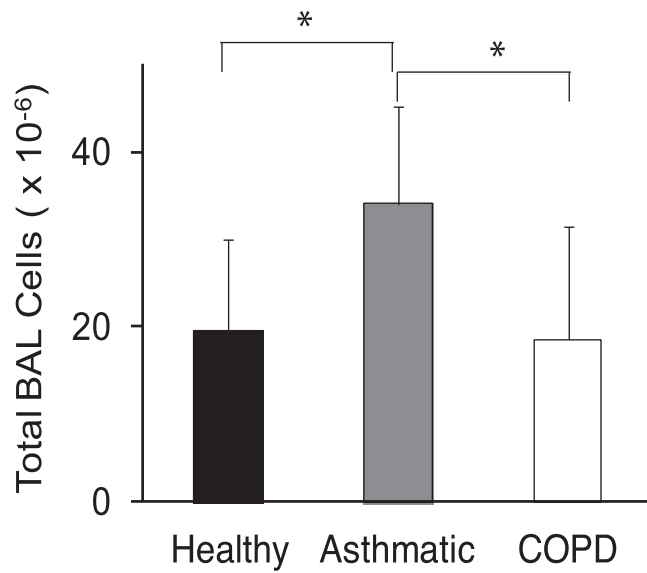


FIG E9. Comparison of total numbers of airway cells recovered by means of BAL from healthy subjects and patients with asthma or COPD. Data shown are mean cell numbers $\pm$ SDs. $*P = .023$. Study groups included 10 healthy subjects, 9 asthmatic patients, and 8 patients with COPD.

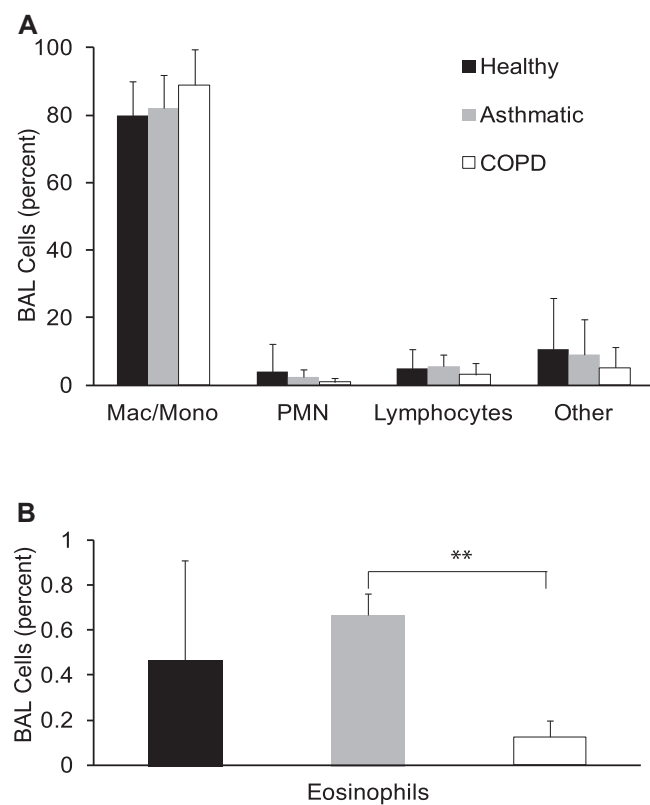


FIG E10. Comparison of leukocyte subsets in BAL fluid from healthy subjects, asthmatic patients, and patients with COPD. **A**, Blinded analysis of BAL cell differentials (expressed as percentages of total) from asthmatic patients, patients with COPD, and healthy subjects quantitated by using Diff-Quik–stained cytospin preparations. No statistically significant differences were observed between the study groups. **B**, Histogram showing percentages of eosinophils in BAL fluid harvested from healthy subjects and patients with asthma or COPD. ** $P = .0017$ for comparison of eosinophils in asthmatic patients versus those with COPD. *Mac*, Macrophages; *Mono*, monocytes.

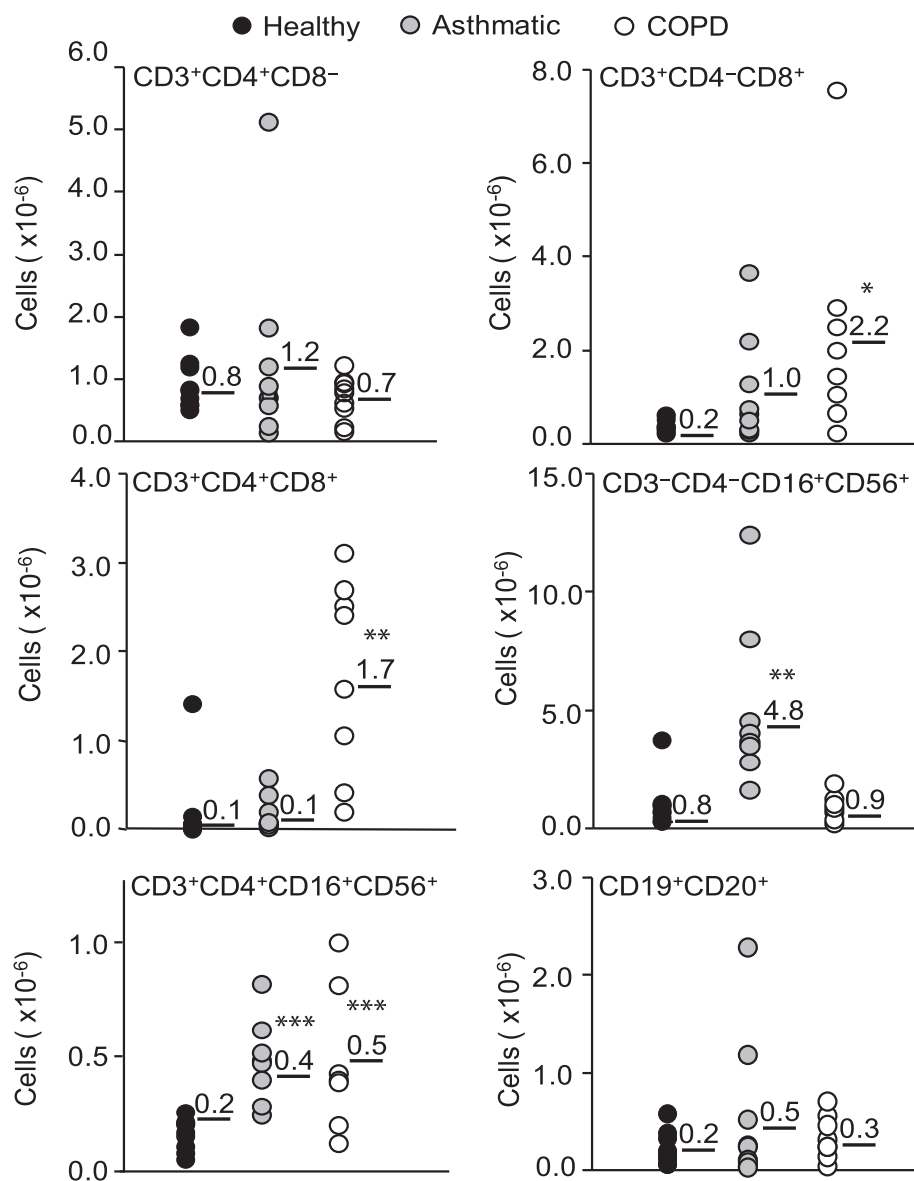


FIG E11. Comparisons of absolute numbers of B lymphocytes, CD4⁺ T lymphocytes, CD8⁺ T lymphocytes, CD4⁺CD8⁺ T lymphocytes, invariant NK T, and NK cells in BAL fluid recovered from healthy subjects and patients with asthma or COPD. BAL cells were analyzed by means of flow cytometry, and absolute numbers of the indicated cell types were determined. **P* = .0029 for CD4⁺CD8⁺ cells in asthmatic patients compared with those with COPD. ***P* < .001 comparing CD4⁺CD8⁺ cells in asthmatic patients, patients with COPD, and healthy subjects. ***P* < .001 for CD3⁻CD4⁻CD16⁺CD56⁺ cells in comparisons between asthmatic patients and patients with COPD and asthmatic patients versus healthy subjects. ****P* = .0091 for CD3⁺CD4⁺CD16⁺CD56⁺ cells in comparisons between asthmatic patients or patients with COPD versus healthy subjects.

TABLE E1. Comparison of proportions of leukocyte subsets in BAL fluid among healthy subjects, asthmatic patients, and patients with COPD

Cell population	Group			P value*
	Healthy subjects (n = 10)	Asthmatic patients (n = 9)	Patients with COPD (n = 8)	
CD19 ⁺ CD20 ⁺	1.1 ± 0.4 [†]	1.4 ± 2.0	1.8 ± 0.1 [‡]	.0108
CD3 ⁺ CD4 [−] CD8 ⁺	1.2 ± 0.4 [‡]	2.5 ± 2.9 [§]	14.4 ± 17.3 ^{‡§}	.0005
CD3 ⁺ CD4 ⁺ CD8 ⁺	0.8 ± 2.0 [‡]	0.4 ± 0.5 [§]	10.4 ± 5.2 ^{‡§}	.0004
CD3 ⁺ CD4 ⁺ CD8 [−]	5.1 ± 3.4	3.3 ± 4.2	5.4 ± 3.2	.0576
CD3 [−] CD4 [−] CD16 ⁺ CD56 ⁺	5.1 ± 7.4 [†]	13.0 ± 5.9 [†]	6.0 ± 3.9	.0097
CD3 ⁺ CD4 ⁺ CD16 ⁺ CD56 ⁺	1.0 ± 0.8 [‡]	1.3 ± 0.5 [§]	3.5 ± 2.0 ^{‡§}	.0004

Values shown represent proportions of individual cell populations in total leukocytes recovered by means of BAL (± SD).

*P values were determined by using the Kruskal-Wallis test to assess whether the distributions differ among the study groups.

[†]Distributions differ between the healthy and asthmatic groups by using a Bonferroni-adjusted type I error rate of 0.0167.

[‡]Distributions differ between the healthy and COPD groups by using a Bonferroni-adjusted type I error rate of 0.0167.

[§]Distributions differ between the asthmatic and COPD groups by using a Bonferroni-adjusted type I error rate of 0.0167.

TABLE E2. Weights of principal components

	Principal component 1	Principal component 2
P1 (CD11b ⁺ DAF-FM-DA ⁺)	−0.3961	0.1018
P2 (population A)	0.1775	0.5000
P3 (population B)	0.4068	0.4166
P4 (population C)	−0.5280	0.0943
P5 (population D)	0.3642	−0.4768
P6 (population E)	0.2859	0.4701
P7 (population F)	0.3910	−0.3297

The first principal component gives negative weights to P1 and P4 while giving positive weights to P2, P3, P5, P6, and P7, which separates each of the 3 groups along the x-axis. The second principal component gives large positive weights to P2 and P6 while giving negative weights to P5 and P7 to distinguish patients with COPD from asthmatic patients and healthy subjects along the y-axis.