

Luminex
complexity simplified.



**Capabilities for Today.
Flexibility for Tomorrow.**

Amnis[®] CellStream[®] Flow Cytometry Systems.

LEARN MORE >



Up-Regulation of Programmed Death-1 Expression on Beryllium-Specific CD4⁺ T Cells in Chronic Beryllium Disease

This information is current as
of November 21, 2019.

Brent E. Palmer, Douglas G. Mack, Allison K. Martin, May
Gillespie, Margaret M. Mroz, Lisa A. Maier and Andrew P.
Fontenot

J Immunol 2008; 180:2704-2712; ;
doi: 10.4049/jimmunol.180.4.2704
<http://www.jimmunol.org/content/180/4/2704>

References This article **cites 41 articles**, 19 of which you can access for free at:
<http://www.jimmunol.org/content/180/4/2704.full#ref-list-1>

Why *The JI*? [Submit online.](#)

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
<http://jimmunol.org/subscription>

Permissions Submit copyright permission requests at:
<http://www.aai.org/About/Publications/JI/copyright.html>

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
<http://jimmunol.org/alerts>

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2008 by The American Association of
Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.



Up-Regulation of Programmed Death-1 Expression on Beryllium-Specific CD4⁺ T Cells in Chronic Beryllium Disease¹

Brent E. Palmer,* Douglas G. Mack,* Allison K. Martin,* May Gillespie,‡ Margaret M. Mroz,‡ Lisa A. Maier,*‡ and Andrew P. Fontenot^{2*†}

Chronic beryllium disease (CBD) is caused by workplace exposure to beryllium and is characterized by the accumulation of memory CD4⁺ T cells in the lung. These cells respond vigorously to beryllium salts in culture by producing proinflammatory Th1-type cytokines. The presence of these inflammatory cytokines leads to the recruitment of alveolar macrophages, alveolitis, and subsequent granuloma development. It has been shown that chronic exposure to conventional Ags leads to up-regulation in the expression of negative regulators of T cells such as programmed death-1 (PD-1). Due to the persistence of beryllium in the lung after the cessation of exposure, aberrant regulation of the PD-1 pathway may play an important role in CBD development. In the present study, PD-1 expression was measured on blood and bronchoalveolar lavage (BAL) CD4⁺ T cells from beryllium-sensitized and CBD subjects. PD-1 expression was significantly higher on BAL CD4⁺ T cells compared with those cells in blood, with the highest expression on the beryllium-specific T cell subset. In addition, the expression of PD-1 on BAL CD4⁺ T cells directly correlated with the severity of the T cell alveolitis. Increased expression of the PD-1 ligands, PD-L1 and PD-L2, on BAL CD14⁺ cells compared with blood was also seen. The addition of anti-PD-1 ligand mAbs augmented beryllium-induced CD4⁺ T cell proliferation, and an inverse correlation was seen between PD-1 expression on beryllium-specific CD4⁺ T cells and beryllium-induced proliferation. Thus, the PD-1 pathway is active in beryllium-induced disease and plays a key role in controlling beryllium-induced T cell proliferation. *The Journal of Immunology*, 2008, 180: 2704–2712.

Chronic beryllium disease (CBD)³ is characterized by the accumulation of memory CD4⁺ T lymphocytes and granulomatous inflammation in the lungs of beryllium-exposed individuals (1, 2). When stimulated with beryllium salts, a large percentage of bronchoalveolar lavage (BAL) CD4⁺ T cells from CBD patients respond vigorously by producing proinflammatory cytokines such as IFN- γ and TNF- α (3, 4). The presence of these inflammatory cytokines leads to the recruitment of alveolar macrophages, alveolitis, and granuloma development. The regulation of this response is in part dependent on a balance between costimulatory and coinhibitory pathways. We have previously demonstrated that memory CD4⁺ T cells in the blood of CBD patients require CD28 costimulation for proliferative and cytokine

responses to beryllium (5). Conversely, proliferation and secretion of Th1-type cytokines by effector memory T (T_{EM}) cells in the lung are functionally independent of CD28 costimulation, suggesting a transition within memory T cell subsets from CD28 dependence in circulating cells to CD28 independence in fully differentiated T_{EM} cells in lung (5). Less is known about the expression of coinhibitory molecules in CBD and other chronic inflammatory lung disorders. It has been shown that chronic exposure to conventional Ags can lead to up-regulation in the expression of negative regulators of CD4⁺ and CD8⁺ T cells such as programmed death-1 (PD-1) (6–10). However, the role of PD-1 in response to nonconventional Ags, such as beryllium, is unknown.

PD-1, a member of the CD28 family, is involved in signaling T cell death and similar to CTLA-4, negatively regulates T cell function (11, 12). Engagement of PD-1 by its ligands, PD-L1 and PD-L2, inhibits T cell proliferation and cytokine production (13–15). When PD-1 is engaged, Src homology phosphatase-1 and -2 block PI3K activation, interfere with glucose uptake, and alter both CD3- and CD28-mediated changes to the T cell transcriptional profile resulting in decreased T cell activation (15, 16). Dysregulation of the PD-1 pathway has been implicated in human autoimmune diseases such as rheumatoid arthritis and type 1 diabetes (17–21). PD-1-deficient mice exhibit hyperactivation of the immune system and subsequently develop autoimmune disease (13). Recently, PD-1 has been shown to be up-regulated on virus-specific T cells during chronic disease, and its expression was directly correlated with viral Ag load (6–9). Taken together, these findings demonstrate that PD-1 is up-regulated on T cells that are responsive to persistent Ag to down-regulate the function of T cells which are aberrantly responding to autoantigens or to shut off a chronic inflammatory response to a pathogen. Due to the persistence of

*Department of Medicine and †Department of Immunology, University of Colorado at Denver and Health Sciences Center, Denver, CO 80262; and ‡Department of Medicine, National Jewish Medical and Research Center, Denver, CO 80206

Received for publication August 24, 2007. Accepted for publication December 11, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by the following National Institutes of Health Grants: HL62410 and ES011810 (to A.P.F.), and the General Clinical Research Center (M01-RR00051) from the Division of Research Resources.

² Address correspondence and reprint requests to Dr. Andrew P. Fontenot, Division of Clinical Immunology, University of Colorado at Denver and Health Sciences Center, 4200 East Ninth Avenue, Denver, CO 80262. E-mail address: andrew.fontenot@uchsc.edu

³ Abbreviations used in this paper: CBD, chronic beryllium disease; BAL, bronchoalveolar lavage; T_{EM} cell, effector memory T cell; PD-1, programmed death-1; BeS, beryllium sensitized; SEB, staphylococcal enterotoxin B; MFI, mean fluorescence intensity; WBC, white blood cell; T_{CM}, central memory T cell.

Table I. Characteristics of BeS and CBD patients^a

Characteristics	BeS Patients (n = 14)	CBD Patients (n = 26)
Age (years)	58 (43–68)	57 (37–78)
Gender (M/F)	4/10	6/20
Race (C/AF/H) ^b	12/1/1	22/1/3
Smoking status (CS/FS/NS) ^c	0/10/4	0/10/16
Industry of exposure (nuclear/ceramic/other)	14/0/0	10/7/6
Treatment (none/prednisone/methotrexate)	14/0/0	16/10/2
Beryllium lymphocyte proliferation test, stimulation index		
Peripheral blood lymphocytes	3.2 (0.7–42)	3.5 (1–122)
BAL	1.4 (1–2.1)	64 (1.2–305) ^d
BAL cells		
WBC count (×10 ⁶)	20 (11–79)	30 (9.5–230)
Lymphocytes (%)	6.7 (2.5–29)	29 (6–84) ^d

^a Data expressed as median (range).
^b C, Caucasian; AF, African American; H, Hispanic.
^c CS, Current smoker; FS, former smoker; NS, never smoker.
^d Value of *p* < 0.0001.

beryllium in the lung long after the cessation of exposure (22–24), aberrant regulation of the PD-1 pathway may play an important role in the development of CBD.

With a known antigenic stimulus and an accessible target organ, CBD is an organ-specific immune-mediated disorder that provides an opportunity to investigate PD-1 expression on Ag-specific

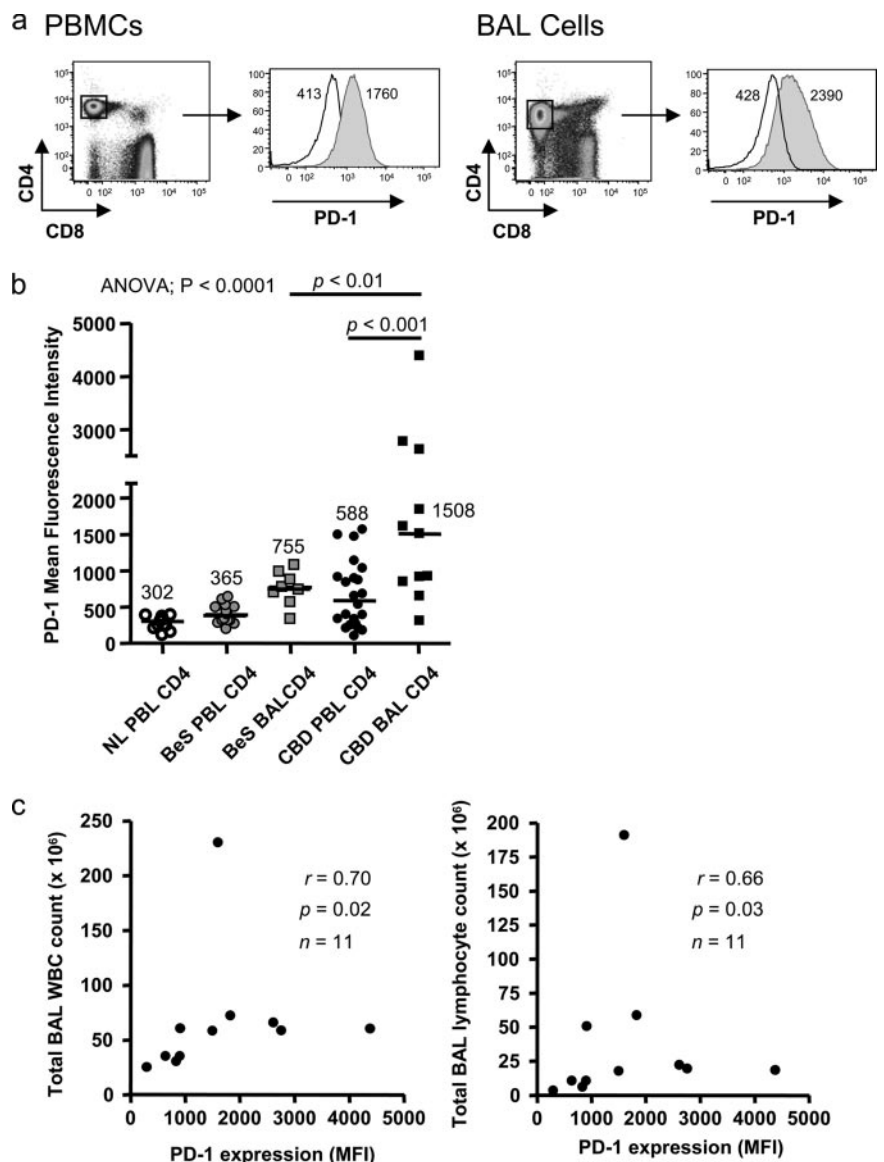


FIGURE 1. PD-1 expression on total CD4⁺ T cells in the BAL of CBD patients is elevated and correlates with T cell alveolitis. *a*, A representative example of PD-1 staining on total CD3⁺CD4⁺ T cells from the blood (*left panel*) and BAL (*right panel*) of a CBD patient. PD-1 expression on CD4⁺ T cells (shaded) compared to the FMO control (open) is shown in the histogram. *b*, MFI of PD-1 expression on total CD3⁺CD4⁺ T cells from the blood of normal controls (*n* = 13), BeS subjects (*n* = 14), and CBD patients (*n* = 22) and the BAL of BeS (*n* = 8) and CBD (*n* = 11) is shown. Median values are indicated with solid lines. Statistical significance was established using the Kruskal-Wallis test. *c*, Correlation between PD-1 expression (MFI) on total BAL CD3⁺CD4⁺ T cells from CBD patients (*n* = 11) and total BAL WBC (*left*) and lymphocyte counts (*right*) is shown.

CD4⁺ T cells from a target organ. During chronic HIV infection, it has recently been shown that PD-1 is up-regulated on CD4⁺ T cells to a greater extent in the lymph node (i.e., the target organ in HIV disease) compared with peripheral blood (7). We examined the expression of PD-1 on beryllium-specific CD4⁺ T cells from the blood and BAL of beryllium-sensitized (BeS) and CBD subjects. PD-1 expression was elevated on total CD4⁺ T cells from the lungs of CBD subjects and was directly correlated with the severity of the CD4⁺ T cell alveolitis. Furthermore, in the lung, PD-1 expression was most elevated on the beryllium-responsive subset of CD4⁺ T cells. Blockade of the PD-1 pathway led to enhanced beryllium-specific CD4⁺ T cell proliferation, indicating the functionality of this pathway. Taken together, these data demonstrate that PD-1 is up-regulated and active on beryllium-specific CD4⁺ T cells and plays an important role in regulating the proliferative response of the beryllium-specific CD4⁺ T cell.

Materials and Methods

Study population

Twenty-six patients with a diagnosis of CBD and 14 BeS patients were enrolled in this study. Thirteen healthy non-beryllium-exposed control subjects were also enrolled. The diagnosis of CBD was established using previously defined criteria, including a history of beryllium exposure, the presence of granulomatous inflammation on lung biopsy, and a positive proliferative response of blood and/or BAL T cells to beryllium sulfate (BeSO₄) in vitro (25, 26). The diagnosis of BeS was established based on a history of beryllium exposure, a positive proliferative response of PBMC to BeSO₄ in vitro, and the absence of granulomatous inflammation or other abnormalities on lung biopsy (27, 28). Active smokers were excluded from enrollment. Informed consent was obtained from each patient and control subject, and the protocol was approved by the Human Subject Institutional Review Boards at the University of Colorado at Denver Health Sciences Center and the National Jewish Medical and Research Center (Denver, CO).

The demographics of the BeS and CBD patients 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 both subject groups were male. Ten CBD patients were treated with oral glucocorticoids and two received oral methotrexate. No difference in beryllium-induced proliferation of blood cells was seen between BeS and CBD patients. In contrast, a significant increase in the proliferation of BAL cells from CBD patients compared with BeS subjects in response to beryllium was seen (median stimulation index, 64 (range, 1.2–230) vs 1.4 (1.0–2.1), $p < 0.0001$). All clinical beryllium lymphocyte proliferation tests were performed in the Clinical Immunology Laboratory (National Jewish Medical and Research Center). CBD subjects had a statistically significant increase in the percentage of BAL lymphocytes (median, 29%; range, 6–84%) compared with BeS patients (median, 6.7%; range, 2.5–29%; $p < 0.0001$).

Preparation of peripheral blood and BAL cells and beryllium-induced cytokine production

PBMCs were isolated from heparinized blood by Ficoll-Hypaque density gradient separation, and BAL was performed as previously described (29, 30). PBMCs ($2.5\text{--}5 \times 10^6$) were resuspended in RPMI 1640 plus 10% heat-inactivated human serum (Gemini Bio-Products), placed in 12- × 75-mm culture tubes, and incubated overnight at 37°C in a humidified 5% CO₂ atmosphere. Following the overnight incubation, 3 μg/ml anti-CD28 and -CD49d mAbs (BD Biosciences) were added, and the cells were stimulated under the following conditions: 100 μM BeSO₄ (Brush Wellman), 10 ng/ml staphylococcal enterotoxin B (SEB) (Toxin Technologies), or medium alone. The cultures were incubated at a 5° slant at 37°C in a humidified 5% CO₂ atmosphere for 6 h with brefeldin A (BD Biosciences) added after 1 h.

Immunofluorescence staining of stimulated CD4⁺ T cells and monocytes

Stimulated PBMCs were washed and stained with anti-CD4 (allophycocyanin-Cy7; BD Biosciences), anti-CD3 (PE-Texas Red; Beckman Coulter), anti-CD8 (Alexa Fluor 405; Caltag Laboratories), and anti-CD279 (PD-1) (FITC; BD Biosciences) mAbs for 30 min at 4°C. Cells were washed with PBS containing 1% BSA, fixed, permeabilized, and stained with anti-IFN-γ (PE-Cy7; BD Biosciences) and anti-IL-2 (allophyc-

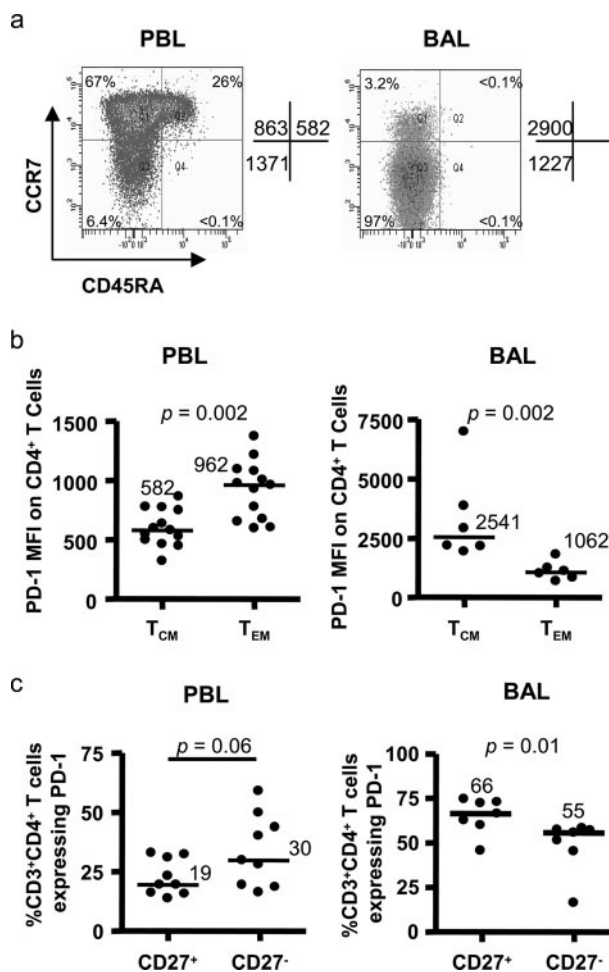


FIGURE 2. PD-1 expression on memory CD4⁺ T cells from the blood and BAL of CBD patients. *a*, Distribution of memory CD4⁺ T cell subsets in PBL and BAL of a representative subject with CBD is shown. The percentage of each memory subset within the total CD4⁺ T cell pool is depicted in each quadrant. PD-1 expression (MFI) for each memory subset is shown to the right of the density plots. *b*, PD-1 expression on T_{CM} (CCR7⁺CD45RA⁻) and T_{EM} (CCR7⁻CD45RA⁻) cells from the PBL ($n = 13$) and BAL ($n = 6$) of CBD patients is shown. The median PD-1 MFI on CD4⁺ T cells expressing each maturation phenotype is indicated as a solid line. *c*, The percentage of CD3⁺CD4⁺ T cells in the PBL ($n = 9$) and BAL ($n = 7$) expressing PD-1 in relation to CD27 is shown. Median values are indicated with solid lines. Statistical comparisons were made using the Mann-Whitney *U* test.

ocyanin; Caltag Laboratories) mAbs for 30 min at 4°C. Fluorescence minus one and isotype controls were used in all staining. In some experiments, the cells were also stained with anti-CD27, -CCR7, and -CD45RA mAbs (all obtained from BD Biosciences). PBMCs or BAL cells were stained with anti-CD14-FITC (BD Biosciences) and PE-labeled anti-PD-L1 or -PD-L2 mAbs (both obtained from BD Biosciences). Cells were first incubated with FcR-blocking reagent (Miltenyi Biotec) to reduce non-specific staining.

Proliferation assay

Proliferation assays were performed using PBMCs and BAL cells (2×10^6 cells/well) labeled with 2.0 μM CFSE (Molecular Probes) (31, 32). The CFSE-labeled cells were cultured at 37°C for 5 days in a humidified 5% CO₂ atmosphere in RPMI 1640 supplemented with 10% heat-inactivated human serum, 20 mM HEPES, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (all from Invitrogen Life Technologies) with the addition of either medium, 10 μg/ml plate-bound anti-CD3, or 100 μM BeSO₄. PD-L1 and/or PD-L2 mAbs (1 μg/ml; eBioscience) were added to the cultures to block PD-1 ligation. Cells were

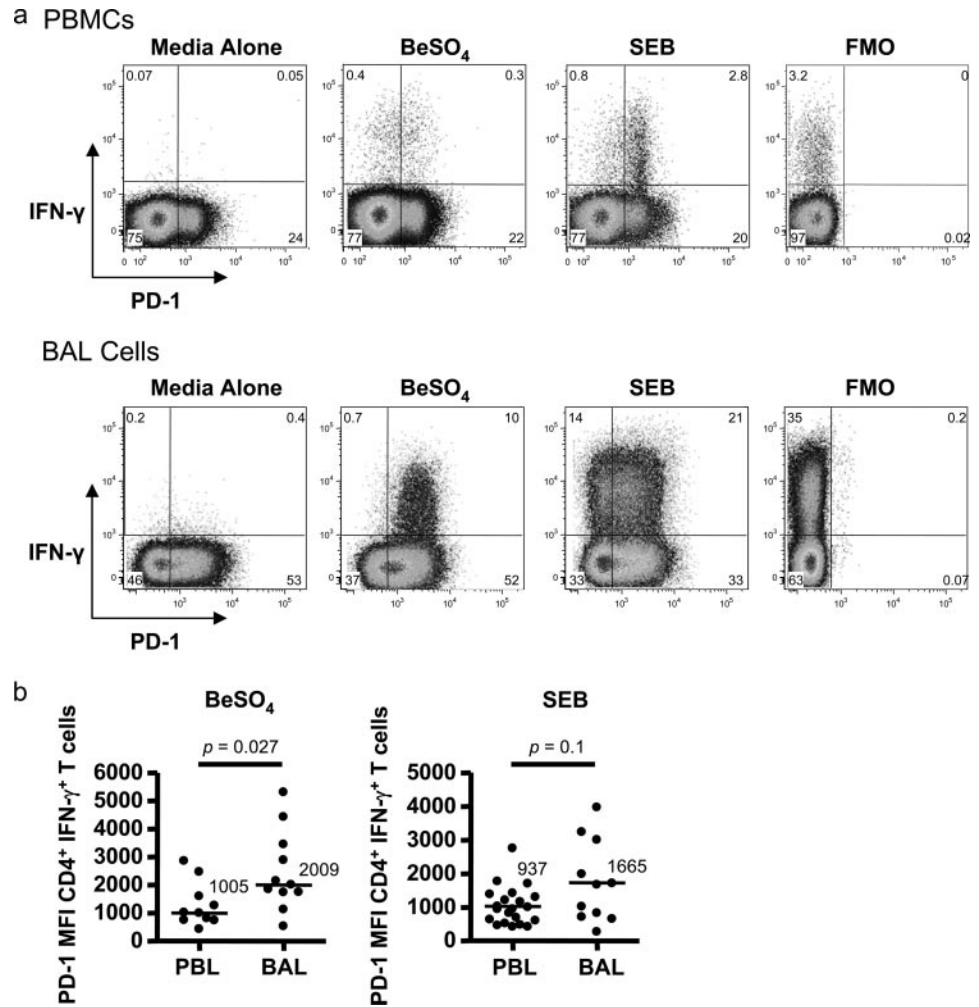


FIGURE 3. PD-1 expression is up-regulated on IFN- γ -producing, beryllium-specific CD4⁺ T cells in the lung. *a*, Representative density plots showing expression of PD-1 on IFN- γ -expressing CD4⁺ T cells from the blood (*upper panel*) and BAL (*lower panel*) in response to Ag stimulation. *b*, PD-1 expression (MFI) on beryllium-specific ($n = 10$ PBL, $n = 11$ BAL) and SEB-reactive ($n = 22$ PBL, $n = 11$ BAL) IFN- γ -expressing CD4⁺ T cells in the blood and lung of CBD patients is depicted. Median values are indicated with solid lines, and statistical comparisons were made using the Mann-Whitney *U* test.

stained with mAbs directed against CD3-PE (BD Biosciences), CD4-allophycocyanin (Caltag Laboratories), and CD8-PerCP (BD Biosciences). Cells were washed and resuspended in 1% formaldehyde.

Flow cytometry

Cells were analyzed using a FACSAria 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 mAbs used in the test samples. The data files were analyzed using Diva software (BD Biosciences). Lymphocytes were gated based on their forward and side scatter profile. CD3⁺ cells were selected, and the expression of CD4 and CD8 was analyzed in a bivariate dot plot. Because PD-1 expression was not bimodal, expression levels on CD4⁺ T cells were determined by subtracting the mean fluorescence intensity (MFI) of the fluorescence minus one control from the PD-1 MFI on the population of interest. Biexponential scaling was used in all dot plots. Because the frequency of beryllium-specific CD4⁺ T cells in blood tends to be low, we only examined the expression of PD-1 on cytokine-producing cells with frequencies $\geq 0.04\%$ to ensure an adequate number of events for analysis as previously described (31–33). The CFSE-blocking experiments were acquired using a FACSCalibur flow cytometer (BD Immunocytometry Systems), and CellQuest Pro software was used to analyze the data.

Statistical analysis

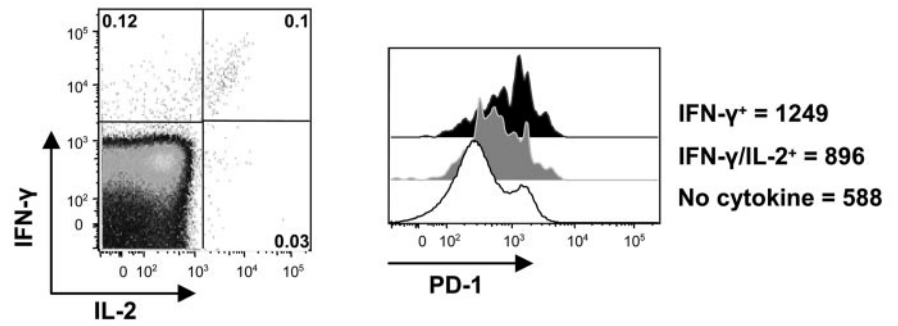
The Mann-Whitney *U* test and the Kruskal-Wallis ANOVA were used to determine significance of differences between subject groups. A Spearman correlation was performed to analyze the association between PD-1 expression on BAL CD4⁺ T cells and continuous variables of CBD. A *p* value of <0.05 was considered statistically significant.

Results

PD-1 expression is elevated on total BAL CD4⁺ T cells from CBD subjects

Because the expression of PD-1 is up-regulated on T cells in response to persistent viral Ag exposure, we initially analyzed PD-1 expression on blood and BAL CD4⁺ T cells from patients with CBD, a disease characterized by persistent exposure to a nonconventional T cell Ag. Freshly isolated PBMCs and BAL cells were obtained from CBD patients ($n = 22$ PBL, 11 BAL), BeS subjects ($n = 14$ PBL, 8 BAL), and normal control subjects ($n = 13$ PBL), and the MFI of PD-1 expression on total CD3⁺CD4⁺ T cells was determined by immunofluorescence staining and cytofluorographic analysis. Representative histograms of PD-1 expression on total CD3⁺CD4⁺ T cells from the peripheral blood and BAL of a CBD subject are shown in Fig. 1*a*. PD-1 expression (MFI) was significantly higher on BAL CD4⁺ T cells (median, 1508; range, 308–4384) from CBD subjects compared with either blood CD4⁺ T cells (588, range 99–1561) from the same disease cohort or BAL CD4⁺ T cells (755; range, 332–1073) from BeS subjects ($p < 0.01$ and $p < 0.001$, respectively) (Fig. 1*b*). Although not statistically significant, PD-1 expression trended higher on total CD4⁺ T cells from the blood of CBD patients (median, 588; range, 99–1561) compared with the expression on blood CD4⁺ T cells from BeS subjects (median, 365; range, 193–635) and normal control subjects (median, 302; range, 110–386).

a PBMCs



BAL Cells

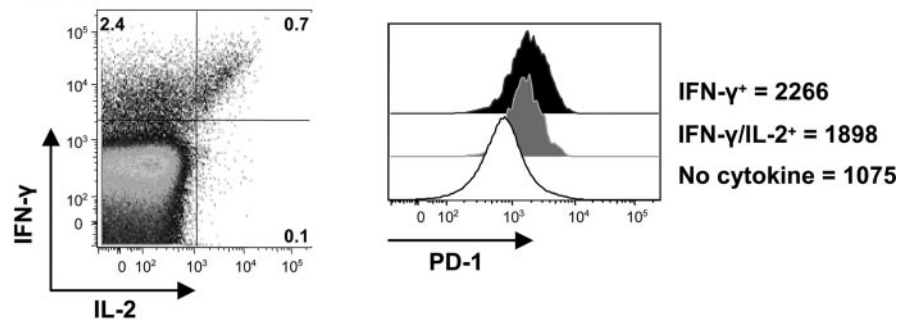
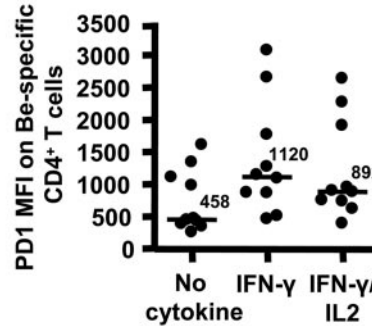
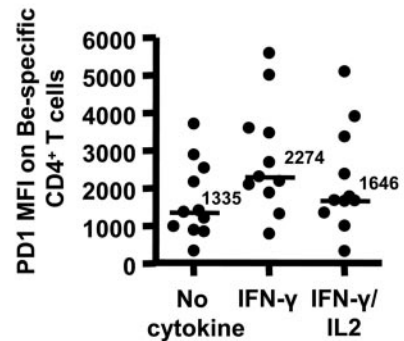


FIGURE 4. Distribution of PD-1 expression on beryllium-specific, Th1-type, cytokine-producing CD4⁺ T cells subsets. *a*, Representative histograms of PD-1 expression (MFI) on beryllium-specific, IFN- γ - and IFN- γ /IL-2-producing CD4⁺ T cell subsets from the blood and BAL of CBD patients is shown. *b*, PD-1 expression (MFI) on the different cytokine-producing subsets of beryllium-specific CD4⁺ T cells from the blood ($n = 10$; left panel) and BAL ($n = 11$; right panel) of CBD patients is shown. Median values are indicated with solid lines.

b PBMCs



BAL Cells



Based on the observation that PD-1 expression on T cells from subjects with chronic viral infections was associated with disease severity (6, 7, 9), we correlated PD-1 expression on BAL CD4⁺ T cells from CBD patients with total BAL white blood cell (WBC) and lymphocyte counts (Fig. 1c). Significant positive correlations were seen for both total BAL WBC count ($r = 0.70$; $p = 0.02$) and lymphocyte count ($r = 0.66$; $p = 0.03$). No correlation was observed, however, between PD-1 expression on BAL CD4⁺ T cells and total BAL macrophage count ($r = -0.34$; $p = 0.29$) (data not shown). Thus, the severity of the CD4⁺ T cell alveolitis appears to be closely tied to PD-1 expression on CD4⁺ T cells in the BAL.

PD-1 expression is higher on less differentiated T cell subsets in the lung

Due to the association of PD-1 expression with T cell exhaustion, we determined whether its expression on CD4⁺ T cells was correlated with the expression of other memory T cell markers. Using CCR7 and CD45RA to identify CD4⁺ memory T cell subsets, we evaluated PD-1 expression on central memory T (T_{CM}) and T_{EM} cells in blood and BAL (Fig. 2). As expected, the median PD-1 expression (MFI) on blood CD4⁺ T cells was higher on T_{EM} cells (962; range, 597–1371) compared with T_{CM} cells (582; range,

321–863; $p = 0.002$). Conversely, BAL CD4⁺ T_{CM} cells expressed higher levels of PD-1 (2541; range, 1925–6980) than the T_{EM} cell subset (1062; range, 676–1810; $p = 0.002$) (Fig. 2, *a* and *b*). Using CD27, another marker used to differentiate T_{CM} from T_{EM} cells, we again observed the same trend with the CD4⁺CD27⁺ T cell subset containing a higher percentage of PD-1-expressing cells in the lung ($p = 0.01$) while CD4⁺CD27⁻ T cells contained a greater percentage of PD-1-expressing CD4⁺ T cells in the blood ($p = 0.06$) (Fig. 2c). These findings suggest that PD-1-expressing CD4⁺ T cells in the circulating pool represent a subset of the differentiated T_{EM} cell population while in the lung these cells are predominately found in the T_{CM} cell subset.

PD-1 expression is up-regulated on beryllium-specific CD4⁺ T cells

To determine whether PD-1 expression is up-regulated on beryllium-specific CD4⁺ T cells, we measured the levels of PD-1 on beryllium-specific and SEB-reactive IFN- γ -producing T cells in CBD subjects. IFN- γ -producing T cells were initially analyzed because we had previously shown that beryllium-specific CD4⁺ T cells in both blood and BAL were skewed toward poorly proliferating, IFN- γ -producing T_{EM} cells (32). Polychromatic flow

cytometry was used to measure PD-1 expression on Ag-specific CD4⁺ (CD3⁺/CD4⁺/CD8⁻) T cells. Representative density plots for blood and BAL CD4⁺ T cells stimulated with either medium, BeSO₄, or SEB are shown in Fig. 3*a*. IFN- γ -secreting, beryllium-specific CD4⁺ T cells from the BAL expressed significantly higher levels of PD-1 than either total CD3⁺CD4⁺ T cells or beryllium-reactive CD4⁺ T cells in blood (Fig. 3). The median PD-1 expression on IFN- γ ⁺, beryllium-specific CD4⁺ T cells from the BAL was 2009 (range, 526–5308) compared with 1005 (median; range, 427–2859; $p = 0.027$) for IFN- γ ⁺, beryllium-reactive CD4⁺ T cells in blood (Fig. 3*b*, left). Although not statistically significant, PD-1 expression (MFI) on beryllium-specific, IFN- γ ⁺, CD4⁺ T cells in BAL was also higher compared with PD-1 expression on SEB-reactive CD4⁺ T cells (median, 1665; range, 264–3962; $p = 0.15$) in the same compartment. This is not altogether surprising because we have previously shown that the beryllium-reactive CD4⁺ T cells that are recruited to the lung in CBD express TCR β -chain V regions (V β) that are responsive to SEB (e.g., V β 3 and V β 17) (29). In addition to MFI, the data were also analyzed as percent positive and the same trends and statistical significance were seen (data not shown).

No difference in PD-1 expression on IFN- γ -only and IFN- γ /IL-2-secreting, beryllium-responsive CD4⁺ T cells

It is widely accepted that IFN- γ -producing CD4⁺ T cells are more differentiated than CD4⁺ T cells that retain the ability to secrete IL-2 (34). However, it is less clear whether differences exist between cells that produce only IFN- γ and those that continue to express IL-2 in addition to IFN- γ . To determine whether the expression of PD-1 differed during the various stages of CD4⁺ T cell differentiation, we measured PD-1 on BeSO₄-responsive CD4⁺ T cells that produced either IFN- γ alone, IFN- γ and IL-2, or no cytokine from the blood and BAL of CBD subjects. Fig. 4*a* shows representative histograms of PD-1 expression on cytokine-producing CD4⁺ T cells from the blood and BAL. Overall, the level of PD-1 expressed on beryllium-specific CD4⁺ T cells in the BAL was higher than on beryllium-specific cells in blood. However, no significant difference between PD-1 expression on IFN- γ -only and IFN- γ /IL-2-secreting CD4⁺ T cells was observed, irrespective of the location of the beryllium-responsive T cell. For example, the median PD-1 expression (MFI) on beryllium-specific, IFN- γ -only producing CD4⁺ T cells in blood was 1120 (range, 464–3079) compared with 892 (range, 391–2639) on IFN- γ /IL-2-producing cells and 458 (range, 261–1611) on cells which do not express either IFN- γ or IL-2 ($p > 0.05$) (Fig. 4*b*). Similar findings were seen for IFN- γ -only and IFN- γ /IL-2-expressing CD4⁺ T cells in the BAL in response to BeSO₄.

Increased expression of PD-L1 and PD-L2 in the lung

Suppression of PD-1-expressing T cells requires that the PD-1 ligands (PD-L1 and PD-L2) are expressed on APCs. To examine this, PD ligand expression was measured by flow cytometry on CD14⁺ monocyte/macrophages in PBMCs and BAL cells from BeS, CBD, and normal control subjects. There was no difference between groups in the expression of PD-L1 and PD-L2 on CD14⁺ cells from blood (Fig. 5). The median PD-L1 expression (MFI) on CD14⁺ cells from the peripheral blood of BeS, CBD, and normal subjects was 6.6, 9.4, and 13, respectively (Fig. 5*a*). The median PD-L2 expression (MFI) on CD14⁺ cells from blood in BeS, CBD, and normal subjects was 2.5, 2.6, and 7, respectively (Fig. 5*b*). However, PD-L1 and PD-L2 expression on CD14⁺ cells from the BAL was significantly elevated. Median PD-L1 expression (MFI) on CD14⁺ cells in the BAL of BeS (125, range 42–192) and CBD (142, range 96–197) subjects was 19- and 15-fold higher,

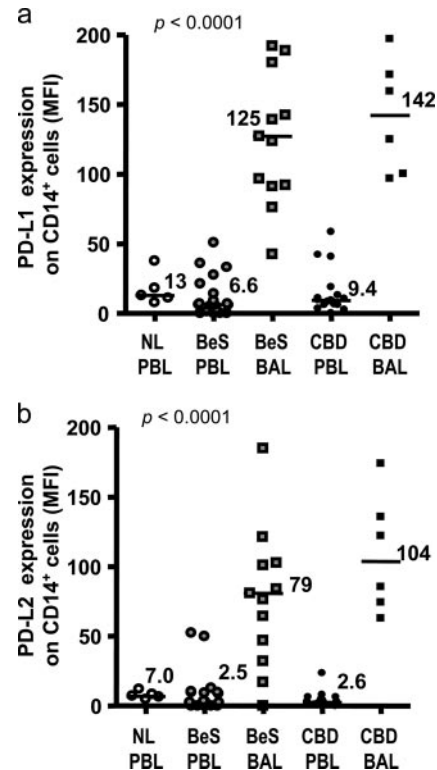


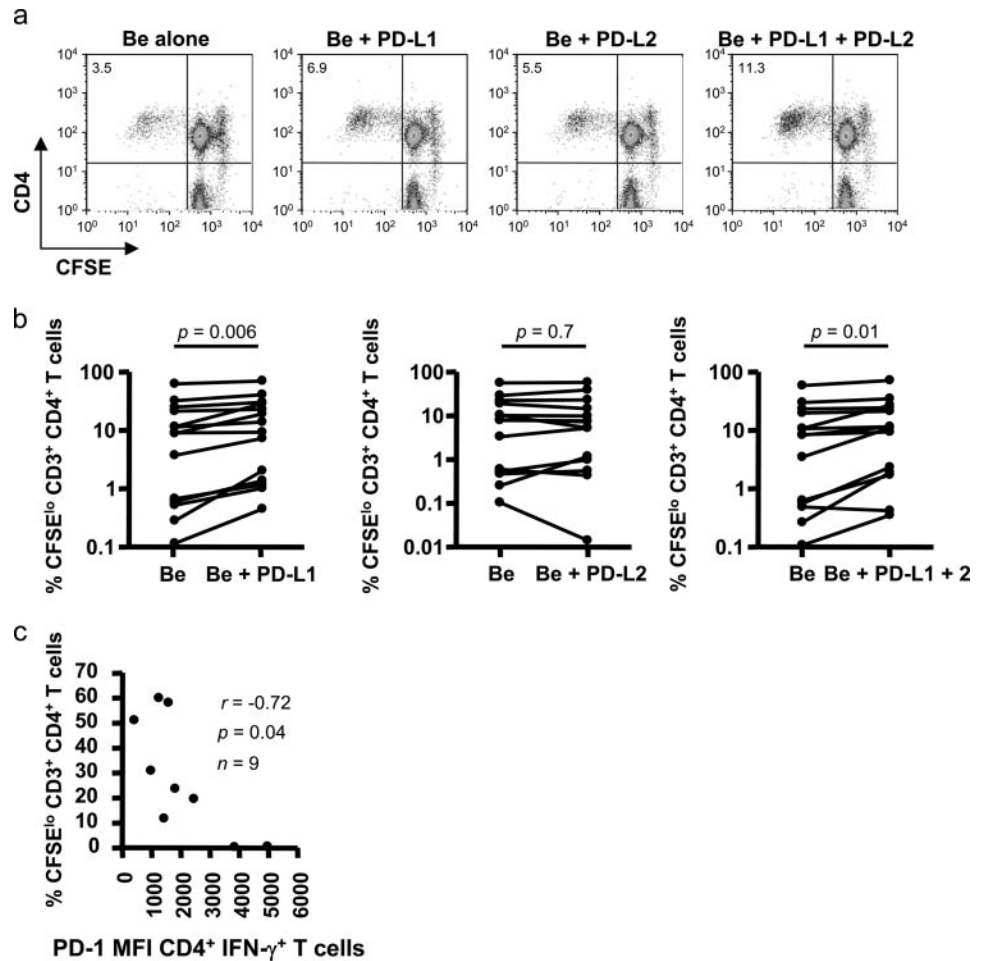
FIGURE 5. PD-1 ligand expression is elevated on CD14⁺ cells in the lung of BeS and CBD patients. PD-L1 (*a*) and PD-L2 (*b*) expression was evaluated on CD14⁺ cells from the peripheral blood of normal ($n = 5$), BeS ($n = 14$), and CBD ($n = 17$) patients and the BAL of BeS ($n = 12$) and CBD ($n = 6$) subjects. Median values are indicated with solid lines. Statistical significance was established using the Kruskal-Wallis test.

respectively, compared with CD14⁺ cells in blood. Median PD-L2 expression (MFI) on CD14⁺ cells from the BAL of BeS (79; range 39–184) and CBD (104; range 63–174) subjects was 32- and 40-fold higher than in blood. These data demonstrate that CD14⁺ APCs in the BAL of BeS and CBD patients have significantly higher levels of PD-1 ligands than those in the blood, suggesting that the PD-1 pathway is active and up-regulated in the BAL and not specific for CD14⁺ cells in the lung of CBD patients.

Blocking the PD-1 pathway enhances beryllium-specific CD4⁺ T cell proliferation

To demonstrate that the PD-1 pathway is functional in CBD, we blocked PD-1 interaction with its ligands using mAbs directed against PD-L1 and PD-L2. PBMCs from 14 CBD patients were labeled with CFSE, and proliferation was measured as the percentage of CFSE^{low}CD3⁺CD4⁺ T cells after 7 days of stimulation with 100 μ M BeSO₄. Representative density plots from a CBD subject showed enhanced BeSO₄-specific CD4⁺ T cell proliferation by blocking the PD-1 pathway using anti-PD-L1, anti-PD-L2, and a combination of both mAbs (Fig. 6*a*). All 14 subjects exhibited an increase in beryllium-specific CD4⁺ T cell proliferation upon blockade with a mAb directed against PD-L1 alone ($p = 0.006$) (Fig. 6*b*, left panel). Blockade with PD-L2 mAbs was not as effective with only 8 of 14 subjects demonstrating increased proliferation compared with BeSO₄ alone ($p = 0.7$) (Fig. 6*b*, center panel). Blocking the pathway with the addition of both anti-PD-L1/L2 mAbs also resulted in a significant increase in beryllium-specific CD4⁺ T cell proliferation ($p = 0.01$) (Fig. 6*b*, right panel). The greatest enhancement of proliferation occurred when the blocking Ab was used at a concentration of 1 μ g/ml (titration

FIGURE 6. Blockade of the PD-1/PD-1 ligand pathway results in increased beryllium-specific CD4⁺ T cell proliferation. *a*, Representative density plots of the percentage of CFSE^{low}CD3⁺CD4⁺ T cells from a CBD subject after 7 days of stimulation with 100 μM BeSO₄ in the presence and absence of anti-PD-L1, anti-PD-L2, or both Abs is shown. *b*, Summary data showing the percentage of CFSE^{low}CD3⁺CD4⁺ T cells in the presence and absence of the individual or combined blocking Abs (*n* = 14). Statistical comparisons were made using the Mann-Whitney *U* test. *c*, Correlation between PD-1 expression (MFI) on beryllium-responsive, IFN-γ-expressing CD4⁺ T cells from nine CBD patients and beryllium-induced proliferation of CD3⁺CD4⁺ T cells as measured by loss of CFSE is shown.



range 0.1–5 μg/ml; data not shown). Thus, the PD-1 pathway is indeed functional in CBD, and blockade of that pathway reinvigorates exhausted beryllium-specific CD4⁺ T_{EM} cells.

To determine whether the reduced lymphocyte proliferation seen in certain CBD subjects was related to the high expression of PD-1, we correlated PD-1 expression on beryllium-responsive, IFN-γ-expressing CD4⁺ T cells with the ability of CD4⁺ T cells from nine subjects to proliferate after BeSO₄ exposure in culture (Fig. 6c). A significant inverse correlation was seen ($r = -0.72$; $p = 0.04$), further emphasizing the importance of the PD-1 pathway in controlling beryllium-induced T cell proliferation.

Discussion

Aberrant proliferation and cytokine production by CD4⁺ T cells in the lungs of subjects exposed to beryllium is central to the immunopathogenesis of beryllium-induced disease. Determining how beryllium-specific CD4⁺ T cells in the lung are regulated represents an important area for understanding the mechanisms responsible for the development and progression of lung fibrosis. We have previously shown that beryllium-specific memory CD4⁺ T cells in blood remain dependent on CD28 engagement for optimal T cell activation while lung CD4⁺ T cells no longer require CD28 costimulation for either proliferation or Th1-type cytokine secretion (5). Another member of the CD28 family, PD-1, is a negative regulator of T cell function and is up-regulated on circulating T cells persistently exposed to Ag. CBD represents an important model of persistent Ag exposure and is characterized by the accumulation of large numbers of beryllium-specific CD4⁺ T cells in the lung (1–3). The present study is the first to demonstrate that

PD-1 is up-regulated on lung CD4⁺ T cells during an inflammatory response and that the PD-1 pathway in the lung is indeed functional. In addition, we demonstrate that persistent exposure to a nonconventional Ag, such as beryllium, leads to increased expression of PD-1 on Ag-specific CD4⁺ T cells. These findings provide at least a partial explanation for the reduction in beryllium-induced lymphoproliferation seen in certain individuals with CBD despite the presence of Th1-type, cytokine-expressing, beryllium-specific CD4⁺ T cells.

T cell regulation is important for initiating protective T cell responses to foreign Ags as well as to “switch off” harmful or aberrant responses to either self or persistent Ags. We have previously demonstrated that a large percentage of lung CD4⁺ T cells from CBD patients had lost CD28 expression and that the loss of CD28 extended to cells that were not responsive to beryllium (5). This is in contrast to the presence of PD-1 on the majority of IFN-γ-expressing, beryllium-responsive CD4⁺ T cells in the BAL. These findings suggest that the up-regulation of PD-1 expression on beryllium-responsive CD4⁺ T cells in the lung was most likely related to the persistence of beryllium in the target organ. Because these cells no longer require a second signal from professional APCs for optimal T cell activation, it is possible that they could respond in an uncontrolled manner to persistent Ag exposure, resulting in exuberant tissue damage. Thus, in the presence of persistent Ags, such as beryllium, the up-regulation of coinhibitory molecules may represent a vital step in controlling the adaptive immune response.

Following activation, T cells express coinhibitory molecules such as PD-1, CTLA-4, or B and T lymphocyte attenuator which

function to down-regulate the immune response (35). We have previously shown that CTLA-4 is expressed following the activation of beryllium-specific CD4⁺ T cells from the lungs of CBD subjects (5). In the present study, we found that PD-1 expression is up-regulated on CD4⁺ T cells from the BAL of subjects with CBD and closely correlated with the severity of the CD4⁺ T cell alveolitis. An important unanswered question is whether the severity of the alveolitis, and thus the up-regulation of PD-1 on CD4⁺ T cells, is tied to a greater beryllium burden in the lung. Blocking of the interaction between PD-1 and its ligands enhanced the proliferative capacity of beryllium-specific CD4⁺ T cells suggesting that this inhibitory pathway is active. The ligands for PD-1 are highly expressed on CD14⁺ alveolar macrophages, but not on blood monocytes, and yet BAL CD4⁺ T cells from CBD patients still produce copious amounts of Th1-type cytokines after beryllium recognition. In addition, there are some CBD subjects who possess large numbers of beryllium-specific CD4⁺ T cells in the lung (e.g., up to 20% of BAL CD4⁺ T cells from CBD patients express IFN- γ and TNF- α after beryllium exposure in culture) which lack a proliferative potential, suggesting that, at least in some subjects, the PD-1 pathway may control the proliferative capacity of these pathogenic effector T cells. At the present time, it remains unclear whether the PD-1 pathway affects just proliferation or whether cytokine production is also affected. However, Petrovas et al. (36) recently showed that the engagement of PD-1 during Ag stimulation had no effect on the ability of HIV- or CMV-specific CD8⁺ T cells to produce either IFN- γ , TNF- α , or IL-2.

The lung represents a unique environment that is constantly exposed to inhaled Ags. For this reason, a mechanism to down-regulate uncontrolled T cell responses in the lung must be in place. Although PD-1 expression was 2-fold higher in the lungs of CBD compared with BeS subjects, its expression in both cohorts was significantly higher than in blood suggesting that the PD-1 pathway may play a role in the down-regulation of T cell responses in the lung. To date, most studies have examined PD-1 expression on CD4⁺ T cells in blood. Thus, little information exists on the expression of PD-1 on Ag-specific CD4⁺ T cells in a target organ. We have recently shown that PD-1 expression on CD4⁺ T cells in the lymph nodes of subjects with chronic HIV infection was significantly higher than on cells from blood (7). Although this is in part a consequence of persistently high levels of viral Ag, it may also be that PD-1 expression in target organs is higher than in the bloodstream because of the activated state of the cells. In the present study, we observed increased PD-1 expression on CD4⁺ T cells in the lung as well as increased expression of its ligands on alveolar macrophages. The expression of PD-L1 and PD-L2 was not only high in the lung of CBD patients, but also in the lung of BeS subjects suggesting that these receptors may be constitutively expressed on alveolar macrophages in the lung. The suppressive effects of alveolar macrophages on T cell functions has been well-documented (37–39), and our data suggest that this could be in part due to the high level of PD-1 ligand expression.

In addition to T cell exhaustion, PD-1 plays a role in the differentiation of T cells. In HIV disease, we have previously demonstrated that PD-1 expression is greater on more differentiated IFN- γ -producing CD4⁺ T cells than less differentiated cells that only produced IL-2 (7). For that reason, we initially hypothesized that CD4⁺ T_{EM} cells in the lung, which represents the major CD4⁺ T cell subset in the BAL (3), would also express the highest levels of PD-1. However, PD-1 was actually expressed at the highest levels on BAL CD4⁺ T_{CM} cells. Investigating CD8⁺ T cells from normal and HIV-infected individuals, Sauce et al. (40) showed that PD-1 expression was highest on cells with an early to

intermediate stage of differentiation, with decreased expression of PD-1 on more differentiated T cell subsets. On influenza-specific CD8⁺ T cells in blood, PD-1 was also expressed on less differentiated T cell subsets (41). These data suggest that there is a direct link between the level of differentiation and the expression of PD-1 on CD8⁺ T cells. Based on these recent studies and our present findings, we hypothesize that the immune system is attempting to down-regulate the function of these recently recruited lung CD4⁺ T_{CM} cells to potentially prevent their differentiation into pathogenic T_{EM} cells. Limiting the capacity of T_{CM} cells to divide and differentiate into the T_{EM} cells which produce the inflammatory cytokines could lead to decreased recruitment of alveolar macrophages, alveolitis, and granuloma development.

Due to the persistent presence of beryllium in the lung (24), CBD is not curable and requires continuous treatment once therapy is initiated. Although corticosteroid treatment provides symptomatic relief, it does not result in a significant drop in the number of CD4⁺ T cells in the lung or the number of circulating beryllium-specific CD4⁺ T cells (29, 42). This study clearly demonstrates that PD-1 expression on beryllium-specific CD4⁺ T cells is up-regulated and that the pathway is active. Although alveolitis persists, it is likely that the PD-1 pathway is leading to some decrease in beryllium-specific CD4⁺ T cell function and severity of disease. Engagement of the PD-1 receptor with either a stimulatory mAb or soluble PD-L1/L2 could lead to additional functional down-regulation of beryllium-specific CD4⁺ T cells in the lung. The development of targeted immunotherapy that would specifically down-regulate the function of pathogenic T cells, not only for CBD, but for other inflammatory lung diseases where Ag persists and T cell regulation is lost, would represent an important advance. A PD-1-based immunotherapeutic treatment may be superior to systemic corticosteroids, which reduce the function of all T cells, because it would directly target Ag-specific CD4⁺ T cells which are responsible for the production of proinflammatory cytokines such as IFN- γ and TNF- α .

In summary, the present study shows, for the first time, that PD-1 expression is significantly up-regulated on Ag-specific CD4⁺ T cells in the lungs of subjects with chronic pulmonary inflammation. Although we have demonstrated that the PD-1 pathway is active by showing increased beryllium-specific CD4⁺ T cell proliferation after blocking PD-1 interaction with its ligands, its expression is directly correlated with the severity of the CD4⁺ T cell alveolitis, suggesting that this pathway alone is not sufficient to completely down-regulate T cell function for control of disease. A better understanding of the role of PD-1 in CBD and other pulmonary inflammatory diseases could provide important insight into the mechanism of disease progression and the development of lung fibrosis.

Disclosures

The authors have no financial conflict of interest.

References

1. Amicosante, M., and A. P. Fontenot. 2006. T cell recognition in chronic beryllium disease. *Clin. Immunol.* 121: 134–143.
2. Fontenot, A. P., and L. A. Maier. 2005. Genetic susceptibility and immune-mediated destruction in beryllium-induced disease. *Trends Immunol.* 26: 543–549.
3. Fontenot, A. P., S. J. Canavera, L. Gharavi, L. S. Newman, and B. L. Kotzin. 2002. Target organ localization of memory CD4⁺ T cells in patients with chronic beryllium disease. *J. Clin. Invest.* 110: 1473–1482.
4. Tinkle, S. S., L. A. Kittle, B. A. Schumacher, and L. S. Newman. 1997. Beryllium induces IL-2 and IFN- γ in berylliosis. *J. Immunol.* 158: 518–526.
5. Fontenot, A. P., L. Gharavi, S. R. Bennett, S. J. Canavera, L. S. Newman, and B. L. Kotzin. 2003. CD28 costimulation independence of target organ versus circulating memory antigen-specific CD4⁺ T cells. *J. Clin. Invest.* 112: 776–784.

6. Day, C. L., D. E. Kaufmann, P. Kiepiela, J. A. Brown, E. S. Moodley, S. Reddy, E. W. Mackey, J. D. Miller, A. J. Leslie, C. DePierres, et al. 2006. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 443: 350–354.
7. D'Souza, M., A. P. Fontenot, D. G. Mack, C. Lozupone, S. Dillon, A. Meditz, C. C. Wilson, E. Connick, and B. E. Palmer. 2007. Programmed death 1 expression on HIV-specific CD4⁺ T cells is driven by viral replication and associated with T cell dysfunction. *J. Immunol.* 179: 1979–1987.
8. Golden-Mason, L., B. Palmer, J. Klarquist, J. A. Mengshol, N. Castelblanco, and H. R. Rosen. 2007. Upregulation of PD-1 expression on circulating and intrahepatic hepatitis C virus-specific CD8⁺ T cells associated with reversible immune dysfunction. *J. Virol.* 81: 9249–9258.
9. Trautmann, L., L. Janbazian, N. Chomont, E. A. Said, S. Gimmig, B. Bessette, M. R. Boulassel, E. Delwart, H. Sepulveda, R. S. Balderas, et al. 2006. Upregulation of PD-1 expression on HIV-specific CD8⁺ T cells leads to reversible immune dysfunction. *Nat. Med.* 12: 1198–1202.
10. Zhang, J. Y., Z. Zhang, X. Wang, J. L. Fu, J. Yao, Y. Jiao, L. Chen, H. Zhang, J. Wei, L. Jin, et al. 2007. PD-1 up-regulation is correlated with HIV-specific memory CD8⁺ T-cell exhaustion in typical progressors but not in long-term nonprogressors. *Blood* 109: 4671–4678.
11. Ishida, Y., Y. Agata, K. Shibahara, and T. Honjo. 1992. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J.* 11: 3887–3895.
12. Sharpe, A. H., and G. J. Freeman. 2002. The B7-CD28 superfamily. *Nat. Rev. Immunol.* 2: 116–126.
13. Freeman, G. J., A. J. Long, Y. Iwai, K. Bourque, T. Chernova, H. Nishimura, L. J. Fitz, N. Malenkovich, T. Okazaki, M. C. Byrne, et al. 2000. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J. Exp. Med.* 192: 1027–1034.
14. Latchman, Y., C. R. Wood, T. Chernova, D. Chaudhary, M. Borde, I. Chernova, Y. Iwai, A. J. Long, J. A. Brown, R. Nunes, et al. 2001. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat. Immunol.* 2: 261–268.
15. Parry, R. V., J. M. Chemnitz, K. A. Frauwirth, A. R. Lanfranco, I. Braunstein, S. V. Kobayashi, P. S. Linsley, C. B. Thompson, and J. L. Riley. 2005. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. *Mol. Cell Biol.* 25: 9543–9553.
16. Chemnitz, J. M., R. V. Parry, K. E. Nichols, C. H. June, and J. L. Riley. 2004. SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. *J. Immunol.* 173: 945–954.
17. Ansari, M. J., A. D. Salama, T. Chitnis, R. N. Smith, H. Yagita, H. Akiba, T. Yamazaki, M. Azuma, H. Iwai, S. J. Khoury, et al. 2003. The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice. *J. Exp. Med.* 198: 63–69.
18. Fife, B. T., I. Guleria, M. Gubbels Bupp, T. N. Eagar, Q. Tang, H. Bour-Jordan, H. Yagita, M. Azuma, M. H. Sayegh, and J. A. Bluestone. 2006. Insulin-induced remission in new-onset NOD mice is maintained by the PD-1-PD-L1 pathway. *J. Exp. Med.* 203: 2737–2747.
19. Hatachi, S., Y. Iwai, S. Kawano, S. Morinobu, M. Kobayashi, M. Koshiba, R. Saura, M. Kurosaka, T. Honjo, and S. Kumagai. 2003. CD4⁺ PD-1⁺ T cells accumulate as unique anergic cells in rheumatoid arthritis synovial fluid. *J. Rheumatol.* 30: 1410–1419.
20. Keir, M. E., S. C. Liang, I. Guleria, Y. E. Latchman, A. Qipo, L. A. Albacker, M. Koulmanda, G. J. Freeman, M. H. Sayegh, and A. H. Sharpe. 2006. Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J. Exp. Med.* 203: 883–895.
21. Wan, B., H. Nie, A. Liu, G. Feng, D. He, R. Xu, Q. Zhang, C. Dong, and J. Z. Zhang. 2006. Aberrant regulation of synovial T cell activation by soluble costimulatory molecules in rheumatoid arthritis. *J. Immunol.* 177: 8844–8850.
22. Jones-Williams, W., and D. Kelland. 1986. New aid for diagnosing chronic beryllium disease (CBD): laser ion mass analysis (LIMA). *J. Clin. Pathol.* 39: 900–901.
23. Jones-Williams, W., and E. R. Wallach. 1989. Laser microprobe mass spectrometry (LAMMS) analysis of beryllium, sarcoidosis, and other granulomatous diseases. *Sarcoidosis* 6: 111–117.
24. Sawyer, R. T., J. L. Abraham, E. Daniloff, and L. S. Newman. 2005. Secondary ion mass spectroscopy demonstrates retention of beryllium in chronic beryllium disease granulomas. *J. Occup. Environ. Med.* 47: 1218–1226.
25. Newman, L. S., K. Kreiss, T. E. King, Jr., S. Seay, and P. A. Campbell. 1989. Pathologic and immunologic alterations in early stages of beryllium disease: re-examination of disease definition and natural history. *Am. Rev. Respir. Dis.* 139: 1479–1486.
26. Rossman, M. D., J. A. Kern, J. A. Elias, M. R. Cullen, P. E. Epstein, O. P. Preuss, T. N. Markham, and R. P. Daniele. 1988. Proliferative response of bronchoalveolar lymphocytes to beryllium. *Ann. Intern. Med.* 108: 687–693.
27. Newman, L. S. 1996. Significance of the blood beryllium lymphocyte proliferation test. *Environ. Health Perspect.* 104: 953S–956S.
28. Mroz, M. M., K. Kreiss, D. C. Lezotte, P. A. Campbell, and L. S. Newman. 1991. Reexamination of the blood lymphocyte transformation test in the diagnosis of chronic beryllium disease. *J. Allergy Clin. Immunol.* 88: 54–60.
29. Fontenot, A. P., B. L. Kotzin, C. E. Comment, and L. S. Newman. 1998. Expansions of T-cell subsets expressing particular T cell receptor variable regions in chronic beryllium disease. *Am. J. Respir. Cell Mol. Biol.* 18: 581–589.
30. Fontenot, A. P., M. T. Falta, B. M. Freed, L. S. Newman, and B. L. Kotzin. 1999. Identification of pathogenic T cells in patients with beryllium-induced lung disease. *J. Immunol.* 163: 1019–1026.
31. Palmer, B. E., N. Blyveis, A. P. Fontenot, and C. C. Wilson. 2005. Functional and phenotypic characterization of CD57⁺CD4⁺ T cells and their association with HIV-1-induced T cell dysfunction. *J. Immunol.* 175: 8415–8423.
32. Fontenot, A. P., B. E. Palmer, A. K. Sullivan, F. G. Joslin, C. C. Wilson, L. A. Maier, L. S. Newman, and B. L. Kotzin. 2005. Frequency of beryllium-specific, central memory CD4⁺ T cells in blood determines proliferative response. *J. Clin. Invest.* 115: 2886–2893.
33. Palmer, B. E., E. Boritz, and C. C. Wilson. 2004. Effects of sustained HIV-1 plasma viremia on HIV-1 gag-specific CD4⁺ T cell maturation and function. *J. Immunol.* 172: 3337–3347.
34. Sallusto, F., and A. Lanzavecchia. 2001. Exploring pathways for memory T cell generation. *J. Clin. Invest.* 108: 805–806.
35. Greenwald, R. J., G. J. Freeman, and A. H. Sharpe. 2005. The B7 family revisited. *Annu. Rev. Immunol.* 23: 515–548.
36. Petrovas, C., J. P. Casazza, J. M. Brenchley, D. A. Price, E. Gostick, W. C. Adams, M. L. Precopio, T. Schacker, M. Roederer, D. C. Douek, and R. A. Koup. 2006. PD-1 is a regulator of virus-specific CD8⁺ T cell survival in HIV infection. *J. Exp. Med.* 203: 2281–2292.
37. Bilyk, N., and P. G. Holt. 1993. Inhibition of the immunosuppressive activity of resident pulmonary alveolar macrophages by granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 177: 1773–1777.
38. Holt, P. G., J. Oliver, N. Bilyk, C. McMenamin, P. G. McMenamin, G. Kraal, and T. Thepen. 1993. Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells in vivo by resident alveolar macrophages. *J. Exp. Med.* 177: 397–407.
39. Strickland, D., U. R. Kees, and P. G. Holt. 1996. Regulation of T-cell activation in the lung: alveolar macrophages induce reversible T-cell anergy in vitro associated with inhibition of interleukin-2 receptor signal transduction. *Immunology* 87: 250–258.
40. Sauce, D., J. R. Almeida, M. Larsen, L. Haro, B. Autran, G. J. Freeman, and V. Appay. 2007. PD-1 expression on human CD8 T cells depends on both state of differentiation and activation status. *AIDS* 21: 2005–2013.
41. He, X. H., Q. T. Jia, F. Y. Li, M. Saltis, Y. Liu, L. H. Xu, and Q. B. Zha. 2007. CD8⁺ T cells specific for both persistent and non-persistent viruses display distinct differentiation phenotypes but have similar level of PD-1 expression in healthy Chinese individuals. *Clin. Immunol. In press.*
42. Pott, G. B., B. E. Palmer, A. K. Sullivan, L. Silveira, L. A. Maier, L. S. Newman, B. L. Kotzin, and A. P. Fontenot. 2005. Frequency of beryllium-specific, TH1-type cytokine-expressing CD4⁺ T cells in patients with beryllium-induced disease. *J. Allergy Clin. Immunol.* 115: 1036–1042.