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Impaired Function of CTLA-4 in the Lungs of Patients with Chronic Beryllium Disease Contributes to Persistent Inflammation

Jennifer L. Chain,* Allison K. Martin,* Douglas G. Mack,* Lisa A. Maier,*† Brent E. Palmer,* and Andrew P. Fontenot*‡

Chronic beryllium disease (CBD) is a granulomatous lung disease that develops in genetically susceptible individuals following workplace exposure to beryllium (1–3). Inflammation in the lung is the end-result of an adaptive immune response to beryllium presented by HLA-DP molecules containing a glutamic acid residue at the 69th position of the β-chain (4–6). CD4+ effector memory T (T EM) cells from blood and bronchoalveolar lavage fluid (BALF) of CBD patients robustly secrete IL-2, IFN-γ, and TNF-α when stimulated with beryllium salts in vitro (7, 8). In vivo, beryllium-responsive T EM cells accumulate in the lung and produce these proinflammatory cytokines in response to persistent beryllium exposure, leading to alveolitis, granulomatous inflammation, and ultimately, fibrosis (1–3).

CD4+ T cells from the lungs of CBD patients proliferate poorly in vitro yet retain the ability to secrete Th1-type cytokines in response to beryllium stimulation (9, 10), suggesting a dysfunctional phenotype with persistent inflammation, despite the absence of CD28-mediated costimulation (10) and increased expression of programmed death 1 (PD-1), a coinhibitory receptor that regulates beryllium-induced Th cell proliferation (11). Aside from PD-1, relatively little is known about negative regulators of inflammation in chronic inflammatory lung disorders. CTLA-4 is a coinhibitory receptor (12, 13) with similar functions to PD-1 (14–16) and antagonistic activities to CD28 (17–20). It is upregulated on CD4+ TEM cells from blood and heart tissue of individuals chronically infected with Trypanosoma cruzi (21). In addition, CTLA-4 and PD-1 are coexpressed on HIV-specific T cells from the blood of HIV-infected subjects (22, 23). These findings suggest that CTLA-4 plays a key role in the regulation of the T cell phenotype associated with chronic infections, as well as other chronic inflammatory disorders.

To define the function of the CTLA-4 pathway in beryllium-induced disease, we examined the expression of CTLA-4 in blood and BALF cells of normal individuals, beryllium-sensitized (BeS) subjects, and CBD patients. CTLA-4 expression was elevated on total CD4+ T cells from the lungs of study subjects compared with blood. Furthermore, CTLA-4 expression was greatest in the beryllium-responsive subset of CD4+ T cells that retained the ability to proliferate and express IL-2. Functional assays show that the induction of CTLA-4 signaling in blood cells inhibited beryllium-induced T cell proliferation while having no effect on the proliferative capacity of beryllium-responsive CD4+ T cells in the lung. Collectively, our findings suggest a dysfunctional CTLA-4 pathway in the lung and its potential contribution to the persistent inflammatory response that characterizes CBD. The Journal of Immunology, 2013, 191:000–000.
presence of granulomatous inflammation on lung biopsy and a positive proliferative response of blood and/or BALF T cells to beryllium sulfate (BeSO₄) in vitro (24, 25). The diagnosis of beryllium sensitization was established based on a positive proliferative response of PBMCs to BeSO₄ in vitro and the absence of granulomatous inflammation or other abnormalities on lung biopsy (26, 27). Active smokers were excluded from enrollment. 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.

The demographics of the study subjects are shown in Table I. No difference was seen in the age of the BeS and CBD patients enrolled in this study. The majority of BeS and CBD subjects were male. Six CBD patients were treated with oral glucocorticoids. All clinical beryllium lymphocyte proliferation tests (BeLPTs) were performed in the Advanced Diagnostics Laboratory at National Jewish Health. No difference in the blood BeLPT was seen between BeS and CBD patients. In contrast, a significant increase was seen in the proliferation of BALF cells from CBD patients compared with BeS subjects in response to beryllium; the median stimulation index for CBD patients was 8.3 (range, 0.8–308) versus 1.2 (range, 0.8–3.8; \( p < 0.001 \)) for BeS subjects. CBD subjects had a statistically significant increase in the percentage of BALF lymphocytes (median, 15%; range, 1–82%) compared with BeS patients (median, 5%; range, 1–15%; \( p < 0.001 \)).

### Preparation of peripheral blood and BALF cells for beryllium-induced cytokine production

PBMCs were isolated from heparinized blood by Ficoll-Hypaque density gradient separation, and bronchoctyrophy with bronchialveolar lavage was performed as previously described (28, 29). PBMCs and BALF cells were stimulated under the following conditions: ~5 × 10⁶ cells were resuspended in RPMI 1640 plus 10% heat-inactivated FBS (HyClone) in 12 × 75-mm culture tubes with anti-CD96 (BD Biosciences) in the presence of medium alone, 100 μM BeSO₄ (Brush Wellman), or staphylococcal enterotoxin B (SEB) for 6 h at 37°C in a humidified 5% CO₂ atmosphere. Brefeldin A (BD Biosciences) was added after 1 h in culture.

### Immunofluorescence staining of CD4⁺ T cells and monocytes/macrophages

PBMCs or BALF cells were washed, incubated with FcR-blocking reagent (Miltenyi Biotec), and stained with anti-CD4 (PerCP-Cy5.5; BD Biosciences), anti-CD3 (PE–Texas Red; Beckman Coulter), anti-CD8 (V-500; BD Biosciences), anti-CD279 (PD-1, FITC; BD Biosciences), and stained with mAbs directed against CD3, CD4, CD8, CD69 (FITC; BD Biosciences), anti-CD45 (eFluor 605NC), anti-CD14 (eFluor 450), anti-CD80 (FITC), anti–IL-2 (AF-700; BioLegend), and anti–Ki-67 (AF-700; BioLegend) mAbs for 30 min at 4°C. For ex vivo staining, PBMCs and BALF cells were washed, blocked with FcR, and stained with anti-CD3 (clone OKT3; eBioscience) for 6, 24, or 48 h. Harvested cells were washed with PBS, and CellTrace Violet–labeled cells were added along with 100 μM BeSO₄, where appropriate, and incubated for 5 d at 37°C in a humidified 5% CO₂ atmosphere. Cells were harvested, incubated with FcR-blocking reagent, stained with anti-CD3 and anti-CD4, and resuspended in 1% PBS, and CellTrace Violet–labeled cells were stimulated with 100 μM BeSO₄ or medium. Wells of a 24-well plate were coated with 50 μg/ml anti-CTLA-4 Ab (clone BNI3; BD Biosciences) or isotype control (IgG₂₃κ; BD Biosciences) and incubated overnight at 4°C (21). Wells were washed with PBS, and CellTrace Violet–labeled cells were added along with 100 μM BeSO₄ where appropriate, and incubated for 5 d at 37°C in a humidified 5% CO₂ atmosphere. Cells were harvested, incubated with FcR-blocking reagent, stained with anti-CD3 and anti-CD4, and resuspended in 1% formaldehyde.

To detect CD69 expression and de novo Ki-67 expression in response to anti-CD3 stimulation, PBMCs were cultured, as described above, in wells of a 24-well plate coated with either medium or 0.1 μg/ml purified anti-human CD3 (clone OKT3; eBioscience) for 6, 24, or 48 h. Harvested cells were stained with mAbs directed against CD3, CD4, CD8, CD69 (FITC; BD Biosciences), and Ki-67, as described above.

### Flow cytometry

Formaldehyde-fixed cells were analyzed using an LSR II flow cytometer (BD Immunocytometry Systems). The number of events collected ranged between 1 and 3 million. Electronic compensation was performed with Ab capture beads (BD Biosciences) stained separately with individual Abs used in the test samples. Data files were analyzed using FlowJo software (TreeStar), and biexponential scaling was used in all dot plots. Lymphocytes were gated based on their forward and side scatter profile. CD4⁺ T cells were selected, and the expression of CD4 and CD8 was analyzed in a bi–variate dot plot. Because the frequency of beryllium-responsive CD4⁺ T cells in blood and BALF tends to be low, we only examined the expression of PD-1, CTLA-4, and Ki-67 on cytokine-producing cells (IFN-γ⁺, IL-2⁺, or both, as described in Results) with frequencies ≥ 0.04% for blood and 0.4% for BALF to ensure an adequate number of events for analysis, as previously described (28–30). SEB-stimulated T cells were analyzed in a similar manner. Dividing cells were identified in proliferation experiments by gating on CellTrace Violet⁺ staining compared with unstimulated control samples.

To control for the accuracy and precision of measurements taken during the course of the study, routine quality control was performed on the LSR II using the Cytometer Setup and Tracking (CS&T) feature within BD FACSDiva software version 6.1.2 (BD Biosciences). Voltage, laser delay, and area scaling were determined using standardized CS&T beads (BD Biosciences), and settings were tracked over time. To verify the laser delay and area scaling determined by CS&T, a manual quality control, using rainbow beads, was performed daily. To further control for quantitative comparisons of surface and intracellular molecule expression levels, collection of data from healthy control subjects was interspersed throughout the study period with acquisition of data from study subjects.

### Statistical analysis

Kruskal–Wallis ANOVA, paired Student \( t \) test, and Spearman correlation analysis were used to determine the significance of differences between subject groups. A \( p \) value < 0.05 was considered statistically significant.

### Results

#### CTLA-4 expression is increased in the lung

To determine whether CTLA-4 expression is increased in the setting of chronic lung inflammation, we initially analyzed its

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AA, African American; CS, current smoker; FS, former smoker; NS, never smoker; O, other; W, white. \( *p < 0.001 \).
expression in blood and BALF CD4+ T cells. Freshly isolated PBMCs and BALF cells were obtained from CBD patients, BeS subjects, and normal controls (Table I), and the mean fluorescence intensity (MFI) of CTLA-4 expression in total CD3+CD4+ T cells was determined by immunofluorescence staining and cytofluorographic analysis. Representative graphs of CTLA-4 expression on CD3+CD4+ T cells from the peripheral blood and BALF of a CBD subject are shown in Fig. 1A. Overall, CTLA-4 expression was significantly increased in CD4+ T cells from the lung compared with blood, regardless of disease status (Fig. 1B). For example, in CBD patients, CTLA-4 expression in BALF CD4+ T cells (median, 1007; range, 559–1246) was 1.7-fold higher than that seen in blood cells (median, 582; range, 310–1284; p < 0.001).

Because CD28 signaling induces the expression of CTLA-4 (32), and CTLA-4 activation in conventional T cells negatively regulates the immune response largely by attenuating CD28-induced signaling (12, 13, 17, 18, 33, 34), we analyzed the coexpression of CTLA-4 and CD28 on CD4+ T cells in blood and BALF. As shown in Fig. 1C and 1D, a significant decrease was seen in CD28 expression, on a per-cell basis, on CD4+ T cells in the lung compared with blood, as measured by MFI, with a significant percentage of BALF CD4+ T cells having lost CD28 expression (Fig. 1C) (10). Gating on CD28+CD4+ T cells, decreased expression of CD28 in the lung occurred in all study groups. When gating on CD28+ and CD28- T cells (representative example shown in Fig. 1E), CTLA-4 expression was significantly higher on CD28-CD4+ T cells in blood and lung of CBD patients compared with their CD28+ counterparts (Fig. 1F). CTLA-4 was also significantly higher on CD28- T cells in BALF compared with CD28+ T cells in blood (1.3-fold higher, p < 0.001), and similar findings were seen in normal control and BeS subjects (data not shown). Thus, CTLA-4 is upregulated on ex vivo BALF CD4+ T cells in the setting of CD28 downregulation; this effect appears to be lung specific as opposed to disease specific because it occurs in all study subjects, regardless of the presence of lung pathology.

**Differential CTLA-4 expression on beryllium-responsive, Th1 cytokine–expressing CD4+ T cells**

As beryllium-responsive T<sub>BM</sub> cells mature into their most terminally differentiated and least proliferative state, they lose the expression of IL-2 and gain the expression of IFN-γ (7, 9, 31). This expression pattern can be used to dissect beryllium-responsive CD4+ T cells into distinct subsets (Fig. 2A), with the least differentiated subset expressing IL-2 alone (IL-2<sup>-IFN-γ</sup>), followed by the upregulation of IFN-γ expression (IL-2<sup>-IFN-γ</sup>); the most terminally differentiated subset is marked by the loss of IL-2 expression (IL-2<sup>-IFN-γ</sup>) (7, 9, 31). To determine whether CTLA-4 expression is upregulated in the context of beryllium exposure, we measured CTLA-4 in these beryllium-responsive, IL-2- and IFN-γ-producing CD4+ T cell subsets in CBD subjects. PBMCs and BALF cells were stimulated with BeSO<sub>4</sub> for 6 h in culture, followed by intracellular cytokine staining to identify the beryllium-responsive T cells in each patient. All subsets of cytokine-secreting, beryllium-responsive CD4+ T cells from blood and BALF expressed significantly higher levels of CTLA-4 compared with unstimulated cells (Fig. 2B). In addition, beryllium-responsive CD4+ cells producing only IL-2 expressed higher levels of CTLA-4 than did cells producing only IFN-γ in blood and BALF (Fig. 2B). Within these distinct cytokine-secreting T cell subsets, CD4<sup>+</sup> T cells in blood and BALF appear to downregulate CTLA-4 expression as they undergo further differentiation (Fig. 2B). For example, median CTLA-4 expression in beryllium-responsive, IL-2-IFN-γ<sup>-CD4<sup>+</sup> T cells in blood (2088; range, 563–3313) was 2-fold lower compared with IL-2-IFN-γ<sup>-</sup> T cells in blood (4278; range, 3501–5793; p < 0.001). Similar findings were seen in BALF, with a 1.7-fold difference in CTLA-4 expression between T cells expressing IL-2 only or IFN-γ only (Fig. 2B). However, unlike CTLA-4, PD-1 expression increased on terminally differentiated beryllium-responsive T cells in the lung (Fig. 2C).

To exclude the possibility that the increased CTLA-4 expression on less-differentiated beryllium-responsive CD4<sup>+</sup> T cells is due to T cell activation, we also stimulated PBMCs and BALF cells from CBD patients with SEB and analyzed intracellular cytokine, CTLA-4, and PD-1 expression. As shown in Fig. 2D, CTLA-4 was upregulated in SEB-responsive cells expressing IL-2 and/or IFN-γ in both blood and BALF. However, the pattern seen in beryllium-
responsive cells in which differentiation to IFN-γ-only producing cells coincided with CTLA-4 downregulation was not seen in response to SEB stimulation. In addition, CTLA-4 expression was significantly increased in beryllium-responsive, IL-2–only–expressing CD4+ T cells from blood and BALF of CBD patients. PD-1 expression (MFI) on beryllium-responsive (C), or SEB-responsive (E) IL-2+IFN-γ−, IL-2+IFN-γ+, and IL-2 IFN-γ− CD4+ T cells from blood and BALF of CBD patients. Data in (B–E) are expressed as the median MFI, with median values represented by horizontal lines. The vertical black line dividing each graph separates datasets into those compared using ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001, Kruskal–Wallis ANOVA.

FIGURE 2. CTLA-4 and PD-1 expression on beryllium-responsive, Th1-type cytokine-expressing CD4+ T cells in blood and BALF of CBD patients. (A) Representative density plots of beryllium-induced IL-2 and IFN-γ expression in blood and BALF cells from a CBD patient. The numbers in the quadrants of each density plot are the percentages of CD4+ T cells that express IFN-γ, IL-2, or both cytokines. CTLA-4 expression (MFI) in beryllium-responsive (B), or SEB-responsive (D) IL-2+IFN-γ−, IL-2+IFN-γ+, and IL-2 IFN-γ− CD4+ T cells from blood and BALF of CBD patients. PD-1 expression (MFI) on beryllium-responsive (C), or SEB-responsive (E) IL-2+IFN-γ−, IL-2+IFN-γ+, and IL-2 IFN-γ− CD4+ T cells from blood and BALF of CBD patients. Data in (B–E) are expressed as the median MFI, with median values represented by horizontal lines. The vertical black line dividing each graph separates datasets into those compared using ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001, Kruskal–Wallis ANOVA.

Beryllium-responsive, IFN-γ−expressing CD4+ T cells do not proliferate in the lungs of CBD patients

Despite the relatively poor proliferative capacity of some beryllium-responsive CD4+ T cells in vitro (9, 10), the proliferative status of the various populations of beryllium-responsive, memory CD4+ T cell subsets in vivo is unknown. Ki-67 is a nuclear protein often analyzed as a marker of in vivo proliferation (35). When cells transition from a resting state, Ki-67 is exposed on the surface of chromosomes and can be detected using standard methods of intracellular flow cytometry (35, 36). To validate that Ki-67 expression is not induced during short-term stimulation, PBMCs were stimulated with plate-bound anti-CD3 for 0, 6, 24, or 48 h and analyzed for changes in Ki-67 expression. As shown in Supplemental Fig. 1A, no differences in the percentage of Ki-67+ T cells were detected at 6 h with anti-CD3 stimulation compared with no stimulation (median, 2.8% versus 1.4%). Conversely, expression of the recent activation marker CD69 was increased significantly as early as 6 h after anti-CD3 stimulation (median, 7.8% versus 1.9% at time 0, p = 0.01) (Supplemental Fig. 1B).

Following a 6-h stimulation with BeSO4 in culture, Ki-67 expression was analyzed in beryllium-responsive T cell subsets from blood and BALF to determine which beryllium-responsive, Th1 cytokine–expressing memory CD4+ T cells were undergoing in vivo proliferation. Fig. 3A shows representative graph overlays of Ki-67 expression in these subsets from blood and lung. Only those cells exclusively producing IL-2 in the blood and BALF were undergoing in vivo proliferation (Fig. 3B). The median percentage of proliferating (Ki-67+)* IL-2+IFN-γ− cells in the blood was 56% (range, 1.6–88%) compared with 15% (range, 2.1–27%) of IL-2+ IFN-γ− cells (p < 0.01) and 7.3% (range 1.1–42%) of IL-2− IFN-
γ⁺ cells (p < 0.001). In BALF, 39% (range, 14–56) of IL-2-IFN-γ CD4⁺ T cells expressed Ki-67 cells compared with 7.5% (range, 5–13; p < 0.001) of IL-2-IFN-γ cells and 4.5% (range, 3–11; p < 0.001) of IFN-γ-only-expressing T cells. Thus, these data suggest that Ki-67 detected in beryllium-responsive cells at 6 h reflects their in vivo proliferative status and that the gain of IFN-γ expression by these cells is associated with a loss of proliferation (Fig. 3B).

**CTLA-4 expression is highest on proliferating, IL-2-secreting CD4⁺ T cells in CBD patients**

We next directly compared the expression of CTLA-4 on populations of proliferating and cytokine-producing CD4⁺ T cells from the blood and lungs of CBD patients. Gating on CD4⁺ T cells, we analyzed CTLA-4 expression based on in vivo proliferation and beryllium-induced Th1 cytokine production. CTLA-4 expression was highest on blood and BALF T cells expressing Ki-67 and IL-2 (Fig. 4A, 4B). Median CTLA-4 expression in blood Ki-67 + IL-2⁺ cells was 3636 (range, 2584–6411) and was 1.7–3-fold higher compared with all other groups (p < 0.001). Median CTLA-4 expression in BALF Ki-67⁺ IL-2⁺ cells was 3089 (range, 2420–3497) and was 1.5–2.2-fold higher compared with all other groups (p < 0.01 or < 0.001). In contrast, PD-1 expression was highest on nonproliferating, IFN-γ-secreting cells in the lung (data not shown).

Based on our findings of increased CTLA-4 and Ki-67 expression on beryllium-responsive, IL-2-expressing CD4⁺ T cells in blood and BALF of CBD patients, we correlated CTLA-4 expression with IFN-γ and IL-2 expression in CD4⁺ T cells from the blood and BALF of a CBD patient. (B) Ki-67 detected in CD4⁺ T cells in blood and BALF CD4⁺ T cells from CBD patients with the following Th1 cytokine-expression patterns: IL-2⁺ IFN-γ⁺, IL-2⁻IFN-γ⁺, IL-2⁻IFN-γ⁻, and IL-2⁺ IFN-γ⁻ CD4⁺ T cells from blood and BALF of a CBD patient.

**Increased expression of CD80 and CD86 on CD14⁺ cells in the lungs of CBD patients**

Suppression of CTLA-4-expressing T cells requires that the CTLA-4 ligands (CD80 and CD86) are expressed on APCs. To examine this, CTLA-4 ligand expression was measured by flow cytometry on monocytes/macrophages, defined by characteristic forward and side scatter profiles and CD14 expression, from blood and BALF of CBD patients. CD80 expression on CD14⁺ and CD14⁻ cells within the macrophage gate of BALF cells was significantly greater than on CD14⁺ monocytes in blood (Fig. 5A). Median CD80 expression was 2120 (range, 713–5970) on CD14⁺ BALF cells and 2522 (range, 1025–5779) on CD14⁺ BALF cells compared with 971 (range, 401–1518; p < 0.001) for CD14⁺ cells in blood (Fig. 5A). Conversely, CD86 expression on BALF cells was only significantly increased on CD14⁺ cells in the macrophage gate compared with CD14⁺ cells in blood (Fig. 5B). These data demonstrate that CD14⁺ cells within the macrophage gate of BALF cells have significantly higher levels of CTLA-4 ligands than do those in the blood, suggesting that this pathway is upregulated in the lung.

**CTLA-4 is not functional in the lungs of CBD patients**

Our data show that CTLA-4 and PD-1 have opposite expression patterns on beryllium-responsive CD4⁺ T cells in blood and BALF, despite the similarity of their proposed functions as inhibitory receptors (14–16). We showed previously that blocking PD-1 engagement with its ligands in culture restored the proliferative capacity of beryllium-responsive lung T cells (11), indicating that the PD-1 pathway is active in the lung of CBD patients. To investigate the functionality of the CTLA-4 pathway, we cross-linked CTLA-4 to induce signaling (21, 33, 37) and measured...
beryllium-induced proliferation in culture, with the expected finding being a decrease in proliferation if the CTLA-4 pathway is active. Cells from CBD patients were labeled with CellTrace Violet and stimulated with BeSO$_4$ in the presence of plate-bound anti–CTLA-4 or isotype control. After 5 d, CD3$^+$CD4$^{hi}$ cells from these cultures were examined for loss of CellTrace Violet as a measurement of beryllium-induced T cell proliferation. Representative examples of proliferating CD4$^+$ T cells (CellTrace Violet$^{lo}$CD4$^{hi}$) from blood and BALF are shown in Fig. 6A. Overall, cross-linking CTLA-4 significantly reduced beryllium-induced CD4$^+$ T cell proliferation in blood ($p = 0.032$) (Fig. 6B, left panel) while having no effect on the proliferation of beryllium-stimulated BALF cells ($p = 0.63$) (Fig. 6B, right panel). Collectively, these data suggest that the CTLA-4 pathway in the lungs of CBD patients is dysfunctional, despite the upregulation of CTLA-4 on beryllium-specific CD4$^+$ T cells and its ligands on APCs in the BALF.

Loss of CD28 expression correlates inversely with an increased frequency of IFN-γ-expressing CD4$^+$ T cells in the lung

Because a primary function of CTLA-4 is inhibition of the CD28-signaling cascade (33), our data suggest that the inability of CTLA-4 to downregulate beryllium-induced T cell proliferation and IFN-γ expression in the lung is related to the independence of beryllium-responsive CD4$^+$ T cells in BALF from CD28-mediated costimulation (10). To determine whether the failure of CTLA-4 to downregulate IFN-γ production is related to decreased CD28 expression, we correlated CD28 expression and the percentage of CD4$^+$ T cells expressing IL-2 alone, IL-2 and IFN-γ, or IFN-γ alone in CBD patients. No correlation was seen between CD28 expression and the percentage of cells expressing IL-2 only ($r = 0.31$, $p = 0.33$, Fig. 7A). However, there was a slight negative correlation between CD28 levels and the percentage of cells producing both IL-2 and IFN-γ ($r = -0.64$, $p = 0.047$, Fig. 7B) and a strong negative correlation with CD4$^+$ T cells expressing IFN-γ alone ($r = -0.73$, $p = 0.010$, Fig. 7C). Conversely, no correlation was seen in the blood between CD28 expression and the percentage of cytokine-secreting T cells ($IL-2^{+}IFN-γ^{-}$, $r = 0.033$, $p = 0.95$; $IL-2^{+}IFN-γ^{-}$, $r = -0.086$, $p = 0.92$; IL-2$^{-}$ IFN-γ$^{-}$, $r = -0.62$, $p = 0.12$) (data not shown). Collectively, these data support an inverse relationship between CD28 expression and the percentage of CD4$^+$ T cells expressing IFN-γ.

FIGURE 5. Expression of CTLA-4 ligands is elevated on APCs in the lung of CBD patients. CD80 (A) and CD86 (B) expression was evaluated on CD14$^{+}$ and CD14$^{-}$ cells within the macrophage/monocyte gate (based on forward and side scatter profiles) from the peripheral blood ($n = 18$) and BALF ($n = 11$) of CBD patients. Median values are indicated by horizontal lines. ***$p < 0.001$, Mann–Whitney U test.

FIGURE 6. Beryllium-induced proliferation in the presence of activating anti–CTLA-4 Ab. (A) Representative density plots of the percentage of CellTrace Violet$^{+}$CD3$^+$CD4$^{hi}$ T cells from blood and BALF of a CBD subject after 5 d of stimulation with 100 μM BeSO$_4$ in the presence of plate-bound anti–CTLA-4 or isotype control. (B) Summary data showing the percentage of CellTrace Violet$^{+}$CD3$^+$CD4$^{hi}$ T cells in the presence and absence of CTLA-4 cross-linking ($n = 7$ for blood and $n = 5$ for BALF). *$p < 0.05$, paired Student $t$ test.

FIGURE 7. Correlation between CD28 expression and IFN-γ expression by beryllium-responsive CD4$^+$ T cells in the lung. CD28 expression (MFI) was correlated with the percentage of IL-2$^{-}$IFN-γ$^{-}$ ($n = 10$) (A), IL-2$^{+}$ IFN-γ$^{-}$ ($n = 10$) (B), and IL-2$^{-}$ IFN-γ$^{-}$ ($n = 11$) (C) beryllium-responsive CD4$^+$ T cells in the BALF of CBD patients.
and IFN-γ expression on beryllium-responsive CD4+ T cells in the lung, contributing to persistent lung inflammation even in the setting of enhanced CTLA-4 expression.

Discussion

CBD represents an important model of persistent Ag exposure and is characterized by the accumulation of large numbers of beryllium-responsive CD4+ T cells in the lung (1, 2, 7). Consequently, Th1-type cytokine secretion by lung T cells plays a central role in the immunopathogenesis of beryllium-induced disease. The regulation of the immune response and the subsequent inflammation in the lung that results from persistent Ag exposure are not well understood. We showed previously that beryllium-responsive CD4+ T cells in the lung predominantly express an effector memory phenotype (7, 31), are CD28 independent (10), express CD57 (a marker of senescence) (38), and upregulate PD-1 (11), a CD28 family member that serves to negatively regulate T cell function. The PD-1 pathway is active in CBD and contributes to the downregulation of proliferation in response to chronic beryllium exposure (11). The present study extends our previous findings by showing that another coinhibitory receptor, CTLA-4, is upregulated on beryllium-responsive CD4+ T cells in blood and lung of CBD patients. In contrast to blood cells, induction of CTLA-4 signaling fails to block beryllium-induced CD4+ T cell proliferation, suggesting a differential functional capacity of CTLA-4 between Ag-specific T cells in blood and lung.

CD28 and CTLA-4 are homologous receptors that share ligands, despite having opposite effects on cellular activation (12, 13, 17, 18, 33, 34). CD28 costimulates T cell activation, whereas CTLA-4 inhibits the T cell response through a blockade of CD28 signaling and activation of CTLA-4–specific signaling pathways (33). We showed previously that beryllium-specific CD4+ T cells in the lung downregulate CD28 and no longer require CD28-mediated costimulation (10, 39), findings consistent with TEM cells in other immune-mediated diseases (40–42). Despite the decreased expression of CD28 on CD4+ T cells in lung compared with blood, CTLA-4 expression on lung T cells was increased. In blood and BALF, CD28+ T cells had lower CTLA-4 levels than did cells expressing CD28, but CTLA-4 was still elevated in lung T cells compared with cells in the blood, suggesting that loss of CD28 expression precedes loss of CTLA-4 in the lung and that even though beryllium-specific TEM cells in the lung are independent of CD28, CTLA-4 expression is still induced.

Because of the inhibitory function of CTLA-4, we were initially surprised that beryllium-responsive, Ki-67+IL-2−CD4+ T cells in both blood and lung expressed the highest levels of CTLA-4. However, previous studies showed that the induction of CTLA-4 expression requires CD28 signaling, IL-2 production, and entry into the cell cycle (13, 32, 43). This is also consistent with published reports of memory T cells containing a relatively large pool of intracellular CTLA-4 (44). The requirement of IL-2 and cell cycle entry for CTLA-4 expression provides an explanation for the highest expression of CTLA-4 on beryllium-responsive T cells in blood that are proliferating and expressing IL-2. We showed previously that a properly functioning PD-1 pathway is likely contributing to the diminished proliferative capacity of Ag-responsive T cells in the lung (11). However, the presence of elevated CTLA-4 expression on beryllium-responsive, IFN-γ–expressing T cells in the lung raises the possibility of an impaired function of this inhibitory receptor to turn off inflammatory cytokine production.

PD-1 and CTLA-4 are both coinhibitory receptors expressed following T cell activation and function to negatively regulate the resulting immune response (14–16). Although these receptors are proposed to have similar functional outcomes (i.e., downregulation of proliferation, cytokine production, cell survival), it is evident that they use distinct signaling pathways to accomplish nonredundant immunomodulatory effects (16). In response to chronic Ag exposure, both of these inhibitory receptors are upregulated (21, 23, 45), contributing to a state of cellular senescence. In this state, cells are more susceptible to death and fail to proliferate or produce cytokines (46). HIV-specific T cells upregulate a series of coinhibitory molecules when chronically stimulated, which results in the inability to mount an effective response against the virus (23, 45). Blocking these receptors restored effector functions to the cells and led to an improved antiviral response. CBD also results in chronic immune activation as a result of persistent exposure to beryllium. Therefore, we originally expected to see PD-1 and CTLA-4 upregulated on the same T cells in the lungs of CBD patients. Instead, we saw an opposite expression pattern, indicating that the expression of each receptor has a distinct role in regulating the immune response to chronic beryllium exposure in the lung.

In this regard, PD-1 predominantly functions to block T cell proliferation of beryllium-responsive cells, having little role in downregulating IFN-γ expression. Our data support this contention, with blockade of the PD-1 pathway restoring proliferation of beryllium-responsive lung T cells while having no effect on intracellular IFN-γ expression (11). In addition to regulating proliferation, one of the major functions of CTLA-4 is to control the secretion of cytokines by activated cells (42–45). In murine models of autoimmunity, blockade of CTLA-4 engagement rapidly induced the onset of diabetes in NOD mice (47, 48) and increased the severity of experimental autoimmune encephalomyelitis in mouse strains susceptible and resistant to the disease (49, 50). Part of the mechanism for the exacerbated disease severity resulting from a lack of CTLA-4 function was increased secretion of IFN-γ by Ag-specific T cells (48, 50). Our data raise the possibility that the continued secretion of IFN-γ by nonproliferating beryllium-responsive CD4+ T cells in the CBD lung is due to the loss of CD28 expression and a resulting ineffective CTLA-4 pathway. In CBD, the continued presence of IFN-γ promotes chronic lung inflammation, including the development of granulomas and fibrosis (1–3).

Our data raise the possibility that a normal consequence of memory cell transition to the most terminally differentiated state in a target organ is a loss of CTLA-4 function. The active mechanism by which CTLA-4 downregulates T cell activation is direct inhibition of the CD28-signaling pathway (17–20). Memory T cells become less dependent on CD28 costimulation as they become more differentiated (40–42), and beryllium-specific TEM cells in the lung no longer require CD28 costimulation, with a subset of these cells having lost CD28 expression (10). In this study, we extend those findings by showing that loss of CD28 directly correlates with increased IFN-γ expression by beryllium-responsive CD4+ T cells in the BALF. Therefore, as memory cells differentiate and lose signaling through CD28, our data suggest that the CTLA-4-mediated signaling pathway is incomplete and cannot downregulate cytokine production. Because beryllium-specific cells in the blood of CBD patients remain CD28 dependent and are less differentiated than their counterparts in lung (7), this could explain the differences in CTLA-4 function between blood and lung observed in this study. We believe that our findings have important implications for other target organ–specific immune-mediated diseases, including cancer, for which CTLA-4-blocking Abs are being used clinically with limited success (51).

In conclusion, our data support the following model of continued inflammation due to chronic beryllium exposure in the lung. In the presence of active alveolitis, beryllium-responsive CD4+ T cells...
are recruited to lung and undergo progressive T cell differentiation, resulting in a loss of CD28 expression and less IL-2 secretion on a per-cell basis compared with their blood counterparts (39, 52). In the process of trafficking to the lung and loss of CD28 expression, beryllium-responsive CD4+ T cells differentiate to gain IFN-γ expression and eventually lose the ability to secrete IL-2. CTLA-4 expression increases on proliferating, IL-2–expressing, beryllium-responsive CD4+ T cells and decreases as cells cease to divide and upregulate PD-1. Therefore, we propose that the major consequence of the loss of CD28 dependence in the lung is an ineffective CTLA-4–signaling pathway, resulting in continued IFN-γ secretion by proliferation-incompetent CD4+ T cells, persistent lung inflammation, and, eventually, lung fibrosis.

Disclosures
The authors have no financial conflicts of interest.

References


