Deficient and Dysfunctional Regulatory T Cells in the Lungs of Chronic Beryllium Disease Subjects

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Rationale: Chronic beryllium disease (CBD) is a CD4⁺ T cell–mediated disorder characterized by persistent lung inflammation. Naturally occurring regulatory T (T_{reg}) cells modulate adaptive immune responses. The role of this T-cell subset in beryllium-induced lung disease is unknown.

Objectives: The aim of this study was to determine whether dysfunctional T_{reg} cells in the lung contribute to the “unchecked” inflammatory response that characterizes CBD.

Methods: Using blood and bronchoalveolar lavage (BAL) cells from normal control subjects and individuals with beryllium-induced disease, we determined the frequency and function of naturally occurring T_{reg} cells.

Measurements and Main Results: A significantly decreased percentage and expression of FoxP3 in BAL CD4⁺ T cells from CBD patients compared with beryllium-sensitized subjects was seen, and the percentage of FoxP3-expressing CD4⁺ T_{reg} cells in BAL inversely correlated with disease severity. In contrast to blood T_{reg} cells derived from beryllium-sensitized subjects and patients with CBD that completely suppressed blood responder T-cell proliferation, BAL FoxP3-expressing T_{reg} cells from patients with CBD are unable to suppress anti-CD3-mediated BAL T-cell proliferation. Mixing studies showed that blood T_{reg} cells are capable of suppressing autologous BAL responder T cells. Conversely, BAL CD4⁺ T_{reg} cells are incapable of suppressing blood T cells, confirming that the failure of BAL T_{reg} cells to suppress T-cell proliferation is caused by a dysfunctional T_{reg} cell subset and not by resistance of BAL effector T cells to suppression.

Conclusions: These findings suggest that the deficient and dysfunctional T_{reg} cells in the lung of patients with CBD contribute to the persistent inflammatory response in this disease.

Keywords: fibrosis; human; granuloma; inflammation

Beryllium exposure in the workplace continues to be a public health concern with approximately 1 million individuals exposed and potentially at risk for developing chronic beryllium disease (CBD) (1). Depending on the nature of the exposure (2–4) and the genetic susceptibility of the individual (5–9), CBD develops in up to 18% of exposed workers. The disease is characterized by noncaseating granulomatous inflammation in the lung and the subsequent development of progressive lung fibrosis (10). CD4⁺ T cells play a vital role in the immunopatho-

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Chronic beryllium disease is characterized by persistent lung inflammation despite removal of individuals from further beryllium exposure and initiation of treatment. No study to date has evaluated the role of naturally occurring regulatory T cells in this persistent inflammatory process.

What This Study Adds to the Field

This study identifies a deficient and dysfunctional regulatory T cell subset in the lung of chronic beryllium disease patients and suggests that the impaired functional capacity of these immune-modulating cells contributes to the “unchecked” beryllium-induced immune response in the lung.

genesis of CBD, and the development of lung granulomas is associated with the accumulation of large numbers of beryllium-specific CD4⁺ T cells in the bronchoalveolar lavage (BAL) (11–14). These beryllium-responsive T cells are compartmentalized to lung (15), secrete Th1-type cytokines (15, 16), and persist at high frequency in patients with active disease (14, 17). Unfortunately, the natural history of beryllium-induced disease is characterized by a gradual decline in lung function (18), with one-third of untreated patients historically progressing to end-stage respiratory insufficiency (19).

Although the role of effector CD4⁺ T cells in driving beryllium-induced inflammation in the lung is well-established, no studies to date have analyzed the presence and functional capacity of regulatory T (T_{reg}) cell subsets in this occupational lung disease. T_{reg} cells play a key role in immunoregulation, in particular suppressing the activation and expansion of effector CD4⁺ and CD8⁺ T cells (20, 21). Naturally occurring T_{reg} cells are derived in the thymus and are characterized as CD4⁺ with a high level of CD25 expression (CD4⁺CD25^{hi}). Depletion of these CD4⁺CD25⁺ T cells, which comprise 5–10% of circulating CD4⁺ T cells, results in the spontaneous onset of multiorgan autoimmunity (20–23). Naturally occurring T_{reg} cells are thought to mediate their effects through cell contact and are anergic to *in vitro* stimulation (24, 25). FoxP3, a member of the forkhead winged helix family of transcription factors, has been identified as a specific molecular marker for T_{reg} cells, and its expression is essential for programming both thymic development and function of T_{reg} cells (26–28). To date, FoxP3 is the most specific molecular marker for naturally occurring T_{reg} cells (27).

Because of the persistent CD4⁺ T-cell alveolitis that characterizes CBD even in the absence of continued beryllium exposure, we hypothesized that either a deficiency or dysfunction of FoxP3-expressing CD4⁺ T_{reg} cells allows for the development

(Received in original form January 1, 2010; accepted in final form March 5, 2010)

Supported by National Institutes of Health grants HL62410, HL92997, AI050864, and ES011810 (A.P.F.), and the Clinical Translational Research Center (UL1 RR025780) from the National Center for Research Resources.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 181, pp 1241–1249, 2010

Originally Published in Press as DOI: 10.1164/rccm.201001-0025OC on March 18, 2010
Internet address: www.atsjournals.org

TABLE 1. DEMOGRAPHICS OF STUDY SUBJECTS*

Characteristics	Normal Controls (n = 25)	BeS Patients (n = 15)	CBD Patients (n = 24)
Age, yr	37 (23–50)	53 (27–77)	59 (44–79)
Gender (M/F)	12/13	13/2	19/5
Race (W/AF/H)	23/0/0	13/1/1	20/2/2
Smoking status (CS/FS/NS)	0/5/20	0/7/8	0/11/13
Industry of exposure (nuclear/ceramic/other)	N/A	11/1/3	18/4/2
Treatment (none/prednisone)	25/0	15/0	20/4
DL _{CO} , % predicted		90 (68–120)	80 (17–94) [†]
BeLPT, stimulation index			
PBL		4.6 (0–24)	3.2 (0.9–162)
BAL		1.5 (1–3.9)	16.9 (0.9–262) [‡]
Bronchoalveolar lavage cells			
WBC count (× 10 ⁶)		18 (11–84)	26 (7–144)
Lymphocytes, %		4.6 (1.5–20)	32 (6.7–52) [‡]

Definition of abbreviations: AF = African American; BAL = bronchoalveolar lavage; BeLPT = beryllium lymphocyte proliferation test; BeS = beryllium-sensitized; CBD = chronic beryllium disease; CS = current smoker; DL_{CO} = diffusing capacity of the lung for carbon monoxide; FS = former smoker; H = Hispanic; NS = never smoker; PBL = peripheral blood lymphocyte; W = white; WBC = white blood cell.

* Data expressed as median (range).

[†] $P < 0.05$.

[‡] $P < 0.0001$.

and perpetuation of an exaggerated beryllium-specific T cell-dependent immune response in the lung, resulting in worsening disease severity. Thus, we compared the frequency and function of FoxP3-expressing CD4⁺ T_{reg} cells in the blood and BAL of subjects with beryllium-induced disease (i.e., CBD and beryllium sensitization) with those of healthy control subjects.

METHODS

A detailed METHODS section is included in the online supplement.

Study Population

Twenty-four patients with CBD and 15 beryllium-sensitized (BeS) patients were enrolled in this study. Twenty-five healthy, non-beryllium-exposed control subjects were also enrolled. Of the subjects enrolled, BAL was performed on 6 control subjects, 15 BeS subjects, and 16 patients with CBD. CBD was diagnosed using previously defined criteria, including the presence of granulomatous inflammation on lung biopsy and a positive proliferative response of blood or BAL T cells to beryllium sulfate (BeSO₄) *in vitro* (12, 29). Beryllium sensitization was diagnosed based on a positive proliferative response of peripheral blood mononuclear cells (PBMCs) to BeSO₄ *in vitro* and the absence of granulomatous inflammation or other abnormalities on lung biopsy (30, 31). The demographics of the study population are shown in Table 1. Informed consent was obtained from each subject, and the protocol was approved by the Human Subject Institutional Review Boards at the University of Colorado Denver and National Jewish Health.

Immunofluorescence Staining and Analysis of Intracellular Cytokine Expression

PBMCs were isolated from heparinized blood, and BAL was obtained as previously described (14, 17). PBMCs and BAL cells were surface stained with anti-CD4 (PerCP; BD, San Jose, CA) and anti-CD25 (allophycocyanin; BD) in phosphate buffered saline supplemented with 1% bovine serum albumin, washed, fixed, and permeabilized before intranuclear staining with anti-FoxP3 (PE; eBioscience, San Diego, CA). Cells were washed and resuspended in 1% formaldehyde. Intracellular cytokine staining was performed as previously described (32). The lymphocyte population was identified using forward and 90-degree light scatter patterns, and fluorescence intensity was analyzed using a FACSCalibur flow cytometer (BD) (32). Analysis was performed using CellQuest Pro (BD) or FlowJo (Tree Star, Ashland, OR) software.

Measurement of the Functional Capacity of CD4⁺CD27⁺CD25⁺ T_{reg} Cells

For purification of T_{reg} cells, CD4⁺ T cells were sorted based on the coexpression of CD25 and CD27 (33). PBMCs and BAL were presorted by negative selection using a CD4⁺ T-cell isolation kit II (Miltenyi Biotec, Auburn, CA) and stained with anti-CD4 (PerCP; BD), anti-CD25 (allophycocyanin; BD), and anti-CD27 (FITC; BD). Cells were sorted on a FACSARIA flow cytometer (BD). Sorted CD4⁺CD25⁺CD27⁺ (T_{reg}) and a fixed number (2.5 × 10³ cells/well) of CD4⁺CD25[−] (responders) T cells were cultured in varying ratios of responder T cells to T_{reg} cells (1:1, 1:1/2, 1:1/4, and 1:1/8) in the presence of 1 × 10⁴ irradiated T cell-depleted PBMC (antigen-presenting cells). Cells were stimulated with plate-bound anti-CD3 (0.1 μg/ml; clone OKT3; Ortho Biotech, Raritan, NJ) and cultured at 37°C for 6 days in RPMI-C as defined in the online supplement. On day 5, 1 μCi of [³H] thymidine (PerkinElmer, Waltham, MA) was added to each well. After 18 hours, cells were harvested, and incorporation of radioactivity was detected by β-emission spectroscopy. For “crossover” experiments, blood T_{reg} cells were mixed with autologous BAL responder T cells and BAL T_{reg} cells were mixed with autologous blood-derived responder T cells.

Statistical Analysis

The Mann-Whitney *U* test and the Kruskal-Wallis analysis of variance with Dunn multiple comparison test were used to determine the significance of differences between subject groups. A Spearman correlation was performed to analyze the association between diffusing capacity of the lung for carbon monoxide (DL_{CO}; % predicted) and FoxP3 expression in CD4⁺ T cells. A *P* value of < 0.05 was considered statistically significant.

RESULTS

CD25 Expression on CD4⁺ T Cells in Blood and BAL

To assess the activation state of CD4⁺ T cells that are recruited to the lung in response to inflammation, we used monoclonal antibodies directed against CD4 and the activation marker, CD25, to determine the frequency of CD4⁺CD25⁺ T cells in blood and BAL of normal control, BeS, and CBD subjects. Representative examples of CD25 expression on the surface of blood and BAL CD4⁺ T cells from these three groups are shown in Figure 1A. Overall, no significant difference in the expression of CD25 was observed in blood of normal control

Figure 1. CD25 expression on CD4⁺ T cells from the blood and bronchoalveolar lavage (BAL) of normal control, beryllium-sensitized (BeS), and chronic beryllium disease (CBD) subjects. (A) Representative density plots of blood and BAL CD4⁺ T cells from normal control, BeS, and CBD subjects for expression of CD25. The percentage of cells in each quadrant of the density plots is shown. (B) The percentage of CD4⁺ T cells expressing CD25 in blood (left) of normal control subjects ($n = 25$), BeS subjects ($n = 15$), and CBD subjects ($n = 24$) and BAL (right) of normal controls ($n = 6$), BeS ($n = 15$), and CBD ($n = 16$) is shown. Median values for each disease group are indicated with solid lines, and statistical comparisons were made using the Kruskal-Wallis test with Dunn multiple comparison test. PBMC = peripheral blood mononuclear cell.

($n = 25$), BeS ($n = 15$), and CBD ($n = 24$) subjects. Conversely, a significantly increased frequency of CD4⁺CD25⁺ T cells was seen in BAL of BeS subjects (median, 12%; range, 2–49%) compared with CBD patients (median, 4.1%; range, 1.7–9.9%; $P < 0.001$) and normal control subjects (median, 3.7%; range, 2.9–5.8%; $P < 0.01$) (Figure 1B). Because CD25 (α -chain of the IL-2 receptor) is expressed on activated T cells, the absence of an expanded CD25-expressing T-cell population in the CBD lung compared with BeS subjects was a surprising finding given the increased BAL white blood cell count and the intense CD4⁺ T-cell alveolitis that characterize CBD.

FoxP3 Expression in Blood and BAL CD4⁺ T Cells from BeS Subjects and Patients with CBD

Because CD25 is coexpressed on naturally occurring T_{reg} cells, we next queried whether a deficiency of FoxP3-expressing CD3⁺CD4⁺ T cells exists in the target organ of patients with

CBD. Representative examples of intracellular FoxP3 expression in blood and BAL cells from a patient with CBD are shown in Figure 2A, with the percentage of CD3⁺CD4⁺ T cells expressing FoxP3 in blood and BAL being 6.6% and 2.6%, respectively. Similar to CD25 expression in blood, no difference in the frequency of FoxP3-expressing CD4⁺ T cells in blood of normal control, BeS, and CBD subjects was seen (Figure 2B). In contrast, a significantly decreased frequency of FoxP3-expressing CD4⁺ T cells was seen in BAL of patients with CBD (median, 2.3%; range, 0.5–9.2%) compared with BeS subjects (median, 8.6%; range, 2.7–14%; $P < 0.001$) and normal controls (median, 12%; range, 9.6–12%; $P < 0.001$). The decreased frequency of FoxP3-expressing CD4⁺ T cells in the BAL of patients with CBD was not caused by a decrease in the absolute number of these cells compared with BeS subjects (Figure 2C). However, in the setting of a dramatic increase in the number of effector CD4⁺ T cells recruited to the lung of patients with CBD, a significantly increased ratio of effector to regulatory T cells was seen in patients with CBD (Figure 2D), indicating a relative deficiency of this immunomodulatory T-cell subset in the face of an intense CD4⁺ T-cell alveolitis.

To determine whether the loss of naturally occurring T_{reg} cells in the lung affects disease severity, we correlated FoxP3 expression on BAL CD4⁺ T cells from BeS subjects and patients with CBD with DL_{co} (percent predicted). Previous studies have shown that DL_{co} is one of the most sensitive static markers of gas exchange in beryllium-induced disease (18), and percent predicted DL_{co} was the only physiologic marker that was significantly different between BeS and CBD patients (Table 1). Eleven BeS and 14 CBD subjects had a DL_{co} performed as part of their clinical evaluation. As shown in Figure 2E, a significant positive correlation was seen between the percentage of BAL CD4⁺ T cells expressing FoxP3 and DL_{co} ($r = 0.65$; $P = 0.0004$). Taken together, these findings raise the possibility that the reduced frequency of naturally occurring CD4⁺ T_{reg} cells in the lung of patients with CBD may play an important role in disease severity.

FoxP3 Expression in CD4⁺CD25^{hi} T Cells in Blood and BAL of CBD Patients

It was previously thought that FoxP3 expression served as an on-and-off switch to positively regulate the suppressive function of T_{reg} cells. However, recent evidence associates decreased FoxP3 expression in T_{reg} cells with diminished function in various immune disorders (34–37). Thus, we compared FoxP3 expression on a per cell basis in CD25^{hi}, CD25^{lo}, and CD25^{neg} T cells from blood and BAL of patients with CBD. The gates for CD25 were set based on CD25 expression on CD3⁺CD4⁺ T cells, where the pattern of CD25 expression is more distinct. CD25^{hi} and CD25^{lo} T cells were defined as the top 1% and next 5% of CD4⁺ T cells based on CD25 expression, respectively. A representative example of FoxP3 expression (MFI) in blood and BAL CD4⁺CD25^{hi}, CD25^{lo}, and CD25^{neg} T cells from a CBD patient (red, blue, and green tracing, respectively) is shown in Figure 3A. The FoxP3 MFI of CD4⁺CD25^{hi} T cells from blood and BAL were 89 and 38, respectively. Overall, no difference in FoxP3 expression in blood T_{reg} cells from normal, BeS, and CBD subjects was seen. However, significantly diminished FoxP3 expression on CD4⁺CD25^{hi} T cells in BAL of patients with CBD was seen compared with blood from normal controls, BeS subjects, and patients with CBD (Figure 3B). FoxP3 expression on BAL CD4⁺CD25^{hi} T cells from patients with CBD was also significantly decreased compared with BAL cells derived from normal control subjects (median, 140; range, 59–165; $P < 0.01$) (Figure 3B). Although not statistically significant,

Figure 2. FoxP3 expression in CD4⁺ T cells from blood and bronchoalveolar lavage (BAL) of normal control, beryllium-sensitized (BeS), and chronic beryllium disease (CBD) subjects. (A) Representative example of intracellular FoxP3 expression in blood and BAL CD4⁺ T cells from a CBD patient is shown. The number in the right upper quadrant of the density plots indicates the percentage of CD4⁺ T cells expressing FoxP3. (B) Percentage of blood and BAL CD4⁺ T cells expressing intracellular FoxP3 is shown. The median percentage of FoxP3-expressing cells in blood (left) and BAL (right) from normal control, BeS, and CBD subjects is shown as a solid line. Statistical comparisons were made using the Kruskal-Wallis test with Dunn multiple comparison test. (C) Absolute number of FoxP3-expressing CD4⁺ T cells in the BAL of BeS and CBD patients is shown, with median values for each disease group indicated with a solid line. (D) Ratio of effector to FoxP3-expressing CD4⁺ T cells in the BAL of BeS subjects and CBD patients is shown. Median values are indicated with solid lines, and statistical comparisons were made using the Mann-Whitney *U* test. (E) Correlation between the percentage of CD4⁺ T cells expressing FoxP3 and diffusing capacity of the lung for carbon monoxide (DL_{CO}; percent predicted) in 11 BeS subjects and 14 CBD patients. PBMC = peripheral blood mononuclear cell.

a trend was noted for decreased FoxP3 MFI in BAL CD4⁺CD25^{hi} T cells from patients with CBD compared with BeS subjects. Similar findings were seen when the MFI of the total FoxP3-expressing CD4⁺ T-cell subset was considered (data not shown).

FoxP3-Expressing T_{reg} Cells Display an Anergic Phenotype

We have previously shown that the coexpression of CD25 and CD27 on CD3⁺CD4⁺ T cells marks most FoxP3-expressing T_{reg} cells in blood and that these cells possess potent suppressive capacity (33). A representative example of our sorting strategy for the purification of blood and lung T_{reg} cells is shown in Figure 4, where 98% and 93% of sorted CD25⁺CD27⁺ T cells

from blood and BAL, respectively, of a patient with CBD expressed FoxP3. Overall, the purity of the sorted T_{reg} cells from blood and BAL of the different patient groups was not statistically significant and was approximately 90% (mean $\pm$ SEM; normal control subjects, $91 \pm 2.7\%$; BeS subjects, $89 \pm 2.8\%$; blood cells from patients with CBD, $89 \pm 2.1\%$; BAL cells from patients with CBD, $86 \pm 2.1\%$).

The failure of natural T_{reg} cells to proliferate after T-cell receptor stimulation *in vitro* suggests that these cells are anergic. To determine whether the same is true for CBD, we investigated two effector functions (i.e., ability to proliferate and secrete Th1-type cytokines) of blood and BAL T cells obtained from patients with CBD. Because of the low frequency of

A. PBMC **BAL** **B.**

Figure 3. Decreased expression of FoxP3 on bronchoalveolar lavage (BAL) CD4⁺ T_{reg} cells. (A) Representative example of FoxP3 expression on CD25^{hi}, CD25^{lo}, and CD25^{neg} CD4⁺ T cells from blood (*top*) and BAL (*bottom*) of a chronic beryllium disease (CBD) patient is shown, with the MFI of FoxP3 expression for each cell population depicted (*red trace*, CD25^{hi}; *blue trace*, CD25^{lo}; *green trace*, CD25^{neg}). CD25^{hi} and CD25^{lo} T cells were defined as the top 1% and 5% of T cells expressing CD25, respectively. (B) MFI of FoxP3 expression on CD4⁺FoxP3⁺ T cells from blood and BAL of normal controls, beryllium-sensitized (BeS) subjects, and CBD patients is shown. The median percentage is shown as a solid line. PBMC = peripheral blood mononuclear cell.

beryllium-responsive T cells in the circulating pool, we used anti-CD3 to assess the ability of blood T_{reg} cells to proliferate in culture. In response to various concentrations of plate-bound anti-CD3 monoclonal antibody (0.1, 0.5, and 2.5 μ g/ml), purified CD4⁺CD25⁺CD27⁺ T_{reg} cells from blood of patients with CBD (*n* = 16) failed to proliferate (Figure 5A). Similar findings were seen with CD4⁺CD25⁺CD27⁺ T_{reg} cells from blood of normal control and BeS subjects (data not shown). Conversely, purified responder T cells demonstrate a vigorous anti-CD3-induced proliferative response at all anti-CD3 concentrations.

PBMC **BAL**

Figure 4. Sorting strategy for the purification of FoxP3-expressing CD4⁺ T_{reg} cells in blood and bronchoalveolar lavage (BAL). In the representative examples shown, 4.1% and 1.5% of blood (*top*) and BAL (*bottom*) CD3⁺CD4⁺ T cells from a chronic beryllium disease (CBD) patient, respectively, coexpressed CD25 and CD27. Analysis of cells after sorting for coexpression of CD4, CD25, and CD27 showed that 98% of blood and 93% of BAL CD25⁺CD27⁺ T cells expressed FoxP3. PBMC = peripheral blood mononuclear cell.

Because the precursor frequency of beryllium-responsive CD4⁺ T cells in BAL is significantly greater than in blood (15), we were able to analyze *ex vivo* BAL CD4⁺ T cells based on BeSO₄-induced IFN- γ secretion and FoxP3 expression, with a representative example shown in Figure 5B. Using *ex vivo* BAL cells from eight subjects with CBD after short-term exposure to BeSO₄, most (mean $\pm$ SEM, 90 $\pm$ 1.9%) beryllium-specific, IFN- γ -expressing CD4⁺ T cells lacked FoxP3 expression (Figure 5C). In addition, FoxP3-expressing CD4⁺ T cells did not express intracellular tumor necrosis factor- α , IL-2, or IL-10 after BeSO₄ stimulation in culture (data not shown). Similar findings were also seen when using the superantigen, SEB, as a positive control.

FoxP3-Expressing T_{reg} Cells Derived from the BAL of Patients with CBD Express a Dysfunctional Regulatory Phenotype

Based on FoxP3 expression (Figure 3B), we speculated that T_{reg} cells in blood of patients with CBD would function normally, whereas those in the CBD lung may have a reduced capacity to suppress effector T-cell functions. Using plate-bound anti-CD3 monoclonal antibody (0.1 μ g/ml) to stimulate responder T cells from blood of normal control, BeS, and CBD subjects, blood T_{reg} cells (CD4⁺CD25⁺CD27⁺) possessed potent suppressor function, completely suppressing effector T-cell proliferation at a responder to suppressor ratio of 1:1, as shown in the representative examples of Figure 6A. Overall, no significant differences were seen in the ability of blood T_{reg} cells from patients with CBD compared with normal control and BeS subjects to suppress effector cell proliferation over the range of responder to suppressor ratios analyzed (Figure 6B). Conversely, the ability of BAL-derived T_{reg} cells from patients with CBD to suppress BAL effector T-cell proliferative responses was impaired (Figures 6A and B). For example, at a responder to suppressor ratio of 1:1, BAL T_{reg} cells from patients with

Figure 5. CD4⁺ T_{reg} cells display an anergic phenotype. (A) Proliferative response of sorted CD25⁺CD27⁺ T_{reg} cells and responder T cells (defined as CD25⁺CD27⁺) from blood of chronic beryllium disease (CBD) patients ($n = 16$) to various concentrations of plate-bound anti-CD3 monoclonal antibody (0.1, 0.5, and 2.5 $\mu\text{g/ml}$) is shown. The data are expressed as the mean counts per minute $\pm$ SEM. (B) A representative experiment is shown for the flow cytometric analysis of bronchoalveolar lavage (BAL) CD4⁺ T cells from a CBD patient stimulated with either BeSO₄ (100 μM ; left density plot) or SEB (10 ng/ml; right density plot) and stained for surface CD4 and intracellular FoxP3 and interferon (IFN)- γ expression. The numbers in the upper quadrants of each density plot are the percentages of CD4⁺FoxP3⁻ and CD4⁺FoxP3⁺ T cells that express IFN- γ . (C) Percentage of *ex vivo* CD4⁺FoxP3⁻ and CD4⁺FoxP3⁺ T cells from the BAL of six CBD patients that express intracellular IFN- γ after short-term exposure to either 100 μM BeSO₄ or 10 ng/ml SEB.

CBD suppressed $61 \pm 7.9\%$ (mean $\pm$ SEM) of anti-CD3-induced effector T-cell proliferation compared with $94 \pm 2\%$ for CBD blood T_{reg} cells ($P < 0.01$). Significant differences between the functional capacity of BAL T_{reg} cells from patients with CBD compared with blood T_{reg} cells from normal control, BeS, and CBD subjects were also seen at responder to suppressor ratios of 1:1/2, 1:1/4, and 1:1/8 (Figure 6B). These findings suggest that T_{reg} cells in BAL of patients with CBD are dysfunctional, thus allowing for an “unchecked” beryllium-

induced immune response in the lung. Because of our inability to purify adequate numbers of T_{reg} cells from the BAL of BeS and normal control subjects despite multiple attempts ($n > 10$), we were unable to address whether this dysfunctional phenotype is specific for the CBD lung or a general feature of BAL T_{reg} cells.

To determine whether the loss of regulatory function by BAL T_{reg} cells results from a decreased T_{reg} cell activity (as suggested by the decreased FoxP3 expression on BAL T_{reg} cells shown in Figure 3) or from resistance of lung effector T cells to inhibition, we performed crossover experiments to examine the ability of blood T_{reg} cells to suppress BAL effector T cells and BAL T_{reg} cells to suppress autologous blood-derived responder T cells. The data from five crossover experiments are summarized in Figure 6C. At a responder to suppressor ratio of 1:1, blood T_{reg} cells and BAL T_{reg} cells from CBD patients suppressed $91 \pm 3.4\%$ and $56 \pm 7.9\%$ (mean $\pm$ SEM), respectively, of anti-CD3-induced BAL effector T-cell proliferation ($P < 0.05$). Conversely, BAL T_{reg} cells failed to suppress the proliferative response of autologous blood effector T cells ($63 \pm 12\%$) compared with the ability of blood T_{reg} cells to suppress blood-derived effector T cells ($94 \pm 2.5\%$; $P < 0.05$). These significant differences persisted at a responder to suppressor ratio of 1:1/2. Taken together, these findings confirm a dysfunctional phenotype of BAL T_{reg} cells from patients with CBD.

DISCUSSION

Our previous work suggests that the beryllium-specific, adaptive immune response is compartmentalized to the lung and persists unabated despite removal of the individual from the workplace and the initiation of corticosteroids. One reason for this persistent immune response is the body’s inability to adequately remove beryllium from the lung of diseased subjects, providing a persistent source of antigen (38, 39). Another potential explanation for the “unchecked” beryllium-induced immune response is provided by the present study, which shows a decreased frequency and expression of FoxP3 in CD4⁺CD25⁺ T cells in the lung of patients with CBD and the inability of these cells to suppress effector T-cell proliferation. This loss of T-cell regulation in the lung has direct physiologic consequences, because it is directly correlated with loss of diffusing capacity and thus disease severity.

The ability of naturally occurring T_{reg} cells to maintain peripheral tolerance is caused by FoxP3, which is required for the development and function of these cells *in vivo* (27, 40). In the absence of FoxP3, such as that seen in scurfy mice and immune dysregulation, polyendocrinopathy, enteropathy, X-linked patients, a loss of T_{reg} cells occurs that results in the development of severe multiorgan autoimmunity (26, 36). Importantly, recent evidence suggests that FoxP3 regulates T_{reg} cell function in a dose-dependent manner. Using a mouse model in which endogenous FoxP3 expression could be manipulated in T_{reg} cells, Wan and Flavell (37) showed that decreased FoxP3 expression resulted in the development of an aggressive autoimmune syndrome similar to that seen in scurfy mice and abolished the *in vitro* and *in vivo* immunosuppressive activities of T cells with attenuated expression of FoxP3. Based on FoxP3 expression in naturally occurring T_{reg} cells derived from the blood and lung of patients with beryllium-induced disease, we predicted and proved that T_{reg} cells in the lung of patients with CBD expressed a dysfunctional phenotype, whereas their counterparts in blood suppressed proliferation of effector T cells derived from both blood and lung. To our knowledge, our study is the first to link diminished FoxP3 expression, as measured by MFI, in T_{reg} cells with their loss of

suppressive capacity in the target organ of human subjects, providing a possible explanation for their dysfunctional phenotype. However, the mechanism responsible for the diminished FoxP3 expression in the CBD lung is unknown. Recent evidence suggests the importance of epigenetic regulation of FoxP3 expression (41). In naturally occurring T_{reg} cells, cytosine phosphodiester guanine dinucleotides at the *Foxp3* locus are completely demethylated. Thus, one possible explanation for the diminished FoxP3 expression in lung T_{reg} cells could be methylation of CpG residues.

In addition to the decreased expression of FoxP3 per cell, we noted a significantly decreased frequency of FoxP3-expressing $CD4^+$ T cells in the BAL of patients with CBD compared with the BAL of normal control and BeS subjects. These findings are similar to a recent study in patients with another granulomatous lung disease, sarcoidosis, where a decreased frequency of FoxP3-expressing $CD4^+$ T cells was seen in blood and BAL of subjects with active disease (42). In contrast, two other studies in sarcoid-

Figure 6. Bronchoalveolar lavage (BAL) $CD4^+$ T_{reg} cells from chronic beryllium disease (CBD) patients are incapable of suppressing anti-CD3-induced responder T cell proliferation. (A) Representative examples of suppression assays using blood of normal control subjects, beryllium-sensitized (BeS) subjects, and blood and BAL of CBD patients is shown. Sorted $CD4^+CD25^+CD27^+$ (T_{reg}) and a fixed number (2.5×10^3 cells/well) of $CD4^+CD25^-$ (responders) T cells were cultured in varying ratios of responder T cells to T_{reg} cells (1:1, 1:1/2, 1:1/4, and 1:1/8) in the presence of 1×10^4 irradiated T cell-depleted PBMC (antigen-presenting cells). Cells were stimulated with 0.1 μ g/ml plate-bound anti-CD3 monoclonal antibody. The percent inhibition of the proliferative response of $CD4^+CD25^-$ T cells by $CD4^+CD27^+CD25^{hi}$ T cells at 6 days is shown. (B) Cumulative data of the ability of T_{reg} cells to suppress proliferation of responder T cells derived from the blood of normal control ($n = 10$), BeS ($n = 13$), and CBD ($n = 16$) subjects and BAL of CBD patients ($n = 8$) are shown, and the mean percentage suppression $\pm$ SEM is depicted. Statistical comparisons were made using the Kruskal-Wallis test with Dunn multiple comparison test. (C) Composite data from five crossover experiments are shown. These experiments were performed as described in B and determine the ability of blood T_{reg} cells to suppress BAL effector T cells and BAL T_{reg} cells to suppress autologous blood-derived responder T cells. Statistical comparisons were made using the Kruskal-Wallis test with Dunn multiple comparison test. PBMC = peripheral blood mononuclear cell.

osis noted an increased frequency of $CD4^+CD25^{hi}$ T cells in the BAL of subjects with active disease (43, 44). Despite the ability of $CD4^+CD25^{hi}$ T cells derived from the sarcoid lung to inhibit T-cell proliferation, these cells failed to suppress effector cytokine secretion (i.e., IFN- γ and tumor necrosis factor- α) even in the presence of a global T_{reg} cell amplification (43). Conversely, the inability of BAL T_{reg} cells from patients with CBD to suppress effector T-cell proliferation suggests an even more profound dysfunctional phenotype than that identified in sarcoidosis, rendering the host unable to control lung inflammation in the presence of persistent beryllium exposure.

The results of the sarcoidosis studies (43, 45) combined with our present work raise the possibility that all lung T_{reg} cells are incapable of suppressing effector T-cell proliferation in the presence of lung inflammation, regardless of the inciting stimulus. Unfortunately, we were unable to address the functional capacity of lung T_{reg} cells in health because of the small number of T cells present in the BAL of healthy control and BeS subjects and inadequate numbers of BAL T_{reg} cells obtained from these subjects as a result of our stringent cell sorting strategy. However, the presence of similar amounts of FoxP3 per T_{reg} cell in the BAL of BeS subjects compared with T_{reg} cells in blood of normal control, BeS, and CBD subjects suggests that these cells might function in a normal suppressive manner. A recent study investigating T_{reg} cells in the blood and BAL of idiopathic pulmonary fibrosis patients and subjects with connective tissue disease with complicating interstitial pneumonia showed a global impairment in T_{reg} cell function that was more marked in the lung and correlated with disease severity (46). Kotsianidis *et al.* (46) also showed that T_{reg} cells derived from the healthy lung retain their suppressive capacity. Collectively, these studies provide strength to the argument that lung T_{reg} cells are incapable of controlling effector T-cell functions in the presence of an inflammatory stimulus.

Transforming growth factor (TGF)- β 1 plays an important role in negatively regulating both innate and adaptive immune responses, in particular suppressing the development of Th1 and Th2 phenotypes (47). Importantly, TGF- β 1 has been shown to support the maintenance of FoxP3 expression, regulatory function, and homeostasis of peripheral $CD4^+CD25^+$ T_{reg} cells in

mice (28). TGF- β 1 is a pleiotropic cytokine with effects on cell growth and differentiation, extracellular matrix formation and fibrosis, and immunomodulation. For example, elevated levels of active TGF- β 1 were seen in the BAL of fibrotic sarcoidosis patients compared with normal control subjects (48). Several functional single nucleotide polymorphisms within the *TGF- β 1* gene have been identified, including C-509T, codon 10 (T + 29C), and codon 25 (G + 74C) (49). In CBD, the -509C and codon 10T single nucleotide polymorphisms have been linked to disease severity (50) and are associated with lower circulating TGF- β 1 levels (51, 52). Although not yet evaluated in CBD, variation within the TGF- β 3 gene has also been linked to lung fibrosis in sarcoidosis (53). The lower TGF- β 1 production expected in those patients with CBD with the -509 C and codon 10T haplotype could result in a deficient FoxP3-expressing T_{reg} cell subset in the lung and ultimately contribute to a proinflammatory microenvironment more conducive to disease progression and collagen deposition.

In summary, we have identified the presence of deficient and dysfunctional T_{reg} cells in the lung of patients with CBD and that the diminution of these FoxP3-expressing cells correlates with disease severity. These findings extend the paradigm that in the presence of persistent antigen exposure (i.e., the sarcoid antigens, beryllium, or an autoantigen) and the compartmentalization of large numbers of antigen-specific T cells in the lung, naturally occurring T_{reg} cells lose their capacity to suppress effector T-cell function, contributing to the persistent inflammatory response.

Conflict of Interest Statement: D.G.M. has no financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.M.L. has no financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.T.F. has received sponsored grants from the Foundation for Sarcoidosis Research over 2 years (\$50,001–\$100,000). B.E.P. has no financial relationship with a commercial entity that has an interest in the subject of this manuscript. L.A.M. provided legal testimony on behalf of patients with community-acquired chronic beryllium disease; she recovered no direct remuneration for this work; she received expert witness fees from Golomb & Honik (up to \$1,000); she received industry-sponsored grants from Centocor (\$5,001–\$10,000, for a beryllium infliximab study: clinical investigational trial); she received a separate industry-sponsored grant from Centocor (\$50,001–\$100,000, for a multicenter, randomized, double-blind, parallel-group, placebo-controlled study evaluating the safety and efficacy of treatment with usteknumab or golimumab in subjects with chronic sarcoidosis); she received an industry-sponsored grant from MondoBiotec (\$10,001–\$50,000, for an *ex vivo* study of Alpha-MSH in chronic beryllium disease); she has submitted a patent with National Jewish for Beryllium ELISPOT to use in diagnosis of beryllium-related health effects; she has received royalties from UpToDate (up to \$1,000); she has received sponsored grants from the Department of Energy (over \$100,000), and various sponsored grants from the National Institutes of Health (over \$350,000); she is employed by National Jewish (up to \$1,000). A.P.F. has received lecture fees from Amgen (up to \$1,000); he has received industry-sponsored grants from Amgen (\$50,001–\$100,000); he has received royalty fees from UpToDate (up to \$1,000); he has received sponsored grants from National Institutes of Health (over \$100,000).

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