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

Douglas G. Mack1, Allison M. Lanham1, Michael T. Falta1, Brent E. Palmer1, Lisa A. Maier1,2, and Andrew P. Fontenot1,3

1Department of Medicine, and 3Department of Immunology, University of Colorado Denver, Aurora, Colorado; and 2Department of Medicine, National Jewish Health, Denver, Colorado

Rationale: Chronic beryllium disease (CBD) is a CD4+ T cell–mediated disorder characterized by persistent lung inflammation. Naturally occurring regulatory T (Treg) 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 Treg 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 Treg 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+ Treg cells in BAL inversely correlated with disease severity. In contrast to blood Treg cells derived from beryllium-sensitized subjects and patients with CBD that completely suppressed blood responder T-cell proliferation, BAL FoxP3-expressing Treg cells from patients with CBD are unable to suppress anti–CD3-mediated BAL T-cell proliferation. Mixing studies showed that blood Treg cells are capable of suppressing autologous BAL responder T cells. Conversely, BAL CD4+ Treg cells are incapable of suppressing blood T cells, confirming that the failure of BAL Treg cells to suppress T-cell proliferation is caused by a dysfunctional Treg cell subset and not by resistance of BAL effector T cells to suppression.

Conclusions: These findings suggest that the deficient and dysfunctional Treg 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 immunopathogenesis 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 (Treg) cell subsets in this occupational lung disease. Treg 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 Treg cells are derived in the thymus and are characterized as CD4+ with a high level of CD25 expression (CD4+CD25hi). 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 Treg 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 Treg cells, and its expression is essential for programming both thymic development and function of Treg cells (26–28). To date, FoxP3 is the most specific molecular marker for naturally occurring Treg 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+ Treg cells allows for the development of unchecked beryllium-induced immune response in the lung.
TABLE 1. DEMOGRAPHICS OF STUDY SUBJECTS*

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Normal Controls (n = 25)</th>
<th>BeS Patients (n = 15)</th>
<th>CBD Patients (n = 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>37 (23–50)</td>
<td>53 (27–77)</td>
<td>59 (44–79)</td>
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<tr>
<td>Gender (M/F)</td>
<td>12/13</td>
<td>13/2</td>
<td>19/5</td>
</tr>
<tr>
<td>Race (W/AF/Hispanic)</td>
<td>8/7/0</td>
<td>13/1/1</td>
<td>20/2/1</td>
</tr>
<tr>
<td>Smoking status (CS/FS/NS)</td>
<td>0/5/20</td>
<td>0/7/8</td>
<td>0/11/13</td>
</tr>
<tr>
<td>Industry of exposure (nuclear/ceramic/other)</td>
<td>N/A</td>
<td>11/1/3</td>
<td>18/4/2</td>
</tr>
<tr>
<td>Treatment (none/prednisone)</td>
<td>25/0</td>
<td>15/0</td>
<td>20/4</td>
</tr>
<tr>
<td>D_{L}_{co}, % predicted</td>
<td>90 (68–120)</td>
<td>80 (17–94)†</td>
<td></td>
</tr>
<tr>
<td>BeLPT, stimulation index</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBL</td>
<td>4.6 (0–24)</td>
<td>3.2 (0.9–162)</td>
<td></td>
</tr>
<tr>
<td>BAL</td>
<td>1.5 (1–3.9)</td>
<td>16.9 (0.9–262)†</td>
<td></td>
</tr>
<tr>
<td>Bronchoalveolar lavage cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBC count (x 10⁶)</td>
<td>18 (11–84)</td>
<td>26 (7–144)</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes, %</td>
<td>4.6 (1.5–20)</td>
<td>32 (6.7–52)†</td>
<td></td>
</tr>
</tbody>
</table>

* Definition of abbreviations: AF = African American; BAL = bronchoalveolar lavage; BeLPT = beryllium lymphocyte proliferation test; BeS = beryllium-sensitized; CBD = chronic beryllium disease; CS = current smoker; D_{L}_{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⁺ Treg 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 T cells to beryllium sulfate (BeSO₄) in vitro (12, 29). Beryllium sensitization was diagnosed based on 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 blood or BAL T cells to beryllium sulfate (BeSO₄) in vitro (12, 29).

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 FACS Calibur 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⁺ Treg Cells
For purification of Treg 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; Cell Sciences). Cells were sorted on a FACS Aria flow cytometer (BD). Sorted CD4⁺CD27⁺CD25⁺ (Treg) and a fixed number (2.5 × 10⁶ cells/well) of CD4⁺CD25⁻ (responders) T cells were cultured in varying ratios of responder T cells to Treg cells (1:1, 1:1/2, 1:1/4, and 1:1/8) in the presence of 1 × 10⁵ 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-1640 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 Treg cells were mixed with autologous BAL responder T cells and BAL Treg 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 (D_{L}_{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
we next queried whether a deficiency of FoxP3-expressing each quadrant of the density plots is shown. (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 the lung compared with BeS subjects was a surprising finding given the increased BAL white blood cell count and the intense CD4\(^+\) T-cell population in the CBD IL-2 receptor) is expressed on activated T cells, the absence of FoxP3 expression in T reg cells from normal control subjects (median, 140; range, 59–121). Because CD25 is coexpressed on naturally occurring T reg cells, 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 on 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 \(^{+}\) 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 D Lco (percent predicted). Previous studies have shown that D Lco is one of the most sensitive static markers of gas exchange in beryllium-induced disease (18), and percent predicted D Lco was the only physiologic marker that was significantly different between BeS and CBD patients (Table 1). Eleven BeS and 14 CBD subjects had a D Lco 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 D Lco \((r = 0.65; P = 0.0004)\). 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 \(^{+}\) T cells, where the pattern of CD25 expression is more distinct. CD25\(^{lo}\) and CD25\(^{hi}\) 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\(^{lo}\) 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 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,
a trend was noted for decreased FoxP3 MFI in BAL CD4<sup>+</sup>CD25<sup>hi</sup> T cells from patients with CBD compared with BeS subjects. Similar findings were seen when the MFI of the total FoxP3-expressing CD4<sup>+</sup> T-cell subset was considered (data not shown).

**FoxP3-Expressing T<sub>reg</sub> Cells Display an Anergic Phenotype**

We have previously shown that the coexpression of CD25 and CD27 on CD3<sup>+</sup>CD4<sup>+</sup> T cells marks most FoxP3-expressing T<sub>reg</sub> 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<sub>reg</sub> cells is shown in Figure 4, where 98% and 93% of sorted CD25<sup>+</sup>CD27<sup>hi</sup> T cells from blood and BAL, respectively, of a patient with CBD expressed FoxP3. Overall, the purity of the sorted T<sub>reg</sub> cells from blood and BAL of the different patient groups was not statistically significant and was approximately 90% (mean ± SEM; normal control subjects, 91 ± 2.7%; BeS subjects, 89 ± 2.8%; blood cells from patients with CBD, 89 ± 2.1%; BAL cells from patients with CBD, 86 ± 2.1%).

The failure of natural T<sub>reg</sub> 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
beryllium-responsive T cells in the circulating pool, we used anti-CD3 to assess the ability of blood Treg cells to proliferate in culture. In response to various concentrations of plate-bound anti-CD3 monoclonal antibody (0.1, 0.5, and 2.5 μg/ml), purified CD4+CD25+CD27+ Treg cells from blood of patients with CBD (n = 16) failed to proliferate (Figure 5A). Similar findings were seen with CD4+CD25+CD27+ Treg 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.

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 ± SEM, 90 ± 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 Treg Cells Derived from the BAL of Patients with CBD Express a Dysfunctional Regulatory Phenotype**

Based on FoxP3 expression (Figure 3B), we speculated that Treg 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 μg/ml) to stimulate responder T cells from blood of normal controls, BeS, and CBD subjects, blood Treg 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 Treg 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 Treg 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 Treg cells from patients with
CBD suppressed 61% ± 7.9% (mean ± SEM) of anti-CD3-induced effector T-cell proliferation compared with 94% ± 2% for CBD blood Treg cells (P < 0.01). Significant differences between the functional capacity of BAL Treg cells from patients with CBD compared with blood Treg 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 Treg 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 Treg 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 Treg cells.

To determine whether the loss of regulatory function by BAL Treg cells results from a decreased Treg cell activity (as suggested by the decreased FoxP3 expression on BAL Treg 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 Treg cells to suppress BAL effector T cells and BAL Treg 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 Treg cells and BAL Treg cells from CBD patients suppressed 91% ± 3.4% and 56% ± 7.9% (mean ± SEM), respectively, of anti-CD3-induced BAL effector T-cell proliferation (P < 0.05). Conversely, BAL Treg cells failed to suppress the proliferative response of autologous blood effector T cells (63% ± 12%) compared with the ability of blood Treg cells to suppress blood-derived effector T cells (94% ± 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 Treg 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 Treg 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 Treg cells occurs that results in the development of severe multiorgan autoimmunity (26, 36). Importantly, recent evidence suggests that FoxP3 regulates Treg cell function in a dose-dependent manner. Using a mouse model in which endogenous FoxP3 expression could be manipulated in Treg 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 vivo and in vitro immunosuppressive activities of T cells with attenuated expression of FoxP3. Based on FoxP3 expression in naturally occurring Treg cells derived from the blood and lung of patients with beryllium-induced disease, we predicted and proved that Treg 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 Treg cells with their loss of
the diminished FoxP3 expression in lung Treg cells could be completely demethylated. Thus, one possible explanation for CD4+ Treg cells in chronic beryllium disease (CBD) patients is incapability of suppressing anti-CD3-induced responder T cell proliferation. Sorted CD4+ CD25+ CD27+ (Treg) and a fixed number (2.5 × 10^4 cells/well) of CD4+ CD25- (responders) T cells were cultured in varying ratios of responder T cells to Treg cells (1:1, 1:1/2, 1:1/4, and 1:1/8) in the presence of 1 × 10^6 irradiated T cell–depleted PBMC (antigen-presenting cells). Cells were stimulated with 0.1 μg/ml plate-bound anti-CD3 monoclonal antibody. The percent inhibition of the proliferative response of CD4+ CD25+ T cells by CD4+ CD27+ CD25hi T cells at 6 days is shown. (B) Cumulative data of the ability of Treg 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 ± 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 Treg cells to suppress BAL effector T cells and BAL Treg 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.

The results of the sarcoidosis studies combined with our present work raise the possibility that all lung Treg cells are incapable of suppressing effector T-cell proliferation in the presence of lung inflammation, regardless of the exciting stimulus. Unfortunately, we were unable to address the functional capacity of lung Treg 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 Treg cells obtained from these subjects as a result of our stringent cell sorting strategy. However, the presence of similar amounts of FoxP3 per Treg cell in the BAL of BeS subjects compared with Treg 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 Treg 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 Treg cell function that was more marked in the lung and correlated with disease severity (46). Kotsianidis et al. (46) also showed that Treg cells derived from the healthy lung retain their suppressive capacity. Collectively, these studies provide strength to the argument that lung Treg cells are incapable of controlling effector T-cell functions in the presence of an inflammatory stimulus.

Figure 6. Bronchoalveolar lavage (BAL) CD4+ Treg 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+ (Treg) and a fixed number (2.5 × 10^4 cells/well) of CD4+ CD25- (responders) T cells were cultured in varying ratios of responder T cells to Treg cells (1:1, 1:1/2, 1:1/4, and 1:1/8) in the presence of 1 × 10^6 irradiated T cell–depleted PBMC (antigen-presenting cells). Cells were stimulated with 0.1 μg/ml plate-bound anti-CD3 monoclonal antibody. The percent inhibition of the proliferative response of CD4+ CD25+ T cells by CD4+ CD27+ CD25hi T cells at 6 days is shown. (B) Cumulative data of the ability of Treg 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 ± 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 Treg cells to suppress BAL effector T cells and BAL Treg 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.

The results of the sarcoidosis studies combined with our present work raise the possibility that all lung Treg cells are incapable of suppressing effector T-cell proliferation in the presence of lung inflammation, regardless of the exciting stimulus. Unfortunately, we were unable to address the functional capacity of lung Treg 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 Treg cells obtained from these subjects as a result of our stringent cell sorting strategy. However, the presence of similar amounts of FoxP3 per Treg cell in the BAL of BeS subjects compared with Treg 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 Treg 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 Treg cell function that was more marked in the lung and correlated with disease severity (46). Kotsianidis et al. (46) also showed that Treg cells derived from the healthy lung retain their suppressive capacity. Collectively, these studies provide strength to the argument that lung Treg cells are incapable of controlling effector T-cell functions in the presence of an inflammatory stimulus.

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TGF-β1 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-β1 were seen in the BAL of fibrotic sarcoidosis patients compared with normal control subjects (48). Several functional single nucleotide polymorphisms within the TGF-β1 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-β1 levels (51, 52). Although not yet evaluated in CBD, variation within the TGF-β3 gene has also been linked to lung fibrosis in sarcoidosis (53). The lower TGF-β1 production expected in those patients with CBD and the −509C 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 the diminution of these FoxP3-expressing cells correlates with disease severity (50) and are associated with lower circulating TGF-β1 production, effector T-cell function, contributing to the persistent inflammation of large numbers of antigen-specific T cells in the lung, 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.

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References


