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# Subsets of airway myeloid-derived regulatory cells distinguish mild asthma from COPD

**Jessy S. Deshane, PhD**[1,2,7,8], **David T. Redden, PhD**[3,8], **Meiqin Zeng, MS**[2], **Marion L. Spell, MS**[1], **Jaroslaw W. Zmijewski, PhD**[1,7], **John T. Anderson, MD**[1], **Rohit J. Deshane, BS**[2], **Amit Gaggar, MD, PhD**[1,5,9], **Gene P. Siegal, MD, PhD**[4,5,6], **Edward Abraham, MD**[1,7,*], **Mark T. Dransfield, MD**[1,9], and **David D. Chaplin, MD, PhD**[2,7,8]

[1]Department of Medicine, Musculoskeletal and Autoimmunity Center, University of Alabama at Birmingham, Birmingham, AL

[2]Department of Microbiology, Musculoskeletal and Autoimmunity Center, University of Alabama at Birmingham, Birmingham, AL

[3]Department of Biostatistics, Musculoskeletal and Autoimmunity Center, University of Alabama at Birmingham, Birmingham, AL

[4]Department of Pathology, Musculoskeletal and Autoimmunity Center, University of Alabama at Birmingham, Birmingham, AL

[5]Department of Cell Developmental and Integrative Biology, Musculoskeletal and Autoimmunity Center, University of Alabama at Birmingham, Birmingham, AL

[6]Department of Surgery, Musculoskeletal and Autoimmunity Center, University of Alabama at Birmingham, Birmingham, AL

[7]Department of Center for Free Radical Biology, Musculoskeletal and Autoimmunity Center, University of Alabama at Birmingham, Birmingham, AL

[8]Department of Comprehensive Arthritis, Musculoskeletal and Autoimmunity Center, University of Alabama at Birmingham, Birmingham, AL

[9]Department of Veterans Administration Medical Center, Birmingham, AL, Birmingham, AL

## Abstract

**BACKGROUND—**Subsets of myeloid-derived regulatory cells (MDRC), phenotypically similar to myeloid-derived suppressor cells found in cancer, have recently been appreciated as critical regulators of airway inflammation in mouse models of asthma.



Co-Corresponding Authors: Jessy S. Deshane, Ph.D., Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, University of Alabama at Birmingham, 1900 University Blvd, Birmingham, AL 35294, Tel: (205) 996-2041, FAX: (205) 934-1721, treena@uab.edu, David D. Chaplin, M.D., Ph.D., Department of Microbiology, University of Alabama at Birmingham, 1825 University Blvd, Birmingham, AL 35294, Tel: (205) 934-3917, Fax:(205) 996-4008, dchaplin@uab.edu.
*current address: Office of the Dean, Wake Forest School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157

**OBJECTIVE—**We test the hypothesis that subsets of airway MDRC contribute differentially to the inflammatory milieu in human asthma and chronic obstructive pulmonary disease (COPD).

**METHODS—**We used BAL to identify and characterize human airway MDRC from 10 normal, 9 mild asthmatic and 8 COPD subjects, none treated with inhaled or systemic corticosteroids. We defined subsets of airway MDRC by 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 normal, asthmatic and COPD subjects, with these differences regulated by the nitrosative and oxidative free radicals and cytokines they produced. Nitric oxide-producing MDRC suppressed and superoxide-producing MDRC enhanced proliferation of polyclonally activated autologous CD4 T-cells. HLA-$DR^+CD11^+CD11c^+CD163^-$ superoxide-producing MDRC, which stimulated proliferation of autologous T-cells, comprised a high fraction of MDRC in airways of subjects with mild asthma or COPD, but not normals. $CD11b^+CD14^+CD16^-$HLA-$DR^-$ nitric oxide-producing MDRC, which suppressed T-cell proliferation, were present in high numbers in airways of subjects with mild asthma, but not subjects with COPD or normals.

**CONCLUSION—**Subsets of airway MDRC conclusively discriminate mild asthmatics, subjects with COPD and normal subjects from each other. The distinctive activities of these MDRC in asthma and COPD may provide novel targets for new therapeutics in these common disorders.

### Keywords

myeloid cell; macrophage; nitric oxide; superoxide; T-regulatory cell

## INTRODUCTION

Myeloid-derived suppressor cells (MDSC) are a heterogeneous group of immature myeloid cells that inhibit lymphocyte function by a range of mechanisms. These include production of reactive oxygen and nitrogen species (ROS & RNS) that are generated by the inducible nitric oxide synthase (iNOS) and NADPH oxidase enzymes, and depletion of key nutrients required for normal function of T-cells, especially arginine by activation of arginase, and tryptophan and cysteine by sequestration in tumor-specific T-cells[1–6]. Additionally, activation of T-cells can be impaired by nitration of their antigen or chemokine receptors[7], or suppressed by induction of T regulatory cells via TGF-β produced by MDSC.[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] MDSC are significant sources of NO and ROS in cancer as well as in 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 MDSC and $O_2^{\cdot -}$-producing pro-inflammatory myeloid cells are major sources of free radicals and are critical regulators of the inflammatory response.[10] NO-producing myeloid cells suppressed airway hyper-responsiveness (AHR) in mice via iNOS-derived NO, arguing for a protective function of NO in attenuation of the inflammatory response in asthma.[10] Superoxide generated by a subpopulation of cells with phenotypic characteristics of MDSC contributed to increased T-cell inflammatory responses and increased AHR in an NADPH

oxidase-dependent fashion.[10] We referred to these NO- and $O_2^{\cdot -}$-producing cell subsets as myeloid-derived regulatory cells (MDRC) due to their broad functions as both up- and down-regulators of the inflammatory response. An imbalance in the ratio of these anti-inflammatory and pro-inflammatory myeloid cell subsets may contribute to many chronic airway inflammatory disorders.

Increased levels of RNS, including NO and its metabolites, and ROS, especially $O_2^{\cdot -}$, are prevalent in human subjects with inflammatory disorders of the lung.[15–18] In asthma, levels of NO produced by iNOS and urea produced by arginase are correlated with the degree of inflammation and with clinical exacerbations.[19–22] The NOS/arginase ratio may also contribute to bronchial tone in subjects with chronic obstructive pulmonary disease (COPD).[23, 24] Although levels of exhaled NO are much lower in patients with stable COPD than in asthmatics, 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 may be differences in the inflammatory patterns as well as the contributions of nitrosative and oxidative stress between bronchial asthma and 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 elevated concentrations of the metabolites of iNOS are localized to the smaller distal airway in human subjects with asthma.[15, 27] This suggests that in asthma the primary cellular sources of iNOS-derived NO may be localized in the bronchiolar and/or alveolar compartments. In contrast, we found that ROS were present in both the proximal and the distal airway compartments.[15] We hypothesize that MDRC subsets contribute importantly to the inflammatory milieu in the airways of subjects with asthma and 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 human asthma,[28, 29] the phenotypic and functional relationships of these cells in the airways of patients with asthma have not been investigated.

In this study, we have analyzed free radical-producing myeloid cells recovered by BAL from 10 normal subjects, 9 subjects with mild asthma, and 8 subjects with moderate COPD. We identified distinct subsets of NO-producing and $O_2^{\cdot -}$-producing MDRC in the airways of subjects with asthma and COPD and showed that these cells can modulate T-cell responses via free radical-dependent mechanisms. Importantly, we found that the proportions of HLA-$DR^+CD11c^+CD163^-$ $O_2^{\cdot -}$-producing MDRC were high in airways of subjects with mild asthma or COPD, but low in normal subjects, and that proportions of $CD11b^+CD14^+CD16^-$HLA-$DR^-$ nitric oxide-producing MDRC were high only in subjects with mild asthma, but not in subjects with COPD or normals. 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 three clinical phenotypes studied here, the MDRC subsets described above contributed prominently to the ability to completely discriminate the asthmatic, COPD and normal populations we analyzed. The tight association of MDRC phenotypes with the discrete inflammatory phenotypes that characterize asthma and COPD suggests that MDRC 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 may lead to identification of new therapeutic targets that focus on unique disease features in asthma and COPD.

## METHODS

### Study Design and Patient Recruitment

BAL fluid collected from asthmatics (n=9), subjects with COPD (n=8) and normal control subjects (n=10) ages 25–65 (Table I) were used for identification and purification of MDRC subpopulations and venous blood was used for purification of peripheral mononuclear cells. In a previous publication,[15] we provided a preliminary characterization of a subgroup of 8 each of these normal and asthmatic subjects, comparing the levels of metabolites of the NO and arginase pathways that were recovered in small volume lavage specimens obtained from large proximal airways with larger volume specimens that sampled the more distal airway compartments. All subjects, including normals and subjects with asthma or COPD, were recruited through the University of Alabama at Birmingham (UAB) Lung Health Center following the exclusion and inclusion criteria described previously.[15] Treatment with inhaled or systemic corticosteroids within the six weeks prior to or during the study was an absolute exclusion criterion. Control subjects had no history of lung disease and had normal spirometry. Asthmatics carried a clinical diagnosis and had mild disease as defined by GINA 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 COPD subjects had an established history of COPD, six of eight with moderate airflow obstruction (50%≤post-bronchodilator FEV1≤80% predicted) and two with severe airflow obstruction (FEV1=33% and 39% predicted). COPD subjects 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 six weeks prior to the study. This study was approved by the Institutional Review Board at UAB (Protocol #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 of cells and the proportion of cells in each subset. To compare among groups the demographics (gender, race) or clinical characteristics (atopic/non-atopic, smoking history) measured as categorical variables, exact p-values for the Chi-Square analyses, instead of asymptotic approximations, were calculated in order to address small sample sizes. Due to the relatively small number of subjects in each of the normal, 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, pre/post-bronchodilator FEV1, body mass index) among groups. Pairwise comparisons were conducted using the Wilcoxon Rank Sum procedure[30] with a Bonferroni correction. To develop two-dimensional graphs based upon numbers of cell types and proportions, the principal components technique was used to calculate the first two principal components. Utilizing these 2 principal components, scatter plots were developed to visualize the clustering of normal, asthmatic and COPD subjects. The first two 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 volume of BAL, total cell numbers, cytokine profiles and T-cell proliferation, the Kruskal Wallis procedure was performed. All procedures were performed using SAS 9.3 and GraphPad Prism.

## RESULTS

### Multiple subsets of airway MDRC can be recovered from subjects with asthma or COPD

The characteristics of the human subjects studied here are described in Table I. None of the study subjects was undergoing treatment with inhaled or systemic corticosteroids at the time of or at any time during the six months prior to their participation in this study. Statistically significant differences existed among the groups with regard to gender, history of remote smoking, atopic/non-atopic status, and pre/post-bronchodilator FEV1. The subjects with mild asthma were predominantly female (8 of 9), and the subjects with COPD were predominantly male (7 of 8). The impact of gender on the biology of free radical-producing myeloid cells or MDRC has not been studied systematically. Feng et al.[31] observed no gender-based differences in numbers of myeloid derived suppressor cells in 44 subjects with renal carcinoma. Studies with much larger sample size will be required to evaluate whether or not gender impacts the phenotype and function of myeloid derived regulatory cells in inflammatory diseases of the airway. As anticipated, all subjects with COPD had a past history of smoking cigarettes. Additionally, a self-reported history of atopy was higher in asthmatic subjects compared to subjects with COPD. Subjects with COPD showed reduced levels of pre- and post-bronchodilator FEV1, differing from both normals and subjects with asthma. This is consistent with the subjects with COPD carrying the designation of moderate-severe disease. The absence of significantly reduced pre-bronchodilator FEV1 measurements in our asthmatic population in association with absence of treatment with inhaled corticosteroids defines this group as mild asthmatics. Obesity was prevalent in all three of the study groups (data not shown). Although there was a trend towards higher body mass index (BMI) in asthmatic subjects, the differences in BMI among the groups did not reach statistical significance. For normal subjects, BMI was 32.3 ± 4.9, for subjects with mild asthma 39.9 ± 12.9, and for subjects with moderate-severe COPD 30.5 ± 12.1 ($p=0.091$).

In order to investigate the characteristics of myeloid cells that may modulate inflammatory responses in the airways, we recovered airway leukocytes by BAL, analyzed them by flow cytometry, purified selected subsets by cell sorting, and analyzed cellular functions using ELISA and assays of cell proliferation. First, we used the fluorescent probe DAF-FM-DA and a selection of cell surface markers to identify NO-producing MDRC in cells recovered

by BAL.[10] When BAL leukocytes from normal subjects were stained with the nitric oxide indicator dye DAF-FM-DA and with the myeloid cell marker CD11b, two DAF-FM-DA$^+$ populations were observed (Figure 1A), one that was CD11b$^-$ and one that was CD11b$^+$. The CD11b$^-$ population was also CD14$^-$CD16$^-$ (not shown) and was designated **population A**. Both the proportions (Figure 1A and Table II) and the absolute numbers of **population A** cells (Figure 2) differed between normal subjects and subjects 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 normal 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 Figure E1 in the Online Repository). Reductions in the percentages of **population B** cells were observed in BAL samples from asthmatic and COPD subjects compared to normal controls (Figure 1A and Table II), whereas reductions in the absolute numbers of **population B** cells were observed only in subjects with COPD (Figure 2).

CD11b$^+$DAF-FM-DA$^+$ cells that were CD14$^+$CD16$^-$ (Figure1A and Table II and CD11c$^-$HLA-DR$^-$ (see Figure E1 in the Online Repository) were designated **population C** and were increased in asthmatic subjects compared to normals (26-fold in proportions and 68-fold in absolute numbers) and decreased in subjects with COPD (7.6-fold in proportions and 34-fold in total numbers compared to subjects with asthma). In asthmatic subjects, **population C** was the predominant DAF-FM-DA$^+$ MDRC population (Figure 1A, Table II and Figure 2). The CD11b$^+$DAF-FM-DA$^+$CD14$^-$CD16$^+$ cells designated as **population D** (Figure 1A and Table II) were also CD11c$^-$HLA-DR$^-$ (see Figure E1 in the Online Repository). **Population D** cells were low in normal subjects compared to those with asthma or COPD (Figure 1A, Table II and Figure 2; 24-fold, and 30-fold difference in percentages and absolute numbers respectively between normal and asthmatic subjects; 23-fold and 16-fold differences in percentages and absolute numbers between normal and COPD subjects).

We next used the fluorescent dye dihydroxyethidium (DHE), as previously reported for murine cells,[10] to identify superoxide-producing airway MDRC. 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 CD163$^+$ were designated **population E** and those that were CD163$^-$ were designated **population F** (Figure 1B and Table II). Both of these ROS-producing subsets were also CD33$^+$ (data not shown) and HLA-DR$^+$ (see Figure E2 in the Online Repository). In asthmatic subjects, **population E** showed a 1.8-fold increase in proportions and 3.4-fold increase in absolute numbers compared to normal and COPD subjects (Figure 1B, Table II and Figure 2). The proportions of **population F** cells were 5.7-fold greater in asthmatic and ~5-fold greater in COPD subjects compared to normals, while the absolute numbers were increased 11-fold in asthmatic and 4.7-fold in COPD samples.

In order to determine the nature of the cytokines and other mediators released by myeloid cells in each of these populations, we sorted the cells by FACS using the same sets of

fluorescently labeled antibodies, as well as DAF-FM-DA and DHE. We first assessed the morphology of cells in those of the sorted populations that were present in sufficient numbers to permit analysis by using DIFF Quick staining of cytospin preparations (Figure E3 in the Online Repository). 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, perhaps more extensive between the normal subjects (who had lower amounts of vacuolization) and those 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 1400 w, **population B** cells from a representative asthmatic subject showed ablation of DAF-FM-DA staining (see Figure E4 in the Online Repository). Similar results were obtained with **population B** cells from all study cohorts (not shown). **Population C** from asthmatic subjects produced the highest levels of nitrate and nitrite in supernatants from cultures of purified MDRC compared to **populations A, B** & **D** (see Figure E5 in the Online Repository). High levels of nitrate and nitrite were also detected in culture supernatants from the $CD163^+$ **population E** which produces ROS via NADPH oxidase, suggesting that a portion of the cells within **population E** could produce both NO and ROS. Stimulation of purified $CD163^+CD11b^+CD11c^+CD33^+HLA\text{-}DR^+$ cells with PMA resulted in increased fluorescence with DHE (see Figure E6A in the Online Repository), which was ablated by treatment with diphenyleneiodonium (DPI; an inhibitor of NADPH oxidase). The time dependent increase in DPI-sensitive 2',7'-dichlorodihydrofluorescein diacetate (DCFH DA) cellular fluorescence (see Figure E6B in the Online Repository) confirmed that **population E** produced $O_2{\cdot}^-$ via the NADPH oxidase complex. The supernatants from cultured BAL MDRC **populations B** and **E** from COPD subjects had reduced levels of nitrate in comparison to asthmatic or normal subjects (see Figure E5 in the Online Repository). 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 COPD subjects.

In the lung microenvironment, when L-arginine, required for TCR-signaling, is depleted by activation of arginase, levels of $O_2{\cdot}^-$ and other ROS can be increased by the uncoupling of NOS enzymes including iNOS.[32–34] We measured urea in culture supernatants of purified MDRC populations to assess arginase activity in these cells, finding that **population E** cells from subjects with COPD showed a >20-fold increase in urea compared to the control group and an ~2-fold increase compared to **population F** from the same study group (see Figure E5 in the Online Repository). Both **populations E** and **F** purified from BAL leukocytes of asthmatic subjects produced urea, indicating that these cells also expressed arginase. Altogether, these data showed that human airway MDRC populations purified from BAL leukocytes of normal, asthmatic and COPD subjects 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 Figure E7 in the Online Repository). The Th2 mediators IL-4, IL-13 and eotaxin, were detected at significantly

higher concentrations in culture supernatants from the $DHE^+$ **populations E** & **F** purified from BAL fluid of both asthmatic and COPD subjects compared to normal subjects. Levels of IL-4 and IL-13 were also significantly higher in **population F** from asthmatic subjects compared to subjects with COPD. In contrast, there were no significant differences noted in the levels of eotaxin comparing subjects with asthma or COPD. The regulatory cytokines IL-10 and TGF-β, implicated in MDRC-mediated immunosuppression and in the induction of T regulatory cells by MDRC, were differentially expressed by **populations A–F**. IL-10 was produced predominantly in $CD14^+$ cells of **populations A, B** & **C.** In contrast, TGF-β showed a more promiscuous pattern, with both the NO- and ROS-producing **populations C, E** & **F** secreting this cytokine. Levels of TGF-β produced by the MDRC subsets **E** and **F** in asthmatic and COPD subjects were increased compared to normal subjects. In asthmatic subjects, the NO-producing MDRC **populations B** & **C** produced ~400-fold more IL-6 than their counterparts purified from COPD or normal samples or compared to ROS-producing MDRC. IL-1β, a proinflammatory signature cytokine for MDRC that has been implicated in the pathogenesis of asthma,[35] was increased in BAL MDRC culture supernatants from asthmatics compared to COPD and normal subjects. IL-17 was produced by the ROS-producing **populations E** & **F** in all study groups, at increased levels compared to the NO-producing MDRC. Additionally, IL-7, which can activate eosinophils and drive eosinophilic inflammation,[36] was >60-fold higher in culture supernatants from $CD163^+$ MDRC (**population E**) purified from subjects with asthma or COPD compared to normal subjects with significantly higher levels produced by cells from subjects with asthma compared to subjects with COPD.

## Free radical pathways regulate heterogeneity in function of MDRC in 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] To define the regulatory behavior of the heterogeneous MDRC subsets in modulating the T-cell response, sorted MDRC populations were co-cultured with autologous $CD4^+$ T-cells in the presence of activating anti-CD3 antibody. **Population A** cells did not suppress proliferation of polyclonally activated T-cells (Figure 3A). In contrast, MDRC **population B** from asthmatic, COPD or normal subjects all suppressed T-cell proliferation (Figures 3A, 3B and 3C) (75% suppression in normals ($p<0.001$) and asthmatics ($p=0.0018$) and 50% suppression in subjects with COPD ($p=0.0041$)). This suppression was iNOS-dependent but $O_2{\cdot}^-$- and arginase-independent as evident by reversal of suppression by the iNOS inhibitor 1400 w and lack of change following addition of superoxide dismutase (SOD) or the arginase inhibitor nor-NOHA.

T-cell proliferation was also suppressed to a similar magnitude in an iNOS-dependent manner by MDRC **population C** ($p<0.001$) (Figure 3B). These cells could be purified in adequate numbers from BAL of only asthmatic subjects. Interestingly, addition of superoxide dismutase, a scavenger of $O_2{\cdot}^-$, enhanced the suppression ($p<0.001$), suggesting that a fraction of **population C** also produce superoxide, which might contribute to MDRC-mediated up-regulation of T-cell proliferation. This was consistent with BAL cells reacting with fluorescent indicators for both NO and ROS (see Figure E8A in the Online Repository).

The arginase inhibitor nor-NOHA, however, did not alter the suppression by **population C** (Figure 3B), suggesting that the arginase pathway did not contribute to the regulatory function of this MDRC subset.

In contrast, **population D** cells from subjects with asthma or COPD did not suppress proliferation when co-cultured with autologous T-cells (Figures 3B and 3C). But addition of the iNOS inhibitor 1400 w or the $O_2^{\cdot -}$ scavenger SOD to co-cultures of **population D** cells from asthmatic subjects and autologous T lymphocytes suppressed T-cell proliferation by 75% (p=0.0025). This inhibition was not due to toxicity of 1400 w or SOD since the inhibitors by themselves did not affect T-cell proliferation (data not shown). In contrast, **population D** cells purified from subjects with COPD did not modulate proliferation of autologous T-cells in the presence of 1400 w or SOD. Adequate numbers of **population D** were not recovered from normal subjects to allow co-culture studies. In an effort to delineate the roles of the ROS and arginase pathways in the function of MDRC, the purified $O_2^{\cdot -}$-producing **populations E** and **F** (from subjects with asthma or COPD only) were co-cultured 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 co-cultures from all three study groups (Figure 3) (normal, p=0.018; asthmatics, p=0.023; COPD, p=0.0034). In co-cultures from normal subjects (Figure 3A), this enhancement of T-cell proliferation was inhibited in a $O_2^{\cdot -}$- and arginase-dependent but iNOS-independent manner as SOD or the arginase inhibitor nor-NOHA, but not 1400 w, ablated the increase in proliferation (p=0.002).

Of interest, while **population E** cells from subjects with asthma, similar to those from normal subjects, enhanced proliferation of polyclonally activated autologous T-cells in a $O_2^{\cdot -}$- and arginase-dependent fashion (p=0.024), addition of 1400 w to these co-cultures further enhanced T-cell proliferation (p=0.016), suggesting that iNOS expressed in this population reduced the enhancement of T-cell proliferation (Figures 3B and 3C). **Population E** cells from subjects with COPD also enhanced T-cell proliferation in a $O_2^{\cdot -}$- and arginase-dependent fashion (p=0.0021), but addition of 1400 w did not show iNOS-dependent regulatory activity of these cells (p=0.69).

The $CD163^-$ **population F** from subjects with asthma or COPD also enhanced polyclonal autologous T-cell proliferation (Figures 3B and 3C). Although an arginase- and superoxide-dependent mechanism modulated this response in subjects with either asthma or COPD (asthmatics, p=0.0032; COPD, p= 0.0095), there was also modest involvement of iNOS-derived NO in COPD samples as 1400 w significantly reduced the increase in T-cell proliferative response (p=0.046). The numbers of **population F** cells recovered from BAL fluid of normal subjects were too low for co-culture experiments.

Altogether, as summarized in Table III, these data show that MDRC can regulate T-cell proliferative responses using free radical pathways; however, the distinct MDRC populations we have defined by 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, $O_2^{\cdot -}$, and arginase pathways. Furthermore, the immunomodulatory mechanisms utilized by these cell populations varied depending on the patient population.

### Alterations in leukocyte numbers in BAL fluid from asthmatics and subjects with COPD

The MDRC numbers are elevated in blood and tumor tissues in humans, 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 MDRC 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 comparing normal to asthmatic subjects, but were significantly greater in asthmatic compared to COPD subjects (see Figure E8B in the Online Repository). Total cell numbers recovered from pooled BAL samples were similar comparing normal and COPD samples, but were significantly higher comparing asthmatics to normal and COPD subjects (see Figure E9 in the Online Repository). The percentages of eosinophils in BAL fluid were increased in asthmatic subjects compared to those with COPD, but not the percentages of monocyte/macrophages, polymorphonuclear leukocytes or lymphocytes (see Figures E10A and E10B in the Online Repository). We compared the absolute numbers and percentages of BAL fluid lymphocytes, NK cells and invariant NK-T-cells which have previously been linked with asthmatic airway inflammation (see Figure E11 and Table E1 in the Online Repository).[40] The numbers of invariant NK-T-cells ($CD3^+CD4^+CD16^+CD56^+$) were higher in both the asthmatic and COPD groups compared to normal subjects. The numbers of $CD3^+CD4^+CD8^+$ cells were increased only in the COPD group while the $CD3^-CD4^-CD16^+CD56^+$ cells were increased only in asthmatic subjects. Significant differences were not noted amongst the study groups for the proportions ($p=0.058$) or absolute numbers ($p=0.96$) of $CD4^+CD3^+CD8^-$ T-cells. The proportions of B-lymphocytes were higher in subjects with COPD compared to normals ($p=0.011$), while the total numbers of B-lymphocytes were not significantly different among the study groups ($p=0.64$).

### 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 (Figure 2) to investigate whether the different MDRC subsets could discriminate and predict whether individuals belonged to the COPD, asthma, or normal group. 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 normals from asthmatics, but did not discriminate normals or asthmatics from subjects with COPD. The proportions of **population B** discriminated normals from both asthmatics and subjects with COPD, but not asthmatics from subjects with COPD. Interestingly, analysis of absolute numbers of cells with the **population B** phenotype (Figure 2) discriminated subjects with COPD from normal subjects and also from subjects with asthma. Analysis of proportions of cells in **population C** distinguished all three groups from each other (Table II); however based on absolute numbers (Figure 2), **population C** discriminated asthmatics from both normals and subjects with COPD, but did not distinguish normal subjects from subjects with COPD. **Population D** discriminated both asthmatics and subjects with COPD from normals based on analysis of either proportions or absolute

numbers. Based on proportions, **population E** discriminated normal subjects and subjects with asthma, whereas analysis of absolute numbers discriminated asthmatics from both normal subjects and subjects with COPD. Based on absolute numbers (Figure 2), **population F** discriminated normal subjects from asthmatics and from subjects with COPD based on either proportions or absolute numbers, but did not discriminate asthmatic subjects from subjects 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 identifies which variables contribute most to this difference, and whether those variables contribute in the same way (i.e. 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 (Figure 4). Weightings for each of the first two principal components are provided in Table E2 in the Online Repository. The first two 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 the 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 the group status (not shown).

## DISCUSSION

This study has established a consistent association between respiratory disease phenotypes in human subjects and subpopulations of airway MDRC by combining cell surface phenotype, profile of molecular mediators produced, and ability to regulate proliferation of polyclonally activated $CD4^+$ T-cells. In order to avoid the potentially confounding effects of treatment with either inhaled or systemic corticosteroids, we limited this study to mild asthmatic subjects who were receiving no corticosteroid medications, and to subjects with moderately severe COPD who also had not received either inhaled or systemic steroids in the six weeks prior to entering our study. Comparing normal subjects and subjects with mild asthma or moderately severe COPD, we show differences between the study groups in the cell surface markers displayed on subsets of MDRC[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 subjects with mild asthma compared to moderate and severe asthma, and how treatment with inhaled or systemic corticosteroids may alter the MDRC repertoire.

Our results indicate that NO- and ROS-producing airway MDRC subsets recovered from lavage fluid from normal subjects are distinctly different compared to those recovered from subjects 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 TGFβ 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 Th2 and pro-inflammatory cytokine bias for the ROS-producing MDRC and a regulatory cytokine profile for the NO-producing cells in both asthmatic and COPD subjects compared to normals. Interestingly, the production of IL-4 and IL-13 was more elevated in the $O_2^{\cdot -}$-producing HLA-DR$^+$CD11c$^+$**CD163**$^-$ subset and the production of IL-7 was higher in the HLA-DR$^+$CD11c$^+$**CD163**$^+$ subset from asthmatic subjects than from subjects with COPD, whereas similarly elevated levels of eotaxin were produced by these cells from asthmatics and subjects with COPD (Figure E7 in the Online Repository). Thus, there were important differences in the production of Th2-associated cytokines in the $O_2^{\cdot -}$-producing cells from the different clinical groups.

These results were consistent with the observed differences in the percent of eosinophils in BAL fluid from asthmatics compared to subjects with COPD (see Figure E10 in the Online Repository). While the numbers of eosinophils recovered in the BAL fluid from subjects with asthma were significantly increased compared to fluid recovered from subjects with COPD, elevated numbers of peripheral blood eosinophils were detected in only a small fraction of subjects (1 of 10 normal subjects, 0 of 9 asthmatics, and 2 of 8 subjects with COPD). The absence of correlation between the numbers of peripheral blood eosinophils, airway eosinophils, and production of IL-7 by MDRC 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.

Based on experiments in which individual MDRC subsets were co-cultured with polyclonally activated autologous CD4$^+$ T-cells, we observed that the CD163$^-$ and the CD163$^+$ HLA-DR$^+$CD11c$^+$ $O_2^{\cdot -}$-producing subsets enhanced proliferation of T-cells, with this proliferation-enhancing activity being dependent on the production of $O_2^{\cdot -}$ by these MDRC (Figure 2). These findings suggest that these MDRC subsets express proinflammatory or immune enhancing activities. It is, thus, significant that the numbers of both of these cell types are substantially elevated in subjects with asthma (Figure 2). With these subsets, cells that were CD163$^-$ were also elevated in subjects with COPD, whereas cells that were CD163$^+$ were elevated only in asthmatics. Perhaps significant in these differing associations is the production by the HLA-DR$^+$CD11c$^+$**CD163**$^-$ subset of high levels of IL17 (Figure E7 in the online repository), likely contributing to the neutrophilic inflammatory response that is typically observed in the airways of subjects 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 Th2-type inflammation that is often present in asthmatic subjects.

While $O_2^{\cdot -}$-producing MDRC showed pro-inflammatory activities supporting enhanced proliferation of activated T-cells, in almost all cases NO-producing MDRC suppressed T-cell proliferation. NO-producing $CD11b^+CD14^+CD16^+$ cells from all study groups suppressed T-cell proliferation in an NO-dependent fashion (Figure 3). The NO-producing $CD11b^+CD14^+CD16^-$ subset that was present in substantial numbers only in airways of asthmatic subjects 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 MDRC can down-modulate 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 auto-regulatory network to attempt to control the inflammatory response and restore homeostasis. In normal individuals, NO-producing MDRC (primarily the $CD11b^+CD14^+CD16^+$ and the $CD11b^-$ subsets) may 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, our data support a model in which airway NO is an indicator of an elevated 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 normal subjects, when recovered from asthmatic subjects suppressed T-cell proliferation, but had no impact on T-cell proliferation when recovered from the airways of subjects with COPD. Whereas the $CD11b^+CD14^+CD16^+$ and $CD11b^+CD14^+CD16^-$ populations produce substantial amounts of either IL-10 or TGFβ or both, the $CD11b^+CD14^-CD16^+$ subset produces extremely low levels of these immunoregulatory cytokines and produces substantial amounts of IL-1β, a cytokine with strong potential to enhance T-cell proliferation. These differences in production of immunoregulatory and proinflammatory cytokines may underlie the absence of T-cell suppressive activity when this subset is purified from subjects with COPD. Additional currently unrecognized factors must contribute to the ability of this subset from asthmatic subjects to suppress T-cell proliferation. It may also be possible that MDRC subsets that appear similar because they share cell surface markers but that have distinct functional characteristics may be differentially activated forms of the same subset. Alternatively, they may be fundamentally different subsets, differentiated along different pathways.

The highest capacity of MDRC subsets to discriminate between normal, asthmatic and COPD subjects depended on consideration of all of the cell subsets that we have 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 subjects 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 $HLA\text{-}DR^+CD11b^+CD11c^+CD163^-$ cells 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 while the numbers and proportions of some of these cell types vary significantly in BAL fluid of subjects with asthma or COPD, in principal component analysis these cell types were not important predictors permitting classification of samples to the asthmatic, COPD or normal groups. This was in striking contrast to our observations that specific phenotypic and functional MDRC subsets were associated with these two 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 normal individuals and subjects with asthma or COPD.

The discrete functional repertoires of the several MDRC phenotypes, together with the physical segregation of $O_2^{\cdot -}$-producing and NO-producing MDRC that we previously observed in proximal and distal airways of asthmatic subjects[15] could indicate that diverse types of immune responses may occur at the same time in discrete tissue compartments, with locally active MDRC subsets providing regional immune regulation. Morphological, phenotypic and functional heterogeneity are accepted hallmarks of MDRC, as is plasticity of the immunosuppressive myeloid compartment.[43] Notably, expression of myeloid cell surface antigens, particularly CD11b, in selected MDRC subsets were lower in subjects with asthma or COPD compared to normals. 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. Our study describes for the first time that heterogeneous subsets of MDRC differentiate two chronic inflammatory lung diseases. Our data are thus consistent with MDRC 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 pro-inflammatory regulatory populations. Investigating the mechanisms underlying the phenotypic differences of MDRC 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 pro-inflammatory 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 proportions and numbers of MDRC subsets and their differing functional profiles discriminate mild asthmatics from subjects with COPD and both disease groups from normal 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

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. Further, they underscore the need to evaluate MDRC subsets as biomarkers for monitoring severity of airway inflammatory diseases and responses to anti-inflammatory therapeutics.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
|---|---|
| **AHR** | airway hyper-responsiveness |
| **COPD** | chronic obstructive pulmonary disease |
| **DAF-FM-DA** | 4-amino-5-methylamino-2’,7’-difluorofluorescein diacetate |
| **DCFH-DA** | 2’,7’-dichlorodihydrofluorescein diacetate |
| **DHE** | dihydroxyethidium |
| **DPI** | diphenyleneiodonium |
| **FACS** | fluorescence-activated cell sorter |
| **FEV1** | forced expiratory volume in 1 second |
| **iNOS** | inducible nitric oxide synthase |
| **MDRC** | myeloid-derived regulatory cells |
| **MDSC** | myeloid-derived suppressor cells |
| **NK** | natural killer |
| **nor-NOHA** | N(omega)-hydroxy-nor-L-arginine |
| **NOS** | nitric oxide synthase |
| **PCA** | principle component analysis |
| **RNS** | reactive nitrogen species |

| | |
|---|---|
| **ROS** | reactive oxygen species |
| **SOD** | superoxide dismutase |

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### Key Messages

- Airway MDRC 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$^{+}$ MDRC enhance proliferation of polyclonally-activated autologous T-cells and express a proinflammatory signature of cytokines, whereas nitric oxide-producing CD11b$^{+}$CD14$^{+}$CD16$^{+}$HLA-DR$^{-}$ MDRC and CD11b$^{+}$CD14$^{+}$CD16$^{-}$HLA-DR$^{-}$MDRC 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 MDRC in normal subjects, but as a substantial fraction in subjects with mild asthma or COPD, whereas CD11b$^{+}$CD14$^{+}$CD16$^{-}$HLA-DR- nitric oxide-producing MDRC were present in high number only in subjects with mild asthma, permitting discrimination of the three clinical groups.

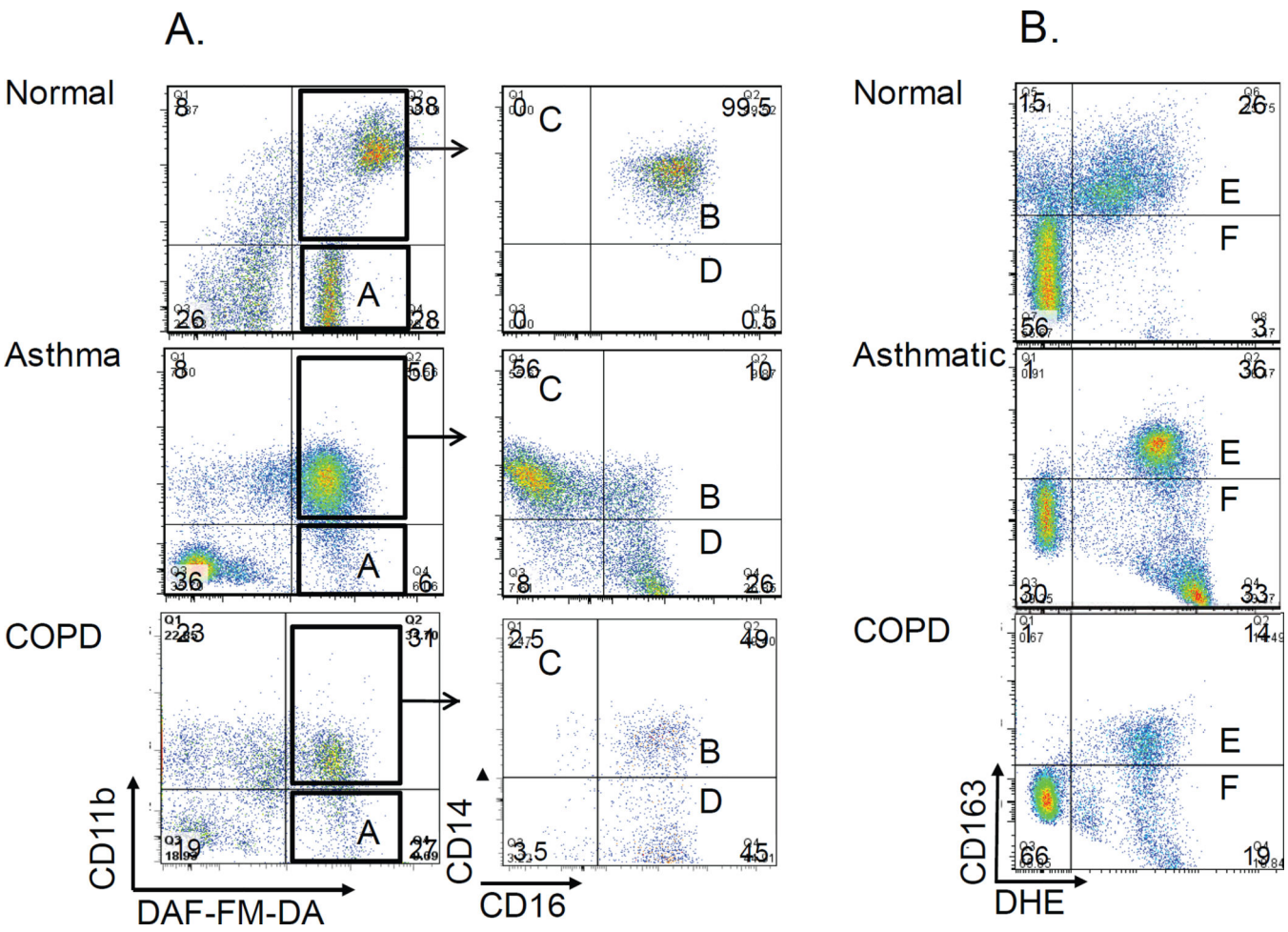


**Figure 1. Different profiles of free radical-producing subsets of myeloid-derived regulatory cell subsets are recovered from the airways of normal subjects and subjects with asthma or COPD**

Cells obtained by BAL were stained with fluorescently labeled antibodies and with the free radical-reactive fluorescent indicator dyes DAF-FM-DA or DHE prior to analysis by flow cytometry. (A) FACS plots of MDRC recovered from BAL fluid demonstrating that the NO-producing $CD11b^{+}$DAF-FM-$DA^{+}$ population can be fractionated into three subsets based on differential expression of CD14 and CD16. These subsets are labeled **B** ($CD11b^{+}$DAF-FM-$DA^{+}CD14^{+}CD16^{+}$), **C** ($CD11b^{+}$DAF-FM-$DA^{+}CD14^{+}CD16^{-}$), & **D** ($CD11b^{+}$DAF-FM-$DA^{+}CD14^{-}CD16^{+}$). The $CD11b^{-}$DAF-FM-$DA^{+}$ population is designated **A.** (B) ROS-producing MDRC, identified by staining with DHE, can be fractionated into $CD163^{+}$ subsets (labeled **E**) and $CD163^{-}$ subsets (labeled **F**).

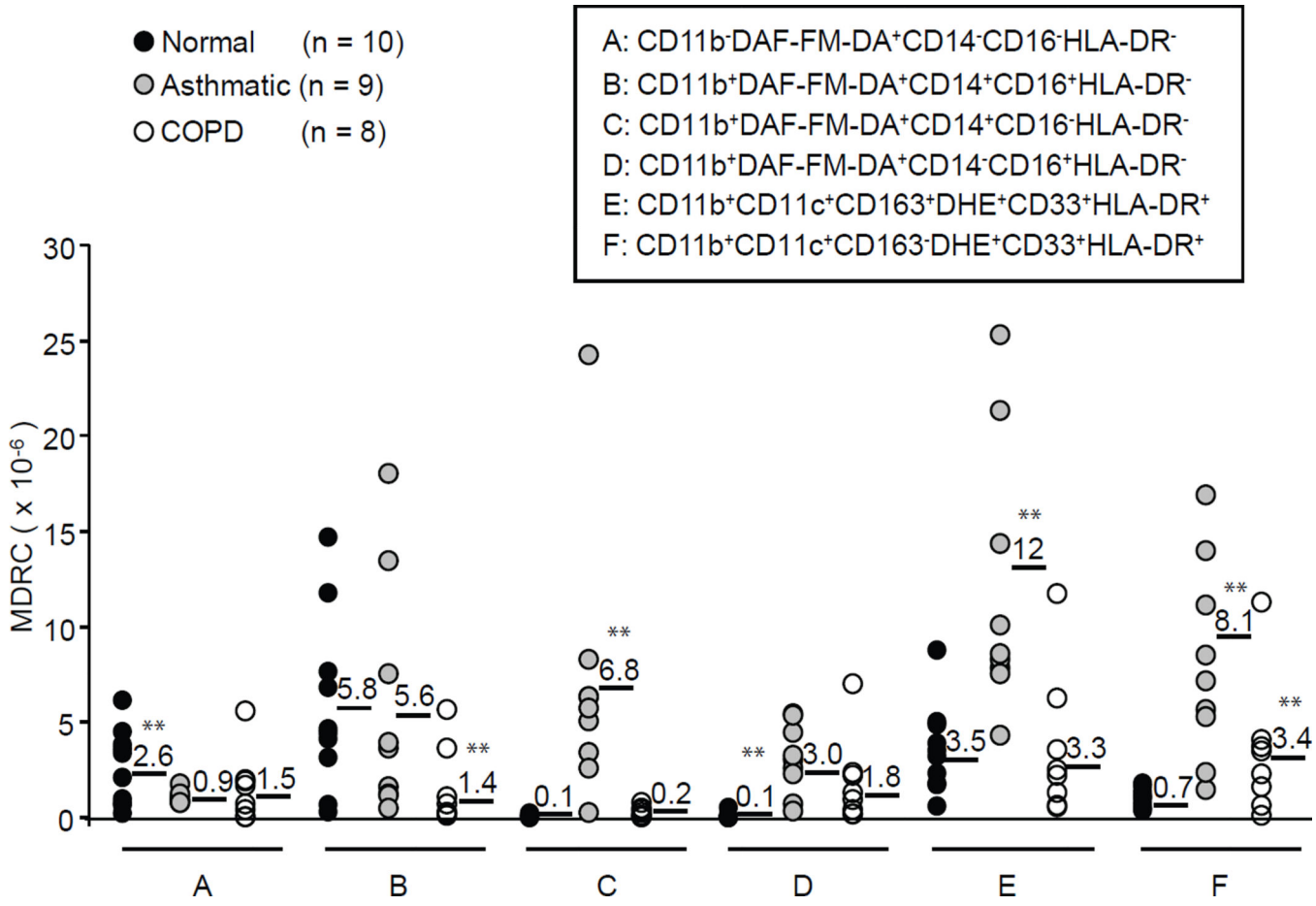


**Figure 2. Absolute numbers of the different MDRC subsets recovered from the airways of subjects with asthma or COPD compared to normal controls**

BAL cells were stained and analyzed as in Figure 1. MDRC populations were identified as **A–F**, as described in Figures 1A & 1B. 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, COPD; grey filled symbols, asthma; and black filled symbols, normal). Numeric data represent pooled means. For **A**: **p=0.046 comparing normals versus asthmatic subjects; for **B**: **p=0.024 comparing COPD versus normal and asthmatic subjects; for **C**: **p<0.001 comparing asthmatic versus COPD and normal subjects; for **D**: **p<0.001 comparing normal versus asthmatic and COPD subjects; for **E**: **p=0.0023 comparing asthmatic versus normal and COPD subjects; for **F**: **p<0.001 comparing asthmatic and COPD versus normal subjects.

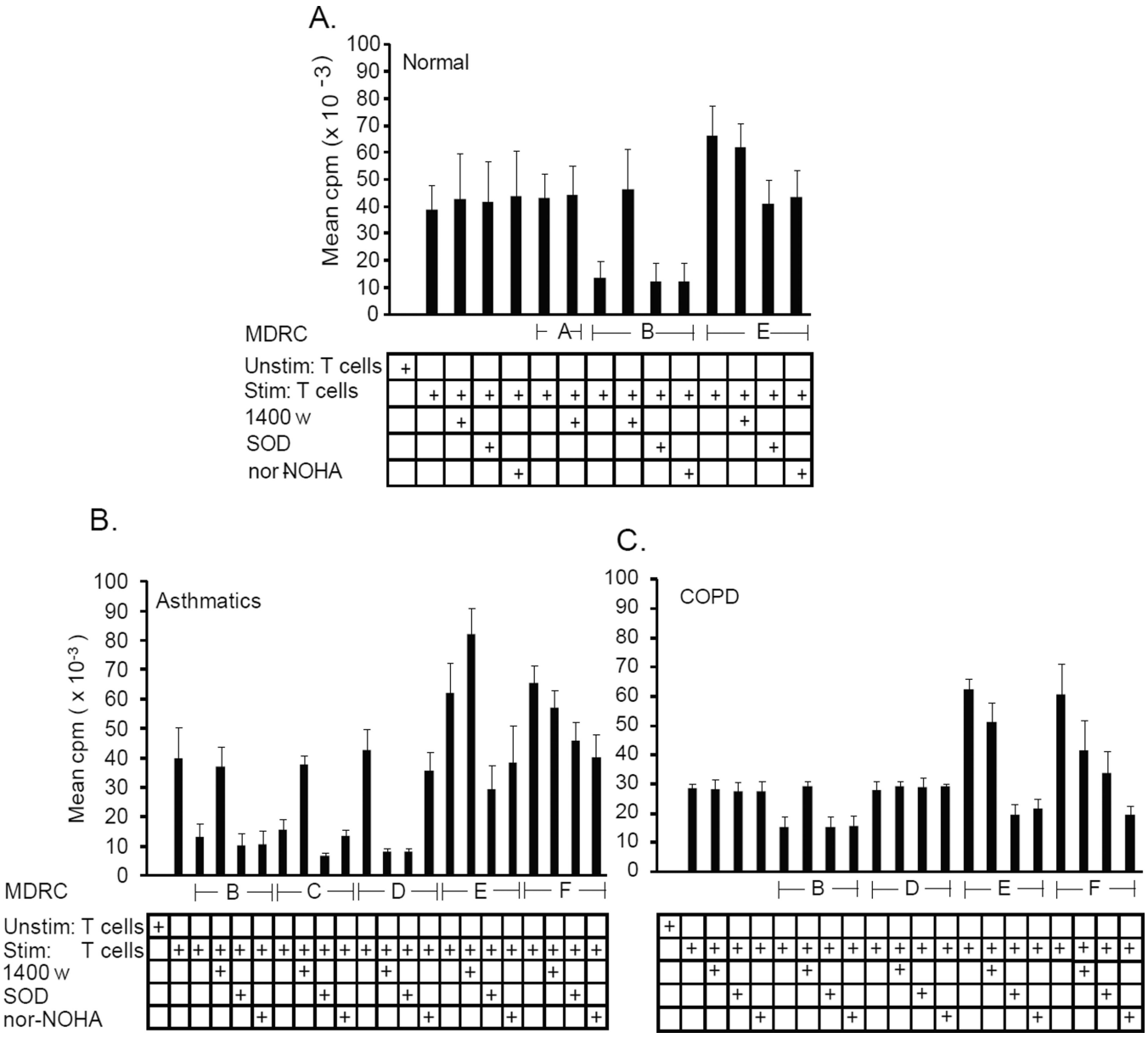


**Figure 3. MDRC subsets show different abilities to modulate T-cell proliferation using the iNOS, arginase and NADPH oxidase pathways**

MDRC subsets ($10^5$ each; **populations A–F** as defined in Figures 1A and 1B) purified by FACS from the BAL fluid of study subjects were cultured at a 1:1 ratio with autologous $CD4^+$ T-cells purified from peripheral blood and activated using plate bound anti-CD3 with or without 1400 w, SOD or nor-NOHA, as indicated. After 48h, $^3$H-thymidine was added and incorporated $^3$H was determined 16h later. Co-culture of anti-CD3-activated autologous T-cells with the indicated MDRC subsets purified from BAL fluid of normal subjects (panel A), asthmatic subjects (panel B), and subjects with COPD (panel C). Means of replicate cpm data for each sample and treatment group for each individual for each study group were pooled and the averages of the means are presented as mean cpm ± SD.

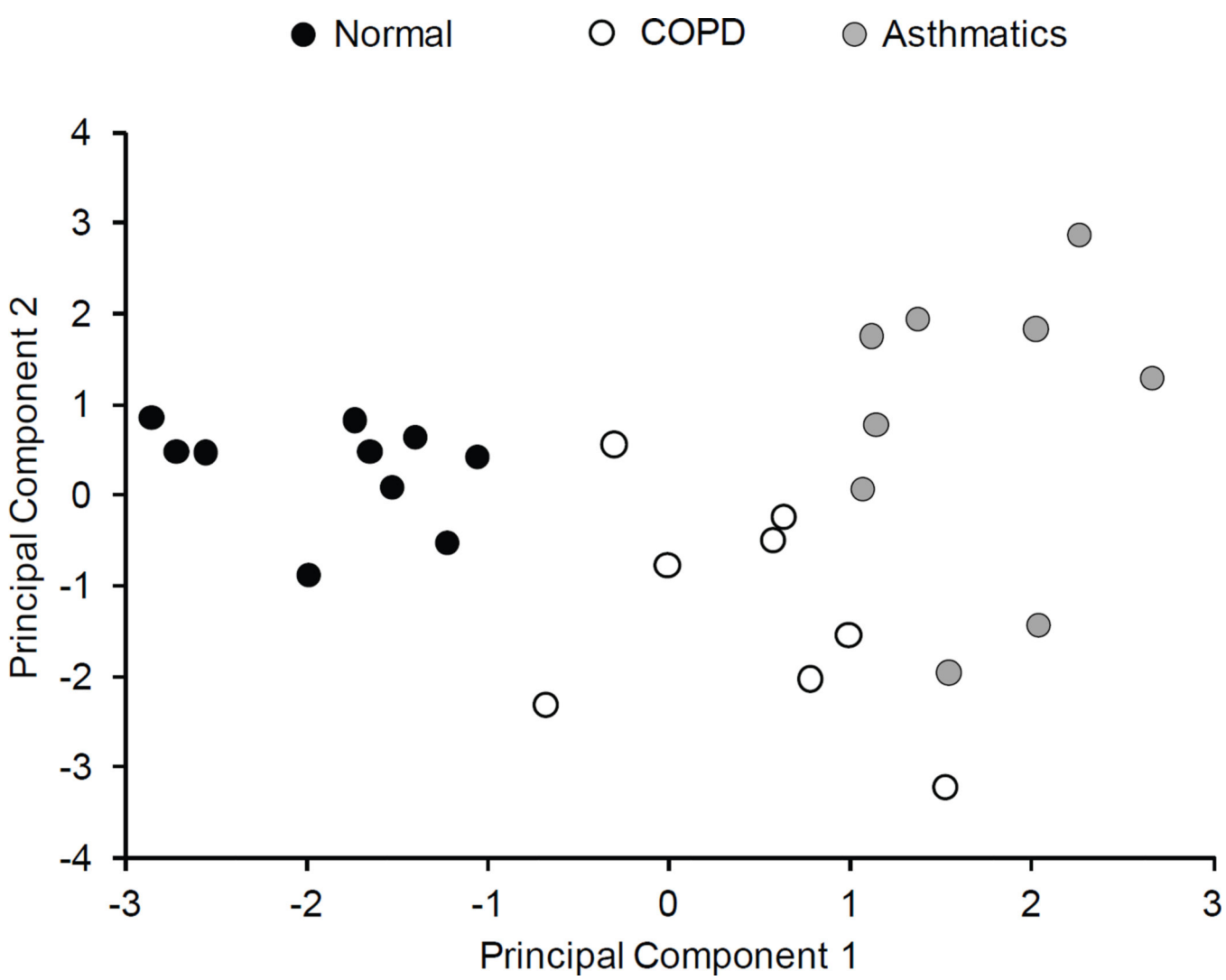


**Figure 4. Multivariate analysis of MDRC subsets discriminates and predicts the group status of the normal, asthmatic and COPD study groups**

A 2-dimensional Principal Component Analysis Plot using the proportions of cells with surface phenotypes defined by flow cytometry, free radical production defined by DAF-FM-DA- or DHE-staining, and ability to regulate T-cell proliferation shows the variance in principal components one and two and separation of asthmatic subjects (grey filled symbols) from COPD subjects (open symbols) and normals (black filled symbols).

## TABLE I

Characteristics of study subjects

| Parameters | Group | | | p-value* |
|---|---|---|---|---|
| | Normal (N= 10) | Asthmatic (N = 9) | COPD (N = 8) | |
| Age (yr) | 52.1±9.4 | 51±10.4 | 56.3±7.9 | 0.3752 |
| Male:Female | 3:7 | 1:8[C] | 7:1[C] | 0.0031 |
| Caucasian:African American | 3:7 | 2:7 | 1:1 | 0.5258 |
| Never smoker | 9:1[B] | 6:3[C] | 0:8[BC] | 0.0002 |
| Atopic:Non-Atopic | 2:8[A] | 7:2[C] | 1:7[C] | 0.0079 |
| Pre-bronchodilator FEV1 (% predicted) | 89±13[B] | 83±26[C] | 55±15[BC] | 0.0006 |
| Post-bronchodilator FEV1(% predicted) | 92±12[B] | 89±11[C] | 56±14[BC] | 0.0007 |

Age and pre- and post-bronchodilator FEV1 are represented as means ± standard deviations.

*For continuous variables, p-values were determined 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's Chi-Square Test

Superscript[A] indicates that the distributions differ between the Normal and Asthmatic Groups, using a Bonferroni adjusted Type I error rate of 0.0167.

Superscript[B] indicates that the distributions differ between the Normal and COPD Groups, using a Bonferroni adjusted Type I error rate of 0.0167.

Superscript[C] indicates that the distributions differ between the Asthmatic and COPD Groups, using a Bonferroni adjusted Type I error rate of 0.0167.

## TABLE II

Proportions of myeloid-derived regulatory cells

| Cell Population | Group | | | p-value* |
|---|---|---|---|---|
| | Normal (N= 10) | Asthmatic (N = 9) | COPD (N = 8) | |
| (A) $CD11b^{-}DAF\text{-}FM\text{-}DA^{+}$ | 14.8 ±12.5[A] | 3.1 ± 1.3[A] | 7.0 ± 5.8 | .0267 |
| $CD11b^{+}DAF\text{-}FM\text{-}DA^{+}$ | 29.0 ± 13.9 | 42.1 ± 11.0[C] | 24.6 ± 11.9[C] | .0211 |
| (B) $CD14^{+}CD16^{+}$ | 91.3 ± 6.0[AB] | 29.8 ± 20.7[A] | 35.2 ± 25.4[B] | .0001 |
| (C) $CD14^{+}CD16^{-}$ | 1.5 ± 0.8[AB] | 40.0 ± 23.0[AC] | 5.2 ± 3.7[BC] | .0001 |
| (D) $CD14^{-}CD16^{+}$ | 1.6 ± 1.7[AB] | 22.6 ± 16.5[A] | 37.3 ± 15.5[B] | .0001 |
| (E) $CD163^{+}DHE^{+}CD11b^{+}CD11C^{+}HLA\text{-}DR^{+}$ | 17.7 ± 6.8[A] | 31.9 ± 11.5[A] | 18.1 ± 12.5 | .0243 |
| (F) $CD163^{-}DHE^{+}CD11b^{+}CD11C^{+}HLA\text{-}DR^{+}$ | 3.9 ± 3.5[AB] | 22.4 ± 14.4[A] | 19.2 ± 15.6[B] | .0008 |

(A) – (F) are subsets described in Figures 1A, 1B and 2.

(B) – (D) are proportions of $CD11b^{+}$ $DAF\text{-}FM\text{-}DA^{+}$ cells.

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

*p-values were determined using the Kruskal-Wallis test, to assess whether the distribution differ among the study groups.

Superscript[A] indicates that the distributions differ between the Normal and Asthmatic Groups, using a Bonferroni adjusted Type I error rate of 0.0167.

Superscript[B] indicates that the distributions differ between the Normal and COPD Groups, using a Bonferroni adjusted Type I error rate of 0.0167.

Superscript[C] indicates that the distributions differ between the Asthmatic and COPD Groups, using a Bonferroni adjusted Type I error rate of 0.0167.

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**TABLE III**

Summary of phenotypic and functional characteristics of myeloid-derived regulatory cell populations

| | Proportions of MDRC | | | Modulation of T cell response | | | Free radical pathway | | |
|---|---|---|---|---|---|---|---|---|---|
| **MDRC Populations** | **Normal** | **Asthmatic** | **COPD** | **Normal** | **Asthmatic** | **COPD** | **Normal** | **Asthmatic** | **COPD** |
| A | **Hi** | Lo | Lo | None | nd | nd | N/A | N/A | N/A |
| B | **Hi** | **Hi** | Lo | ↓ | ↓ | ↓ | iNOS | iNOS | iNOS |
| C | Lo | **Hi** | Lo | nd | ↓ | nd | N/A | iNOS ROS | N/A |
| D | Lo | **Hi** | **Hi** | nd | None | None | N/A | N/A | N/A |
| E | Lo | **Hi** | Lo | ↑ | ↑ | ↑ | ROS Arg | iNOS ROS Arg | ROS Arg |
| F | Lo | **Hi** | **Hi** | nd | ↑ | ↑ | N/A | iNOS ROS Arg | iNOS ROS Arg |

Populations A–F are as described in Figures 1A, 1B and 2

nd, not determined
N/A, not applicable
iNOS, inducible isoform of nitric oxide synthase
ROS, reactive oxygen species
Arg, Arginase