T-Regulatory Cells and Programmed Death 1⁺ T Cells Contribute to Effector T-Cell Dysfunction in Patients with Chronic Obstructive Pulmonary Disease

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Abstract

Rationale: Previous studies from our laboratory have shown that peripheral blood mononuclear cells (PBMCs) from patients with chronic obstructive pulmonary disease (COPD) prone to exacerbations with nontypeable *Haemophilus influenzae* have impaired responses to lipoprotein P6. We hypothesized that an underlying immunosuppressive network could be responsible for the defective antibacterial immunity observed in these patients. We evaluated T regulatory cells (Tregs), myeloid-derived suppressor cells (MDSC), and exhausted T effector cells (programmed death 1 [PD-1]⁺) in patients with COPD, because these cells are known to play a pivotal role in suppressing immune responses.

Objectives: We performed an in-depth characterization of Tregs, T effector cells, and MDSC in COPD and correlated their levels and function with disease severity.

Methods: Treg, effector T cell, and MDSC frequency from patients with COPD and healthy subjects' PBMCs were analyzed by flow cytometry. Treg immunosuppressive capacity was measured by

in vitro suppression assay. The frequency of interferon- γ producing T cells and T-cell proliferation were measured after blocking CTLA-4 and PD-1. Plasma proinflammatory and immunosuppressive cytokine levels were measured.

Measurements and Main Results: Significantly increased levels of Tregs, MDSC, and PD-1⁺ exhausted effector T cells were present in patients with COPD compared with healthy subjects. Tregs from patients with COPD suppressed P6-specific T-cell proliferation to a greater extent than Tregs from healthy subjects. Plasma levels of Treg-generated cytokines, IL-10, and transforming growth factor- β were elevated. Blockade of CTLA-4 resulted in significant augmentation of T-cell IFN- γ production in patients with COPD.

Conclusions: Functionally suppressive Tregs, MDSCs, and exhausted PD-1⁺ T cells contribute to effector T-cell dysfunction in COPD.

Keywords: Foxp3⁺ Tregs; myeloid-derived suppressor cells; cytokines; lung function; T effector cells

Chronic obstructive pulmonary disease (COPD), a leading cause of death worldwide, is initiated mainly by tobacco smoking. Acute and chronic infections with bacteria and viruses are observed in COPD

and contribute to the inflammation and the pathologic manifestations of COPD (1). Viruses or bacteria are responsible for lymphocyte activation in COPD and tobacco smoke also contains particles that

induce Th1 responses in susceptible individuals (2). The role of innate immune responses mediated by macrophages and neutrophils in the pathogenesis of COPD is well established as is the role of adaptive

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At a Glance Commentary

Scientific Knowledge on the

Subject: Our previous work has shown that peripheral blood mononuclear cells from patients with chronic obstructive pulmonary disease (COPD) have impaired responsiveness to bacterial antigens. There have been no studies to date that examined the effects of T regulatory cells (Tregs) on antigen-specific effector T-cell responses. Furthermore, the role of myeloid-derived suppressor cells in COPD remains to be elucidated.

What This Study Adds to the

Field: This study demonstrates for the first time that Tregs in patients with COPD are highly suppressive. The frequency of myeloid-derived suppressor cells is significantly higher in patients with COPD, which may also suppress immune responses by the induction of Tregs or secretion of immunosuppressive cytokines. CD4⁺CD127⁺ T effector cells in patients with COPD express an exhausted phenotype, which may be an additional cause for T-cell dysfunction.

immunity mediated by CD8⁺ T cells (3). We have previously shown that lymphocytes from patients with COPD with frequent nontypeable *Haemophilus influenzae* (NTHI) exacerbations responded poorly when stimulated *in vitro* with lipoprotein P6, an outer membrane protein of NTHI (4). We therefore hypothesized that this could be caused by the high prevalence of functional suppressor cells, such as T regulatory cells (Tregs), myeloid-derived suppressor cells (MDSCs), or functionally exhausted effector T cells (programmed death 1 [PD-1]⁺) in these patients.

Tregs are a subset of CD4⁺ T cells that play a key role in controlling inflammatory immune responses (5) and effector T-cell function by secretion of inhibitory cytokine, such as transforming growth factor (TGF)- β 1 and IL-10 (6). Altered Treg numbers have been observed in a variety of inflammatory diseases, such as inflammatory bowel disease (7, 8) and rheumatoid arthritis (9, 10). Only a limited

number of studies have investigated the presence of Tregs in COPD and reported different findings in lung tissue, bronchoalveolar lavage (BAL), or peripheral blood. Increased numbers of Foxp3⁺ Tregs in the bronchus-associated lymphoid tissue, CD25^{bright} Tregs in the BAL (11, 12), or peripheral blood of patients with COPD have been reported previously (13). In contrast, decreased number of CD25⁺ Tregs in the BAL of patients with COPD and nonsmokers was observed when compared with healthy smokers (14). Importantly, none of these studies evaluated Treg function in patients with COPD.

CTLA-4 expression on Tregs is essential to suppress immune responses by blocking the interactions between CD86/CD80 molecules on the antigen-presenting cells and CD28 on T cells (15). CTLA-4⁺ Tregs thus represent a highly immunosuppressive population and the potential involvement of circulating Foxp3⁺CTLA-4⁺ Tregs in COPD has not been examined previously.

PD-1, a negative costimulatory molecule expressed on immune effector cells, is up-regulated during a sustained inflammatory immune response. PD-1 impairs immune response by escalating IL-10 production, inducing apoptosis, and by causing functional exhaustion of T cells (16). We therefore examined whether exhausted T cells could be an additional source of T-cell dysfunction in patients with COPD.

Perturbations in the number, phenotype, and functional properties of both myeloid dendritic cells (mDCs) and plasmacytoid DC (pDCs) have been reported in chronic inflammatory immune diseases, such as inflammatory bowel disease, celiac disease (17), and COPD (18). Because there is a paucity of data on potential involvement of DCs in the pathogenesis of COPD, we evaluated pDC in the circulation of these patients.

MDSCs are elevated during chronic inflammation and malignancies (19). MDSCs cause profound suppression of both innate and acquired immunity. No studies have thus far examined the role of MDSCs in the pathogenesis of COPD. With the knowledge that MDSC can generate an immunosuppressive milieu and facilitate the up-regulation of Tregs, we investigated whether these cells could be involved in dampening immune responses in patients with COPD.

In the present study, an exhaustive multiparametric evaluation of Tregs, MDSC,

PD-1⁺ T cells, pDC, and effector T cells was performed in patients with COPD to correlate their levels with spirometrically defined severity of the disease. Furthermore, we measured peripheral blood cytokines and Treg functionality.

Methods

Blood Samples

This study was approved by the institutional review board of the VA Western New York Healthcare System and informed consent was obtained from all participants. Heparinized peripheral blood samples were obtained from stable patients with COPD (n = 49) attending the Buffalo Veterans Affairs Medical Center and from age-matched normal healthy donors (n = 43). Patients with COPD were participating in a longitudinal study of bacterial infection in COPD that has been described before (1, 20), and were free of exacerbation for at least 4 weeks at the time of blood draw. Criteria for inclusion in the longitudinal study was the presence of chronic bronchitis; smoking history of at least 10 pack-years; absence of other lung disease, such as asthma, bronchiectasis, and so forth; and ability to comply with monthly visits and study instructions. Healthy control subjects were nonsmokers with normal lung function, no respiratory symptoms, and were free of any respiratory disease. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) density gradient centrifugation of blood samples as described elsewhere (21). This study was conducted in two phases; immunophenotyping was performed in a cohort of patients with COPD (n = 24) and healthy subjects (n = 31), and cytokine mRNA profiles were studied in a subset of these patients (10 of 24), depending on the availability of cells. Treg suppressive function was examined in a different cohort of patients (n = 25) and healthy subjects (n = 12). Insufficient cell recovery after fluorescence-activated cell sorter (FACS) of Tregs and T effector cells in these samples precluded concurrent immunophenotyping.

Tregs, PD-1⁺ T Cells, pDC, and MDSC

FACS measurement of circulating CD25⁺CD127[−] Foxp3⁺CTLA-4⁺GARP⁺ Tregs, PD-1⁺ T cells, T effector cells, pDC,

and MDSC was performed by multicolor staining as described elsewhere (22).

Treg Suppression Assay

P6 lipoprotein of NTHI was purified as described before (23). FACS-sorted T effector cells ($CD4^+CD25^-CD127^+$) were stimulated with 2.5 μ g or 5 μ g endotoxin-free P6 lipoprotein in the presence or absence of FACS-sorted autologous Tregs ($CD4^+CD25^+CD127^-$) at a 2:1 ratio of T effectors to Tregs; lymphocyte proliferation was quantified as described before (4).

Lymphocyte Proliferation Assay and Intracellular Cytokine-Staining Assay

Carboxyfluorescein succinimidyl ester staining of PBMC was performed as described earlier (22). Labeled cells (5×10^4 cells per well) were incubated in the presence or absence of 5 μ g/ml PHA (Sigma, St. Louis, MO) in a 96-well flat bottom plate. Cells were either treated with 10 μ g/ml of anti-CTLA-4 antibody (clone L3D10)/anti-PD-1 antibody (clone EH12.2H7) (Biolegend, San Diego, CA) or untreated. After 3 days of stimulation, harvested cells were stained with APC-H7 anti-CD3, V450 anti-CD4 and V500 anti-CD8 (BD Biosciences, San Jose, CA). After fixation and permeabilization, intracellular staining was performed using PE anti-IFN- γ and PE isotype control (BD Biosciences). Proliferation of CD4 and CD8 T cells was analyzed by calculating proliferation index using the Flowjo Version 9 proliferation platform (TreeStar Inc., Ashland, OR).

Quantitative Real-Time Polymerase Chain Reaction

RNA extraction and cDNA synthesis was performed using RNeasy Protect Mini Kit (Qiagen) and Omni script reverse transcription kit (Qiagen) as described elsewhere (21). Quantitative polymerase chain reaction was performed on Bio-Rad CFX96 Real-Time system using gene-specific TaqMan probes and primers for Foxp3 (Hs01085835), CTLA-4 (Hs034418), PD-1 (Hs01550089), GARP (Hs00194136), and endogenous control glyceraldehyde phosphate dehydrogenase (Applied Biosystems, Foster City, CA) as described elsewhere (21).

Cytokine ELISA

Plasma isolated during PBMC separation and the culture supernatants from the *in vitro* proliferation assay were used to quantify the level of IFN- γ , IL-12p70,

Table 1. Characteristics of Patients with COPD Used for Immunophenotyping (n = 24)

Characteristics	Value	Range
Age, mean	68.5	56–81
Sex		
Male	23	
Female	1	
Race		
White	22	
African American	2	
Pack-years of smoking, mean	67.5	54–80
Smoking status		
Current smokers	10	
Ex-smokers	14	
FEV ₁ , L, mean	1.79	0.53–3.96
FVC, L, mean	3.45	1.76–5.62
FEV ₁ %, mean	49.4	26.77–77.5
FEV ₁ % predicted, mean	53.4	13–96
GOLD severity of airflow obstruction		
GOLD 1 (mild)	3	
GOLD 2 (moderate)	12	
GOLD 3 (severe)	6	
GOLD 4 (very severe)	3	

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; GOLD = Global Initiative for Chronic Obstructive Lung Disease.

IL-17A, tumor necrosis factor- α , IL-6, TGF- β 1, and IL-10 using ELISA kits according to the manufacturer's instructions (eBiosciences, San Diego, CA).

Statistical Analysis

Data were analyzed by permutation *t* test and Pearson correlation. Per comparison

two-sided *P* values less than 0.05 were considered as statistically significant. With 24 patients in each group, similarly conducted experiments have 80% power to detect a minimum difference in mean expression of 0.8 standard deviations. Given the number of comparisons conducted, we

Table 2. Characteristics of Patients with COPD Used for Functional Studies (n = 25)

Characteristics	Value	Range
Age	69.6	51–83
Sex		
Male	25	
Female	0	
Race		
White	24	
African American	1	
Pack-years of smoking, mean	41.9	25–58
Smoking status		
Current smokers	11	
Ex-smokers	14	
FEV ₁ , L, mean	1.78	0.7–4.07
FVC, L, mean	3.22	1.76–5.14
FEV ₁ %, mean	54.27	39.77–83.06
FEV ₁ % predicted, mean	49.7	20–95
GOLD severity of airflow obstruction		
GOLD 1 (mild)	1	
GOLD 2 (moderate)	13	
GOLD 3 (severe)	8	
GOLD 4 (very severe)	3	

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; GOLD = Global Initiative for Chronic Obstructive Lung Disease.

corrected for multiple testing using the methods developed by Hochberg (24). All analyses were done using SAS/STAT software, version 9.4 (SAS Institute Inc., Cary, NC).

Results

Subject Demographics

Clinical characteristics of patients are presented in Tables 1 and 2.

Patients with COPD Exhibit Elevated Levels of Circulating Immunosuppressive Cells

Using our previously defined markers to discriminate Tregs from effector T cells (see Figure E1 in online supplement) (22), we found that PBMCs from COPD contained both an elevated frequency and absolute number of $CD4^+Foxp3^+$ / $CD4^+CD25^+CD127^-Foxp3^+$ Tregs compared with healthy control subjects (Figures 1A, 1B, 1E, and 1F). In addition to

an increased number of Foxp3 mRNA copies in COPD (Figure 1C), there was a significant correlation between Foxp3 gene expression and $CD4^+Foxp3^+$ T-cell frequency (Figure 1D). Thus, not only is the frequency of $Foxp3^+$ Tregs elevated in patients with COPD, these cells also express more of the Foxp3 transcription factor than healthy control subjects.

Alongside elevated Treg levels, the frequency (Figure 1I) and absolute number of circulating

Figure 1. Accumulation of immunosuppressive cells in patients with chronic obstructive pulmonary disease (COPD). (A) Frequency and (B) absolute number of $CD4^+Foxp3^+$ T regulatory cells (Tregs) in the peripheral blood of patients with COPD ($n = 24$) and healthy control subjects ($n = 31$). (C) Quantitative polymerase chain reaction analysis of Foxp3 gene expression in peripheral blood mononuclear cells of patients with COPD ($n = 10$) and healthy donors ($n = 10$). Lines represent mean values of Foxp3 mRNA copies normalized to glyceraldehyde phosphate dehydrogenase (GAPDH) control. (D) Correlation between Foxp3 mRNA copies and $CD4^+Foxp3^+$ Treg frequency. (E) Frequency and (F) absolute number of $CD3^+CD4^+CD25^+CD127^-Foxp3^+$ Tregs in the peripheral blood of patients with COPD ($n = 24$) and healthy control subjects ($n = 31$). (G) Frequency and (H) absolute number of $CD3^+CD4^+CD127^-Foxp3^+$ effector T cells in patients with COPD and healthy control subjects. (I) Frequency and (J) absolute number of cells per milliliter of myeloid-derived suppressor cells (MDSCs) in the peripheral blood of patients with COPD ($n = 24$) and healthy donors ($n = 31$). (K) Correlation of $CD11b^+CD33^+$ MDSC frequency and $CD4^+Foxp3^+$ Treg frequency. Each symbol represents an individual COPD patient (black dots) or healthy control subject (grey squares); lines represent mean values for the group. *** $P < 0.001$, permutation t test; † $P < 0.05$ Hochberg adjustment for multiple comparison.

CD14⁺HLA-DR⁺CD11b⁺CD33⁺ MDSC (Figure 1J) was significantly increased in patients with COPD compared with control subjects. The frequency of MDSC in patients with COPD displayed a high degree of correlation with the percentage of Foxp3⁺ Tregs (Figure 1K). Importantly, elevated levels of MDSC was not indicative of dysregulation of all myeloid cells, because the frequency of CD11c⁺CD123⁺ pDC in patients with COPD was equivalent to levels observed in healthy control subjects (see Figure E2).

Tregs from Patients with COPD Express Elevated Levels of CTLA-4

To determine whether Tregs from patients with COPD exhibited the potential for high immunosuppressive capacity, expression of GARP and CTLA-4 was examined because these two markers are known to be expressed on highly immunosuppressive Tregs (22). The frequency of GARP⁺Foxp3⁺ Tregs and surface GARP expression level in COPD was comparable with healthy donors (Figures 2A and 2B). Interestingly, GARP

mRNA transcripts were significantly higher in patients with COPD (Figure 2C), but did not correlate with GARP⁺ Treg frequency (see Figure E3A).

Importantly, in contrast to GARP, the frequency of Foxp3⁺ Tregs that expressed surface CTLA-4 was significantly greater in patients with COPD (Figure 2D). The CTLA-4 protein (Figure 2E) and mRNA (Figure 2F) were also significantly higher in Tregs from patients with COPD compared with healthy subjects. Correlation of CTLA-4 mRNA transcripts

Figure 2. GARP and CTLA-4 expressing T regulatory cells (Tregs) in patients with chronic obstructive pulmonary disease (COPD). (A) Frequency of GARP⁺ Tregs and (B) GARP expression levels measured by mean fluorescent intensity (MFI) on Tregs from COPD (n = 24) and healthy subjects (n = 31). (C) Quantitative polymerase chain reaction analysis of GARP gene expression in peripheral blood mononuclear cells of patients with COPD (n = 10) and healthy donors (n = 10). Lines represent mean values of GARP mRNA copies normalized to glyceraldehyde phosphate dehydrogenase (GAPDH) control. (D) Frequency of CTLA-4⁺ Tregs and (E) CTLA-4 expression levels. (F) Quantitative polymerase chain reaction analysis of CTLA-4 gene expression in peripheral blood mononuclear cells of patients with COPD (n = 10) and healthy donors (n = 10). Lines represent mean values of CTLA-4 mRNA copies normalized to GAPDH control. (G) Correlation of either GARP⁺ Treg frequency or (H) CTLA-4⁺ Treg frequency to CD25⁺CD127⁺Foxp3⁺ Treg frequency in patients with COPD (n = 24) and healthy control subjects (n = 31). Each symbol represents an individual COPD patient (black dots) or healthy control subject (grey squares); lines represent mean values for the group. ***P < 0.001, permutation t test; †P < 0.05 Hochberg adjustment for multiple comparison.

and Foxp3⁺CTLA-4⁺ Treg frequency demonstrated that patients with COPD had both elevated levels of this immunosuppressive cell and expressed more CTLA-4 mRNA on a per cell basis compared with healthy control subjects (see Figure E3B). Frequency of GARP⁺ Tregs did not correlate with frequency of CD25⁺CD127⁻Foxp3⁺ T cells (Figure 2G). In contrast, CTLA-4⁺ T-cell frequency displayed high degree of correlation with frequency of CD4⁺CD25⁺CD127⁻Foxp3⁺ Tregs in patients with COPD and healthy control subjects (Figure 2H). Thus, accumulation of cells with this phenotype may play an important role in immune dysregulation in COPD.

COPD Patient Tregs Exhibit Enhanced Suppressive Activity on Effector T Cells

The presence of significantly elevated levels of immunosuppressive cells prompted us to examine the frequency of effector T cells in patients with COPD and the functional ability of Tregs to suppress autologous effector cells. Effector CD4⁺ T cells were distinguished from CD4⁺ Tregs by the combined absence of Foxp3 expression and presence of CD127 expression (CD4⁺CD127⁺Foxp3⁻ effector cells vs. CD4⁺CD127⁻Foxp3⁺Tregs) (see Figures E4A and E4B).

Although effector CD4⁺ T-cell frequency was similar between patients with COPD and healthy control subjects (Figure 1H), we additionally evaluated the expression of PD-1 on CD127⁺CD4⁺ effector T cells (see Figures E4C and E4D) to determine whether an exhausted T-cell phenotype could further account for T-cell dysfunction in the pathogenesis of COPD. Both the frequency of PD-1⁺CD127⁺ T cells (Figure 3A) and the level of PD-1 expression on these cells (Figure 3B) was significantly higher in patients with COPD than in healthy donors. PD-1⁺CD127⁺ effector T cells from patients with COPD (see Figure E4C) and healthy control subjects (see Figure E4D) did not express Foxp3; conversely, Foxp3 expression was only observed in PD-1⁻CD127⁻ Tregs (see Figures E4E and E4F).

The immunosuppressive capacity of COPD patient Tregs was tested by their ability to suppress the proliferation of NTHI-specific autologous effector T cells. CD4⁺CD25⁻CD127⁺ effector T cells were sorted from PBMCs of patients with COPD

Figure 3. High suppressive potential of T regulatory cells (Tregs) and functional exhaustion of effector T cells in patients with chronic obstructive pulmonary disease (COPD). (A and B) Frequency of CD4⁺PD-1⁺CD127⁺ T cells and PD-1 expression level on CD4⁺ T cells measured as mean fluorescent intensity (MFI). (C and D) Proliferative response of sorted CD4⁺CD25⁻CD127⁺ effector cells to P6 protein in the presence or absence of purified CD4⁺CD25⁺CD127⁻ autologous Tregs. Each symbol represents an individual COPD patient (black dots) or healthy control subject (grey squares); lines represent mean values for the group. **P* < 0.05, ****P* < 0.001, permutation *t* test; †*P* < 0.05 Hochberg adjustment for multiple comparison. PD-1 = programmed death 1.

and healthy control subjects and stimulated with 2.5 or 5 µg/ml of purified lipoprotein P6. The effector cells from both groups were cultured in the presence or absence of sorted autologous CD4⁺CD25⁺CD127⁻ Tregs at 2:1 T effector/Treg ratio (Figures 3C and 3D). At both concentrations of P6, COPD effector T cells proliferated to a lesser extent than effector T cells from healthy control subjects (Figures 3C and 3D, solid black box vs. solid gray box). Although Tregs isolated from both patients with COPD and healthy control subjects suppressed autologous antigen-stimulated effector T-cell proliferation, COPD effector T-cell proliferation was suppressed to a greater extent (67%, 63%) than in healthy control subjects (46%, 49%) (Figures 3C and 3D, open black box vs. open gray box). Not only do COPD effector T cells respond poorly to bacterial antigens, they are suppressed to a greater extent by their own Tregs than observed using Tregs from healthy control subjects. Thus overall the effector T-cell dysfunction in patients with COPD includes the accumulation of PD-1⁺ exhausted cells and a depressed proliferative response to bacterial antigens.

Dysregulated Cytokine Profile in Patients with COPD

Augmented immunosuppression in patients with COPD was also reflected by significant increases in the peripheral blood levels of two Treg-generated immunosuppressive cytokines, IL-10 and TGF-β1 (Figures 4A and 4B). The high frequency of Foxp3⁺ Tregs demonstrated significant correlation with levels of TGF-β1 in patients with COPD (Figure 4C). In addition to this immunosuppressive milieu, the levels of proinflammatory Th1-associated cytokines IFN-γ and IL-12 were significantly increased in patients with COPD (Figures 4D and 4E). Therefore, our results demonstrate that the plasma cytokine milieu in patients with COPD is skewed toward an immunosuppressive and proinflammatory phenotype.

Correlation of Immune Parameters with Clinical Outcome of Patients with COPD

We observed that levels of GARP⁺ Tregs in patients with COPD displayed a positive correlation with improved lung function (FEV₁% predicted) (Figure 5A), suggesting

Figure 4. Elevated levels of immunosuppressive cytokines in patients with chronic obstructive pulmonary disease (COPD). Cytokine-specific sandwich ELISA of plasma from patients with COPD and healthy normal subjects were performed to measure the levels of circulating (A) IL-10, (B) transforming growth factor (TGF)-β1, (D) IFN-γ, and (E) IL-12. (C) Correlation of plasma TGF-β1 levels and frequency of CD4⁺Foxp3⁺ T regulatory cells (Tregs). Each symbol represents an individual patient with COPD (black dots) or healthy control subject (grey squares); lines represent mean values for the group. **P < 0.01, ***P < 0.001, permutation t test; †P < 0.05 Hochberg adjustment for multiple comparison.

that these highly suppressive Tregs may have a beneficial effect on lung function but attenuate pathogen-specific T-cell responses. Frequencies of Foxp3⁺/CTLA-4⁺ Tregs, PD-1⁺ T cells, MDSC, or pDC did not show any correlation with lung function. Elevated levels of the proinflammatory cytokines IFN-γ and IL-12 exhibited an inverse relationship with lung function (FEV₁) (Figures 5B and 5C), suggesting that in patients with COPD with elevated levels of these two potent Th1 cytokines, the proinflammatory immune response worsens their lung function.

Functionality of CD4⁺ and CD8⁺ T Cells after Blocking Immune Checkpoint Receptors

The combined elevated levels of CD25⁺Foxp3⁺ Tregs expressing CTLA-4 and PD-1⁺ effector T cells prompted us to test whether blockade of these checkpoints could enhance effector T-cell function. Proliferation (see Figure E5A) and cytokine production (see Figure E5B) of antigen-stimulated COPD patient effector T cells was examined in the presence or absence of anti-CTLA-4 or anti-PD-1. These receptors were targeted individually to

elucidate the relative contribution of either Treg CTLA-4 blockade or exhausted effector T-cell PD-1 blockade for uncovering beneficial effector T-cell function. The proliferation index of CD4⁺ T cells and CD8⁺ T cells (Figures 6A and 6B, *left graph*) was enhanced in the presence of anti-CTLA-4 or anti-PD-1 as compared with stimulated cells without either blockade. In the presence of anti-CTLA-4, a prominent increase in the frequency of IFN-γ-producing cells (Figures 6A and 6B, *middle graph*) and the level of IFN-γ produced (Figures 6A and 6B, *right graph*) was observed in both CD4⁺ T cells and CD8⁺ T cells. Interestingly, anti-PD-1 treatment resulted in a moderate enhancement in IFN-γ production, suggesting that targeting Treg suppression via CTLA-4 blockade could beneficially enhance antibacterial immunity in patients with COPD. Additionally, detection of secreted cytokines revealed significantly higher levels of IFN-γ in culture supernatants from PBMC treated with anti-CTLA-4 (Figure 6C). Although IFN-γ levels were elevated by anti-CTLA-4 treatment, there was no reduction in production of the Treg-associated

immunosuppressive cytokines IL-10 or TGF-β1. These results therefore confirm that immune dysfunction in COPD is associated with expression of CTLA-4 and PD-1, which represent independent targetable sources of immunosuppression.

Discussion

In this study, we report for the first time that functionally suppressive Tregs and exhausted PD-1⁺ T cells are two major factors contributing to effector T-cell dysfunction in patients with COPD. Previous studies of Treg in COPD have reported varied findings in the lung, BAL, and peripheral blood (12–14, 25). However, Treg functionality has not been studied in patients with COPD. Our analysis has used the dual expression of Foxp3 and CTLA-4 to define suppressive Tregs. We determined that this population was significantly elevated in patients with COPD compared with healthy control subjects. Importantly, we have demonstrated that Tregs in COPD are highly suppressive in function and could inhibit autologous effector T-cell responses

Figure 5. Correlation of immune parameters with lung functions of patients with chronic obstructive pulmonary disease (COPD). (A) Correlation of GARP⁺ Foxp3⁺ T regulatory cells (Tregs), (B) levels of plasma IFN-γ, and (C) levels of plasma IL-12 with lung functions (FEV₁) of patients with COPD. Each symbol represents an individual COPD patient (black dots).

against the P6 lipoprotein of NTHI. We, however, recognize that the differential effector cell proliferative response in patients with COPD versus healthy control subjects makes the overall direct comparison of the Treg suppressive effect difficult. Lee and coworkers (25) conducted functional analysis of Tregs in patients with emphysema and control subjects and reported that Tregs from both groups markedly inhibited the proliferation of autologous T cells in response to anti-CD3/CD28 stimulation. Importantly, we also have shown for the first time that blockade of immune check-point receptors CTLA-4/PD-1 resulted in the enhancement of effector T-cell function in patients with COPD. These findings support the conclusion that Tregs may be at least partially responsible for compromised antipathogen immunity in patients with COPD and may account for chronic pulmonary infection and frequent exacerbations.

Potentially suppressive Tregs may attenuate T effector cell-mediated

destruction of lung epithelium, which if unregulated may eventually lead to emphysematous condition. Importantly, the frequency of Foxp3⁺GARP⁺ Tregs demonstrated a direct relationship with lung function of patients with COPD, which may represent a beneficial effect of Tregs. Consistent with this idea, smokers who do not develop COPD also have elevated number of Tregs in their lungs (14, 26) and these cells may be efficient in attenuating smoke-induced inflammatory responses in the lungs. Antiinflammatory drugs salmeterol and fluticasone or increased indoleamine 2,3-dioxygenase activity have been reported to reduce severity of COPD symptoms by enhancing Treg activity (27, 28). A recent study in a mouse model of chronic cigarette smoke exposure has shown that therapeutic inhibition of effector T cells protected mice from alveolar destruction and the development of emphysema; a similar approach may be efficacious for COPD (29). Our studies suggest the dual nature of

Tregs in COPD. On one hand, Tregs limit cell-mediated immune responses that cause detrimental inflammation and subsequent pathogenesis; on the other hand, Tregs inhibit T effector cell-mediated immune responses, which may facilitate pathogen persistence. A recent report on the dual nature of Tregs in *Mycobacterium tuberculosis* infection of lung corroborates with our findings (30).

Our data are the first demonstration that Foxp3⁺ Tregs from patients with COPD are effective at suppressing NTHI-specific effector T cells. Although the levels of CD127⁺CD25⁻ effector T cells were similar between COPD and healthy subjects, these cells from patients with COPD exhibited a reduced capacity to respond to P6. This weakened response was further diminished by the potent suppressive capacity of the Tregs. Thus, antibacterial immunity in patients with COPD is limited by two equally important factors: the inability of effector T cells to robustly respond to bacterial antigens, and the increased accumulation of Tregs. One of the factors that may account for each of these suppressive aspects is the elevated expression of PD-1 on effector T cells and CTLA-4 on Tregs. By examining the relative contribution of blocking PD-1 or CTLA-4, this might reveal an optimal target for uncovering effector T-cell function. Our finding of enhanced proliferation and IFN-γ production in cells treated with anti-CTLA-4 suggests that dampening Treg function may represent the dominant immunosuppressive mechanism. Although PD-1 blockade resulted in only a modest enhancement of effector T-cell function, its use for further uncovering effector responses may require simultaneous treatment with CTLA-4 blockade. The GARP⁺CTLA-4⁺ Tregs in patients with COPD may represent a targetable source for alleviating immunosuppression of antibacterial immunity. Our study is the first demonstration of the differential effect of CTLA-4 versus PD-1 blockade on T-cell responses in COPD. In contrast to our findings in patients with COPD, in hepatitis C virus infection it has been reported that the frequency of CD127⁺ hepatitis C virus-specific T cells and not the expression of exhaustion markers on hepatitis C virus-specific T cells or the intrahepatic levels of their ligands predicted the outcome of acute infection (31). Similarly, in a mouse model of fungal sepsis no

Figure 6. Enhanced T-cells functionality after blocking immune check-points in patients with chronic obstructive pulmonary disease. (A) Proliferation of CD4⁺ T cells (left), frequency of CD4⁺ T cells producing IFN- γ (middle) and expression levels of IFN- γ on CD4⁺ T cells (right) measured as mean fluorescence intensity (MFI). (B) Proliferation of CD8⁺ T cells (left), frequency of CD8⁺ T cells producing IFN- γ (middle), and expression levels of IFN- γ on CD8⁺ T cells (right) measured as MFI. Peripheral blood mononuclear cells from patients with chronic obstructive pulmonary disease ($n = 3$) were stimulated *in vitro* in the presence or absence of anti-CTLA-4 or programmed death 1 (PD-1) antibody. T-cell proliferation was measured based on dilution of carboxyfluorescein succinimidyl ester and proliferation index was calculated using the Flowjo Version 9 proliferation platform (TreeStar). Intracellular cytokine staining was performed to measure IFN- γ . (C) Cytokine-specific sandwich ELISA of the culture supernatants from proliferation assay was performed to measure the levels of secretory IFN- γ , IL-6, IL-10, IL-17-A, transforming growth factor (TGF)- β 1, and tumor necrosis factor (TNF)- α . * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, permutation t test; † $P < 0.05$ Hochberg adjustment for multiple comparison.

significant changes in splenic T-cell IFN- γ production were found in mice treated with either anti-PD-1 or anti-CTLA-4 antibodies (32).

In addition to examining T cells, we have also provided the first evidence of MDSC accumulation in patients with COPD. Although the presence of MDSCs has been extensively studied in patients with cancer, their role in COPD has not been investigated thus far. In our study, elevated levels of MDSC correlated with elevated levels of Tregs, which is in agreement with studies that suggest reciprocal control of these two cell types. Inflammation promotes the accumulation of MDSC, which induce Tregs producing TGF- β 1 and IL-10, which results in effector T-cell suppression (19). In support of these findings, we have demonstrated that the frequency of circulating Tregs in patients with COPD shows an excellent correlation

with the percentage of MDSC and levels of plasma TGF- β 1. LPS-induced CD11b⁺Gr1⁺F4/80⁺ regulatory myeloid cells have been reported to suppress allergen-induced airway inflammation in a mouse model (33–35). Elevated numbers of potentially suppressive MDSC may cause a blunted immune response in COPD and this could be a potential link between chronic inflammation and cancer (19).

In conclusion, our studies provide clear evidence for the accumulation of functionally suppressive circulating Tregs and MDSCs in patients with COPD, which may counteract inflammatory immune reactions occurring in the lungs. T effector cells in patients with COPD are not only suppressed by Tregs but they also express an exhausted phenotype, which may mitigate their ability to carry out efficacious antiviral and antibacterial

effector functions. Blockade of immune check-points, CTLA-4, and PD-1 resulted in the enhancement of effector T-cell responses. Therefore, CTLA-4⁺/PD-1⁺ cells could also comprise a novel therapeutic target for the treatment of COPD. In this patient population, a fine balance must be achieved between proinflammatory responses that clear pathogens but damage lung tissue and immunosuppressive cells and cytokines that attenuate proinflammatory responses but hinder antibacterial immunity. ■

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