

Beryllium-specific CD4⁺ T cells in blood as a biomarker of disease progression

Allison K. Martin, BS,^a Douglas G. Mack, PhD,^a Michael T. Falta, PhD,^a Margaret M. Mroz, MSPH,^d Lee S. Newman, MD,^{a,c} Lisa A. Maier, MD,^{a,c,d} and Andrew P. Fontenot, MD^{a,b,d} Aurora and Denver, Colo

Background: CD4⁺ T cells are responsible for the progressive lung damage seen in patients with chronic beryllium disease (CBD), a granulomatous lung disorder in which antigen-specific, T_H1-type, cytokine-secreting T cells have been characterized. Compared with those seen in beryllium (Be)-sensitized subjects, increased numbers of Be-responsive T cells are present in the blood of patients with CBD.

Objective: The aim of this study was to determine whether the number of Be-specific T cells in blood predicted the development of CBD in a cohort of Be-exposed subjects.

Methods: Using IFN- γ ELISpot and proliferation-based assays, we determined the frequency and proliferative capacity of Be-responsive T cells in blood.

Results: Compared with the Be lymphocyte proliferation test, which detected an abnormal Be-induced proliferative response in 11 (4.2%) of 260 workers from a Be-machining facility, the IFN- γ ELISpot detected a sensitization rate of 10% ($\chi^2 = 55.7$, $P < .0001$). A significant positive correlation was also noted between the number of Be-responsive CD4⁺ T cells in the blood and lung tissue of patients with CBD. Importantly, the transition from Be sensitization to CBD was associated with an increased number of antigen-specific T cells in blood.

Conclusion: These findings have important implications for Be-induced disease and potentially other immune-mediated disorders, suggesting that the frequency of antigen-specific T cells in blood can serve as a noninvasive biomarker to predict disease development and severity of the Be-specific CD4⁺ T-cell alveolitis. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: Human, lung, CD4⁺ T lymphocytes, beryllium, cytokines, granuloma, ELISpot

Chronic beryllium disease (CBD) is an organ-specific immune-mediated disorder characterized by the presence of granulomatous inflammation in the lung.¹ Depending on a subject's exposure and genetic susceptibility, the disorder occurs in 1% to 18% of beryllium (Be)-exposed workers.²⁻⁵ Sensitization to Be is detected based on the presence of Be-specific CD4⁺ T cells in blood, which proliferate on exposure to Be salts *in vitro*. This proliferative response forms the basis of the beryllium lymphocyte proliferation test (BeLPT), which is the standard assay for the detection of Be sensitization in the workplace.⁶ Importantly, the BeLPT is incapable of distinguishing between Be sensitization and CBD.⁷ Although sensitization precedes disease, not all beryllium-sensitized (BeS) subjects progress to CBD, and predicting which subjects will advance is currently not possible.⁸ Thus the identification of markers of disease progression would greatly advance our understanding of the immunopathogenesis of Be-induced disease, as well as the effect on patient care.

Despite similar levels of Be-induced proliferation of blood T cells, significantly increased numbers of Be-specific T cells are present in the blood of patients with CBD compared with those seen in BeS subjects.⁹ These findings suggest the presence of a proliferative defect in blood T cells of patients with CBD. We have previously shown that certain Be-specific CD4⁺ T cells proliferate poorly after antigen exposure while maintaining the ability to secrete T_H1-type cytokines.^{9,10} By using ELISpot analysis, IFN- γ -secreting CD4⁺ T cells were present in the blood of some patients with CBD at a time when Be-induced T-cell proliferation was undetectable.⁹ In addition, the ability of Be-responsive T cells to divide was closely linked to the memory maturation state of the T cell, as well as the expression of the coinhibitory receptor programmed death 1 (PD-1).^{10,11} Thus the lack of Be-induced proliferation seen in certain subjects was not due to the absence of circulating Be-specific CD4⁺ T cells but rather to a high fraction of PD-1-expressing effector memory T (T_{EM}) cells that were incapable of vigorous proliferation.^{10,11} Thus we hypothesized that proliferation-based assays might underestimate the frequency of Be sensitization in the workplace.

In the present study we show that the IFN- γ ELISpot has a greater ability to detect a Be-specific immune response in blood compared with the BeLPT and identify a significant positive correlation between the number of Be-responsive T cells in blood and in the target organ. In addition, the transition from Be sensitization to CBD is associated with an increased number of Be-specific T cells in blood. Our findings suggest that an antigen-specific T-cell subset in blood can be used as a biomarker to predict disease development in Be-exposed workers.

From the Departments of ^aMedicine and ^bImmunology and ^cthe School of Public Health, University of Colorado Denver, Aurora, and ^dthe Department of Medicine, National Jewish Health, Denver.

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Corresponding author: Andrew P. Fontenot, MD, Division of Clinical Immunology (B164), University of Colorado Denver, 12700 E 19th Ave, Aurora, CO 80045. E-mail: andrew.fontenot@ucdenver.edu.

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Abbreviations used

BAL:	Bronchoalveolar lavage
Be:	Beryllium
BeLPT:	Beryllium lymphocyte proliferation test
BeS:	Beryllium sensitized
BeSO ₄ :	Beryllium sulfate
CBD:	Chronic beryllium disease
PD-1:	Programmed death 1
SFU:	Spot-forming unit
T _{EM} :	Effector memory T

METHODS**Study population**

We prospectively enrolled 150 current and 110 former workers of a Be-machining facility,^{12,13} with current workers being enrolled during biannual workplace surveillance and former workers enrolled during a 1-time screening program. The demographics of the study population are shown in Table I. An additional cohort of 27 non-Be-exposed control subjects, 139 BeS subjects, and 31 patients with CBD were separately enrolled. The demographics of these study subjects are shown in Table E1 in this article's Online Repository at www.jacionline.org. CBD was diagnosed based on a history of Be exposure; the presence of a Be-specific immune response, as measured by an abnormal blood BeLPT result, bronchoalveolar lavage (BAL) BeLPT result, or both; and the presence of granulomatous inflammation, mononuclear cell infiltration, or both on lung biopsy.^{6,14} Diagnosis of a BeS subject was based on a history of Be exposure, a positive proliferative response of PBMCs to beryllium sulfate (BeSO₄) *in vitro*, and the absence of granulomatous inflammation or other abnormalities on lung biopsy.^{7,15} Progressors were defined as those subjects who advanced from BeS to CBD at the time of the current clinical evaluation based on the development of a Be-specific immune response in BAL and granulomatous inflammation, mononuclear cell infiltration, or both on lung biopsy.⁸

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.

Lymphocyte proliferation assay

PBMCs were isolated from heparinized blood, as previously described.¹⁶ Proliferation assays were performed in quadruplicate in the Clinical Immunology Laboratory at National Jewish Health, as previously described.^{6,14} Data are presented as a stimulation index (SI), with a positive response defined as an SI of 2.5 or greater.⁹

IFN- γ and IL-2 ELISpot assays

ELISpot assays were performed in triplicate by using plates (ImmunoSpot M200; BD Biosciences PharMingen) that were coated with IFN- γ or IL-2 capture mAb (BD Biosciences PharMingen, San Jose, Calif) and blocked with RPMI 1640 supplemented with 10% heat-inactivated FBS (HyClone Laboratories, Inc, Logan, Utah) for 2 hours at room temperature.⁹ PBMCs (5×10^5 cells per well) were added to wells and incubated overnight at 37°C with medium, PHA, and 10 or 100 $\mu\text{mol/L}$ BeSO₄. IFN- γ or IL-2 detection mAbs (BD Biosciences PharMingen) were added, and spots were visualized as previously described.⁹ The ELISpot plates were analyzed with a CTL Immunospot Analyzer (Cellular Technology Ltd, Shaker Heights, Ohio), and results are reported as means $\pm$ SDs of spot-forming units (SFUs) per well minus background SFUs.

As a quality control measure, we assessed variability in the ELISpot assay by splitting PBMCs from 13 patients with CBD and determining the number of SFUs in different plates. As shown in Fig E1 in this article's Online Repository at www.jacionline.org, less than 10% variability in the number of IFN- γ SFUs in response to 100 $\mu\text{mol/L}$ BeSO₄ was seen ($P = .94$).

TABLE I. Characteristics of former and current workers*

Characteristic	Former workers (n = 110)	Current workers (n = 150)
Age (y)	56 (32-78) [†]	46 (23-65)
Sex (M/F)	98/12	139/11
Race (W/AF/H)	109/1/0	149/1/0
Smoking status (CS/FS/NS)	23/41/43	13/51/84
Time since first exposure (y)	30 (2.4-48) [†]	14 (1.9-42)
Be tenure (y)	6.0 (0.4-35)	14 (1.9-42) [†]
HLA-DPB1 Glu69 (% positive)	38	37

AF, African American; CS, current smoker; F, female; FS, former smoker; H, Hispanic; M, male; NS, never smoker; W, white.

*Data are expressed as medians (ranges).

[†] $P < .001$.

Immunofluorescence staining and analysis of intracellular cytokine expression

BAL was obtained as previously described.^{17,18} For stimulation of cytokine expression, 1×10^6 BAL cells were exposed to either medium or 100 $\mu\text{mol/L}$ BeSO₄ for 6 hours, with 10 $\mu\text{g/mL}$ brefeldin A added after the first hour of stimulation.^{19,20} Cells were stained with mAbs directed against CD3 and CD4 (BD Biosciences PharMingen), followed by fixation, permeabilization, and staining with mAbs directed against IFN- γ and IL-2 (Caltag, Burlingame, Calif).^{19,20} The lymphocyte population was identified by using forward and 90° light scatter patterns, and fluorescence intensity was analyzed with an LSR-II flow cytometer (BD Immunocytometry Systems, San Jose, Calif).¹⁷⁻²⁰ Electronic compensation was performed with antibody capture beads (BD Biosciences) stained separately with individual mAbs used in the test samples. Analysis was performed with FlowJo software (Tree Star, Inc, Ashland, Ore).

Statistical analysis

The Mann-Whitney U test and Kruskal-Wallis ANOVA were used to determine the significance of differences between subject groups. The comparison of the stability of IFN- γ SFUs over time within subjects was performed with a Wilcoxon signed-rank test. A Spearman correlation was performed to analyze the association between IFN- γ SFUs in blood and the frequency of T_H1-type, cytokine-expressing CD4⁺ T cells in BAL. A receiver operating characteristic (ROC) analysis was used to determine the optimal threshold value, as measured by sensitivity and specificity. A P value of less than .05 was considered statistically significant.

RESULTS**Delineation of a positive ELISpot response**

To determine a cutoff for a positive IFN- γ and IL-2 ELISpot assay response, we prospectively enrolled 27 non-Be-exposed healthy control subjects and 31 patients with CBD. Demographics of these subjects are shown in Table E1. In response to 100 $\mu\text{mol/L}$ BeSO₄, the median number of IFN- γ -producing cells in the blood of patients with CBD (44 SFUs/ 5×10^5 cells; range, 1.3-500 SFUs/ 5×10^5 cells) was significantly higher compared with that seen in healthy control subjects (0 SFUs; range, 0-3 SFUs; $P < .0001$; Fig 1, A). Similar findings were seen at the lower concentration of Be (Fig 1, A). In addition, the median number of IL-2-producing cells in response to 100 $\mu\text{mol/L}$ BeSO₄ was significantly higher in patients with CBD (17 SFUs; range, 0-500 SFUs) compared with that seen in control subjects (0 SFUs; range, 0-1 SFU; $P < .0001$; Fig 1, A). Similar to our previous study,⁹ 100 $\mu\text{mol/L}$ BeSO₄ was the optimal antigen concentration for the induction of IFN- γ and IL-2 secretion.

By using an ROC curve to distinguish healthy control subjects from patients with CBD, a threshold of 10 or more IFN- γ SFUs

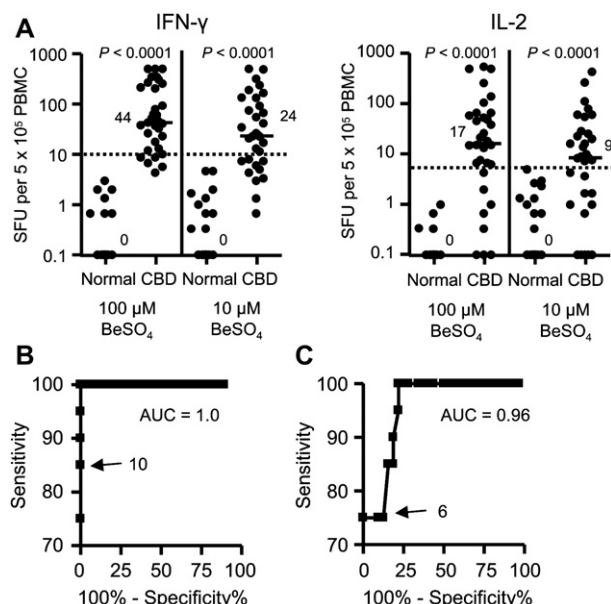


FIG 1. Frequency of Be-specific T cells in blood of healthy control subjects and patients with CBD. **A**, IFN- γ and IL-2 ELISpot analysis. Data are expressed as the mean SFUs, with median values indicated with solid lines. Values above the dotted line represent a positive cytokine response. **B** and **C**, ROC curves for IFN- γ and IL-2 SFUs in blood of patients with CBD (true-positive results) and control subjects (true-negative results). The area under the curve (AUC) and the chosen cutoff value for each ROC curve are shown.

and 6 or more IL-2 SFUs was chosen as an abnormal response (Fig 1, B and C). With this cut-point, the IFN- γ ELISpot had a sensitivity of 85% (95% CI, 67% to 95%) and a specificity of 100% (95% CI, 83% to 100%), with a positive and negative predictive value of 100% and 81%, respectively. Of the 5 subjects with a negative ELISpot response, 4 had a negative BeLPT result. For IL-2, there were 8 patients with CBD who had less than 6 SFUs in response to 100 μ mol/L BeSO₄, resulting in a sensitivity of 76% and a specificity of 100% for the IL-2 ELISpot. On the basis of the ROC analyses, an abnormal IFN- γ and IL-2 ELISpot assay response for this study will be defined as 10 or more and 6 or more SFUs, respectively.

Quantitation of Be-specific, IFN- γ -producing, and IL-2-producing PBMCs

To compare the ability of the ELISpot assay and the BeLPT to detect Be sensitization in the workplace, we prospectively enrolled 150 current and 110 former workers from a single Be-machining facility. None of these subjects had previously been identified as BeS. The majority of Be-exposed workers did not demonstrate an adaptive immune response to Be (Fig 2, A and B). However, an abnormal IFN- γ ELISpot response to 100 μ mol/L BeSO₄ was detected in 15 current and 11 former workers, resulting in rates of Be sensitization of 10% and 9.9%, respectively. This rate of sensitization is similar to the 9.4% sensitization rate that was previously reported for this facility.¹³ Because of the identical rates of sensitization detected in current and former workers, we have grouped the workers together (Fig 2, A). In response to 10 μ mol/L BeSO₄, 20 subjects had an abnormal IFN- γ

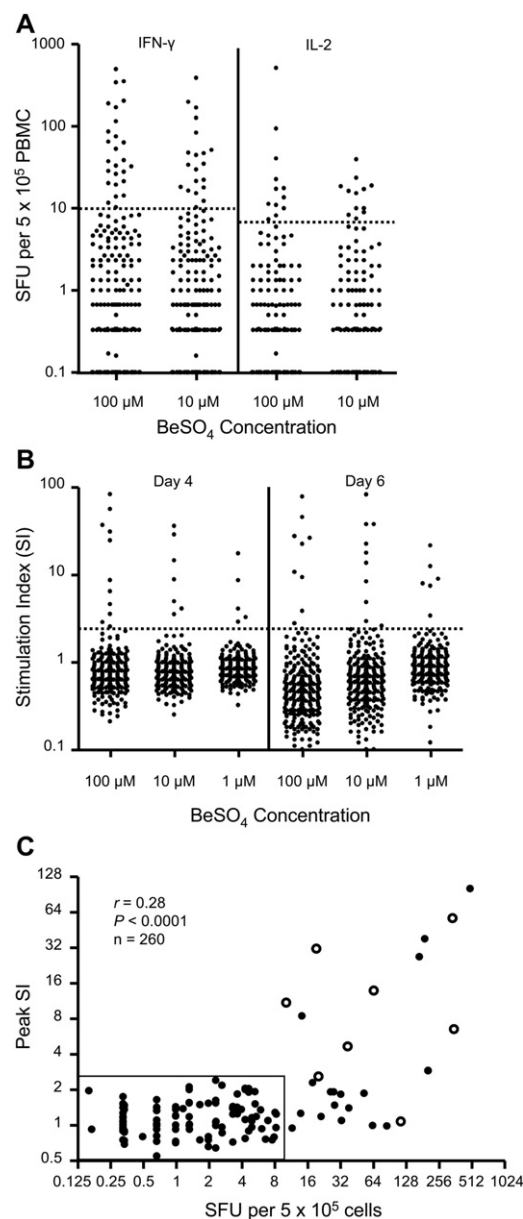


FIG 2. Identification of a Be-induced adaptive immune response. **A**, Frequency of Be-specific T cells in blood, as determined by using the IFN- γ and IL-2 ELISpot assays. Data are expressed as the mean SFUs for each subject. Cytokine values above the dotted line represent a positive cytokine response. **B**, Be-induced proliferative response. An SI indicative of a positive response is defined as 2.5 or greater (dotted line). **C**, Correlation between the number of Be-responsive T cells in blood and peak SI. Subjects with confirmed CBD on clinical evaluation are shown as open circles. The rectangle surrounds subjects with a negative IFN- γ ELISpot response and BeLPT result.

ELISpot response, and all of these subjects had a positive response to the higher concentration of Be.

Twelve subjects demonstrated an abnormal IL-2 ELISpot response to 100 and 10 μ mol/L BeSO₄ (Fig 2, A). All subjects with an abnormal IL-2 ELISpot response also had 10 or more IFN- γ SFUs in response to 100 μ mol/L BeSO₄. Because IFN- γ is the predominant T_H1-type cytokine secreted by Be-specific CD4⁺ T cells derived from either blood or lung tissue,¹⁹ we

have chosen to focus on the IFN- γ response of PBMCs to 100 $\mu\text{mol/L}$ BeSO₄ for our comparison of the ability of the ELISpot assay versus the BeLPT to detect an adaptive immune response in Be-exposed workers.

Be-induced proliferation of PBMCs

PBMCs from the same 260 Be-exposed workers were examined for Be-induced proliferation at the same time as the ELISpot assays were performed. As shown in Fig 2, B, 11 (4.2%) of 260 subjects demonstrated a significant proliferative response (denoted as an SI ≥ 2.5) to 100 $\mu\text{mol/L}$ BeSO₄ after 4 days of culture. Although similar findings were seen in response to different BeSO₄ concentrations and days of culture, 100 $\mu\text{mol/L}$ BeSO₄ after 4 days induced the greatest proliferative response (Fig 2, B). In addition, all subjects with a positive BeLPT result to 1 and 10 $\mu\text{mol/L}$ BeSO₄ had a significant proliferative response to the higher dose of Be. Compared with the ELISpot assay, in which similar rates of sensitization were seen in current and former workers, 9 former and 2 current workers had abnormal BeLPT results, resulting in rates of Be sensitization of 8.1% and 1.3%, respectively. Overall, 10% of the workers had an abnormal IFN- γ ELISpot response compared with 4.2% with a positive BeLPT result ($\chi^2 = 55.7$, $P < .0001$).

To investigate the relationship between Be-specific T-cell proliferation and cytokine secretion, we correlated the peak SI as detected by using the BeLPT and the number of IFN- γ SFUs per 5×10^5 cells in the cohort of 260 subjects. Despite a low r value, a significant positive correlation was noted ($r = 0.28$, $P < .0001$; Fig 2, C). However, this correlation was lost when only those subjects with a detectable Be-specific immune response were included in the analysis ($r = 0.27$, $P = .2$). Fig 2, C, also shows the 15 subjects with greater than 10 IFN- γ SFUs did not proliferate in the presence of Be salts *in vitro* (SI, <2.5). No subject had an abnormal BeLPT result in the presence of less than 10 IFN- γ SFUs, as detected by using the ELISpot assay (Fig 2, C).

Assessment of the IFN- γ ELISpot assay in relation to HLA-DPB1 Glu69

Because HLA-DPB1 alleles with a glutamic acid at position 69 (HLA-DPB1 Glu69) are the most important genetic susceptibility marker for the development of Be-induced disease,^{5,21,22} we analyzed the IFN- γ ELISpot data based on the expression of HLA-DPB1 Glu69 in this cohort of subjects. HLA-DP genetic analysis was available for 212 subjects, including 23 subjects with greater than 10 IFN- γ SFUs. As shown in Fig E2 in this article's Online Repository at www.jacionline.org, a significantly greater number of SFUs were present in the Glu69-positive subjects (median, 2.3 SFUs; range, 0-500 SFUs) compared with that seen in the Glu69-negative subjects (median, 0 SFUs; range, 0-8.3 SFUs; $P < .0001$). More importantly, all subjects with a positive IFN- γ ELISpot response possessed at least 1 HLA-DPB1 Glu69-containing allele.

Frequency of Be-responsive T cells in blood as a biomarker of disease progression

Eight of 26 subjects who demonstrated evidence of Be sensitization in the current study have been clinically evaluated for the

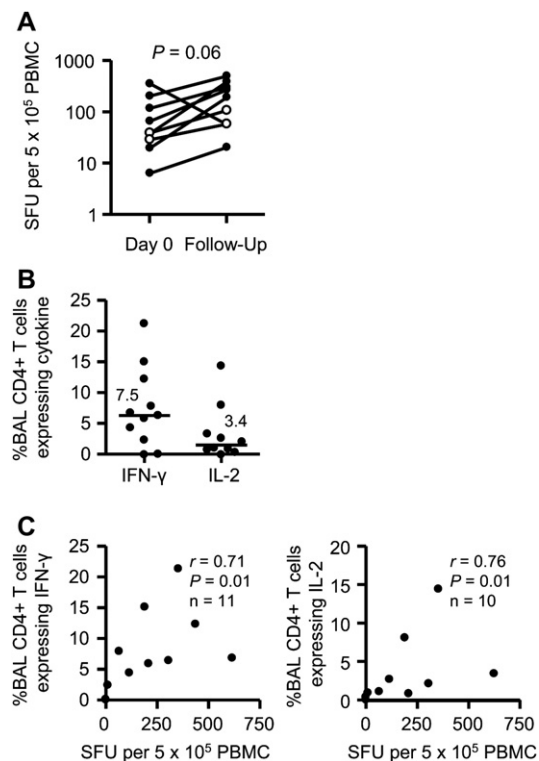


FIG 3. Frequency of Be-responsive CD4⁺ T cells in blood and BAL of patients with CBD. **A**, Be-specific T cells in blood over time. Data are expressed as the mean SFUs per 5×10^5 cells per well. *Open circles* show 2 subjects who had a positive ELISpot response and a negative BeLPT result at first evaluation. **B**, Percentage of BAL CD4⁺ T cells that express IFN- γ ($n = 11$) and IL-2 ($n = 10$) after stimulation with 100 $\mu\text{mol/L}$ BeSO₄. Mean percentage of T_H1 cytokines produced by BAL cells from patients with CBD is shown as a *solid line*. **C**, Correlation between the number of Be-responsive T cells in blood and the frequency of Be-responsive BAL CD4⁺ T cells expressing either IFN- γ (*left panel*) or IL-2 (*right panel*).

presence of granulomatous lung inflammation, and all have been given a diagnosis of CBD. Of the 8 subjects (shown as open circles in Fig 2, C), 7 had both an abnormal IFN- γ ELISpot response and BeLPT result, and 1 had an abnormal IFN- γ ELISpot response and a negative BeLPT result. That subject had 116 IFN- γ SFUs and a peak SI of 1.06. IFN- γ ELISpot assays were repeated on 7 subjects at the time of clinical evaluation and 2 of the 15 subjects with an abnormal ELISpot response and a negative BeLPT result (denoted by open circles in Fig 3, A). Although a trend was noted for an increase in the number of circulating Be-responsive T cells at the second time point, no statistically significant differences were seen ($P = .06$). Importantly, the 2 subjects with an abnormal ELISpot response in the absence of Be-induced proliferation retained a Be-specific immune response in blood.

Because Be-responsive T cells present in blood are likely trafficking to the lung, we queried whether the frequency of IFN- γ -secreting T cells in blood could serve as a biomarker of T-cell alveolitis severity. We obtained simultaneous blood and BAL samples from 11 patients with CBD (6 from the initial study and enrolled at the time of their clinical evaluation and 5 from the same Be-machining facility with a previous diagnosis). In the representative examples shown in Fig E3 in this article's Online Repository at www.jacionline.org, 7.9% and 2.7% of BAL

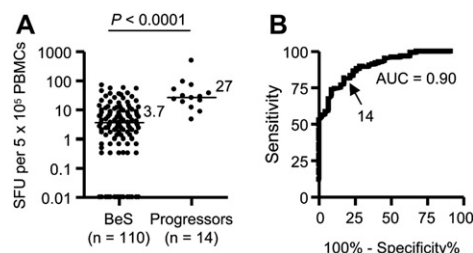


FIG 4. Frequency of Be-specific T cells in blood. **A**, Number of Be-responsive T cells in blood of BeS subjects and subjects whose disease status changed at the time of the clinical evaluation (*Progressors*). Data are expressed as the mean number of IFN- γ SFUs in response to 100 μ mol/L BeSO₄, and median values are indicated with *solid lines*. **B**, ROC curves for IFN- γ SFUs in blood of patients with CBD and BeS subjects. The area under the curve (AUC) and the chosen cutoff value are shown.

CD4⁺ T cells expressed IFN- γ and IL-2, respectively, after short-term culture with BeSO₄. Overall, the frequency of IFN- γ - and IL-2-expressing, Be-specific CD4⁺ T cells in BAL of the patients with CBD was $7.5\% \pm 2.0\%$ and $3.4\% \pm 1.4\%$ (mean $\pm$ SEM), respectively (Fig 3, B). Comparing the frequency of Be-specific T cells in blood and lung tissue, a significant positive correlation was observed between the number of IFN- γ -secreting T cells in blood and the frequency of IFN- γ -expressing CD4⁺ T cells in lung tissue ($r = 0.71$, $P = .01$; Fig 3, C). A positive correlation was also seen between Be-responsive T cells in blood and IL-2-expressing CD4⁺ T cells in lung tissue ($r = 0.76$, $P = .01$; Fig 3, C).

On the basis of the association between the frequency of Be-specific T cells in blood and lung tissue shown here and the CD4⁺ T-cell alveolitis that characterizes CBD, we queried whether the IFN- γ ELISpot assay could predict the development of granulomatous inflammation in BeS subjects who were returning for follow-up evaluation. We consecutively enrolled 139 BeS subjects. The median number of IFN- γ -producing cells in blood of BeS subjects was 3.7 SFUs (range, 0-97 SFUs) in response to 100 μ mol/L BeSO₄ (Fig 4, A). More importantly, 14 BeS subjects progressed to CBD at the time of that clinical evaluation based on the development of a Be-specific immune response in BAL, granulomatous inflammation on lung biopsy, or both, and these progressors had a significantly increased number of Be-responsive, IFN- γ -secreting cells in blood (median, 27 SFUs; range, 4.7-500 SFUs) compared with those BeS subjects whose disease status remained unchanged ($P < .0001$; Fig 4, A). In addition, progressors had a significantly greater number of lymphocytes in BAL compared with stable BeS subjects ($P < .01$; see Table E1).

To determine whether the frequency of Be-specific T cells in blood can distinguish between patients with CBD (combining the 14 progressors in Fig 4, A, and the 31 patients with CBD from Fig 1, A) and subjects with stable Be sensitization, we generated an ROC curve (Fig 4, B). Progressors and patients with CBD were combined because these subjects all have CBD and possess a similar frequency of Be-responsive T cells in blood (compare Figs 1, A, and 4, A). By using a threshold value of greater than 14 IFN- γ SFUs to differentiate CBD from BeS, sensitivity and specificity of 78% and 86%, respectively, were seen, with a positive and negative predictive value of 67% and 91%, respectively. Taken together, these findings suggest that the frequency of

Be-specific T cells in blood can be used as a biomarker of disease progression.

DISCUSSION

The onset of Be sensitization is heralded by the development of a Be-specific adaptive immune response that is typically detected by the presence of Be-induced proliferation of blood T cells. Our previous studies show that the majority of Be-responsive CD4⁺ T cells in blood and lung tissue express an effector memory phenotype, and the ability of CD4⁺ T cells to proliferate after Be exposure in culture is linked to the maturation state of the memory T cell.¹⁰ The retention of Be in the lung and the resultant persistent antigen exposure are associated with the upregulation of co-inhibitory receptors, such as PD-1, on Be-specific CD4⁺ T cells that is also associated with reduced proliferation.¹¹ Collectively, these findings suggest that Be-responsive CD4⁺ T cells in blood and lung tissue represent differentiated T_{EM} cells that have sacrificed proliferative capacity while retaining the ability to express T_H1-type cytokines. Thus we hypothesized that a cytokine-based assay, such as the IFN- γ ELISpot assay, would be better able to detect Be sensitization in the workplace than the BeLPT. By using blood from current and former workers of a Be-machining facility, the current study confirms our hypothesis and shows that the number of Be-responsive T cells in blood correlates with the frequency of IFN- γ - and IL-2-expressing, Be-specific CD4⁺ T cells in lung tissue. We found that those subjects who progressed to CBD had a significantly increased number of Be-responsive T cells in blood compared with those BeS subjects whose disease status remained unchanged, suggesting that quantitation of these cells in blood is an effective means of predicting disease development.

Comparing the rates of abnormal IFN- γ ELISpot responses and BeLPT results, similar rates were seen in former workers (10% vs 8.1%, respectively). Conversely, the rates of sensitization in current workers were 9.9% for the IFN- γ ELISpot assay and 1.3% for the BeLPT. One potential explanation for this observation is that the former workers are no longer actively exposed to Be, and their compressed Be-specific CD4⁺ memory T-cell pool in blood has regained its full proliferative capacity in the absence of current antigenic exposure. In contrast, with continued exposure, the predominance of Be-specific CD4⁺ T_{EM} cells expressing an exhausted or even senescent phenotype limits the ability of these cells to mount a proliferative response.^{11,23} Similar findings have been seen in other diseases characterized by persistent antigen exposure, such as HIV, in which the inability of HIV-1-specific CD4⁺ T cells to proliferate was one of the earliest reported immunologic manifestations of HIV-1 infection.²⁴

Eight of 26 subjects with an abnormal ELISpot response in our cohort of 260 Be-exposed workers have subsequently been given a diagnosis of CBD. Seven of those subjects also had an abnormal proliferative response to Be salts in culture. One caveat is that the 1 patient with CBD with a positive ELISpot response and a normal BeLPT result had an abnormal Be-induced proliferative response at another facility, which prompted initiation of the clinical evaluation. Although the BeLPT was equivalent to the IFN- γ ELISpot assay in the detection of Be sensitization in workers with greater than 100 IFN- γ SFUs per 5×10^5 cells (detecting 7/8 subjects), it has a decreased sensitivity in subjects whose ELISpot response ranged between 10 and 100 SFUs. It remains possible that some of the 15 subjects with a negative

BeLPT result and a positive ELISpot response represent false-positive test results. To date, 2 of these 15 subjects had repeat ELISpot assays and BeLPTs performed, and both had an abnormal ELISpot response in the setting of a negative BeLPT result. Other evidence supporting the validity of our findings includes a subject with a negative BeLPT result and a positive ELISpot response who has been given a diagnosis of CBD and the fact that all subjects with an abnormal ELISpot response possessed HLA-DPB1 Glu69, the major genetic susceptibility marker in this disease. Thus our findings show that the IFN- γ ELISpot assay is more sensitive than the BeLPT for the detection of CBD in this workplace. Additional advantages of the ELISpot assay include a shorter duration (24 hours vs 4 or 6 days), lack of radioactivity, and use of FBS as opposed to human serum.

One uncertainty of the current study is whether our results will translate to other Be facilities where the level of exposure is not as significant and rates of Be sensitization are lower. It is well established that machinists have the greatest levels of Be exposure and the highest rates of Be sensitization.^{12,25} Workplace surveillance in the Be-machining facility tested in the current study has shown that the majority of subjects with Be sensitization had already progressed to CBD at the time of evaluation. In industries with less or intermittent Be exposure, it is possible that the 2 assays will perform in a similar manner or detect Be-induced proliferation in the absence of a cytokine response, which was not the case in this highly exposed cohort. Because the IFN- γ ELISpot assay and the BeLPT are measuring different T-cell functions (ie, IFN- γ secretion and proliferation), it is also possible that these assays might have complementary roles in workplace surveillance.

Our findings suggest that the Be-specific adaptive immune response in blood is maintained over time in patients with CBD. We have previously shown that the BAL T-cell receptor V β expansions and the Be-responsive T-cell clones that comprise those expansions in patients with CBD persist in serial lavages.^{17,18} However, it remains unknown whether the same is true for Be sensitization. In this regard we failed to demonstrate either a Be-induced cytokine or proliferative response in more than 70% of the BeS subjects in this study, raising the possibility that in some BeS subjects the Be-induced immune response might be a transient phenomenon. Conversely, of the 95 BeS subjects who underwent BAL, 7 demonstrated a Be-specific proliferative response in BAL but did not show evidence of granulomatous inflammation or BAL lymphocytosis, thus not fulfilling the criteria for CBD. Of those subjects, 86% possessed greater than 10 IFN- γ SFUs, suggesting that these subjects might be at high risk to progress to CBD or have already progressed despite the absence of granulomas on lung biopsy. These subjects also contribute to the overlap seen between the number of IFN- γ -secreting T cells in the progressors compared with that seen in those with stable Be sensitization.

A biomarker that can predict disease progression has important clinical implications, especially in Be-induced disease, where uncertainty exists regarding who will progress to disease and when progression will occur. In the present study we identified a cutoff of greater than 14 SFUs to differentiate patients with CBD from BeS subjects. This is the identical value identified in our previous study investigating a different patient cohort,⁹ adding further validity to its use in separating stages of disease. However, because of its low positive predictive value, this cutoff might not be clinically useful in determining who has CBD or who needs to

undergo an invasive procedure for diagnostic purposes. In this regard our data suggest that the use of a lower and an upper cutoff might be clinically more useful. For example, in BeS subjects with less than 10 SFUs, the probability of having progressed to CBD would be less than 7% (see Table E2 in this article's Online Repository at www.jacionline.org) and might not warrant the risk of bronchoscopy unless dictated by clinical, radiographic, or physiologic abnormalities. Conversely, subjects with greater than 40 SFUs have an 81% probability of having progressed to CBD (see Table E2). BeS subjects with between 10 and 40 SFUs will likely warrant bronchoscopy, as well as close clinical follow-up. Thus the addition of the IFN- γ ELISpot assay to the diagnostic algorithm of Be-induced disease is a significant advancement over that provided by the BeLPT, which only detects whether beryllium sensitization is present.

In summary, we have shown that the number of Be-responsive T cells in blood can serve as a biomarker of disease development and as an estimate of the severity of Be-induced CD4⁺ T-cell alveolitis. Thus finding an increased quantity of Be-specific CD4⁺ T cells in the blood of workers might signify a sentinel event heralding the development of a Be-induced T-cell alveolitis and the subsequent development of granulomatous inflammation.

Clinical implications: These findings suggest that the number of Be-responsive T cells in the circulation can serve as a biomarker of disease progression and as an estimate of the severity of Be-induced lung inflammation.

REFERENCES

1. Fontenot AP, Maier LA. Genetic susceptibility and immune-mediated destruction in beryllium-induced disease. *Trends Immunol* 2005;26:543-9.
2. Kreiss K, Mroz MM, Newman LS, Martyny J, Zhen B. Machining risk of beryllium disease and sensitization with median exposures below 2 mg/m³. *Am J Ind Med* 1996;30:16-25.
3. Kreiss K, Mroz MM, Zhen B, Martyny JW, Newman LS. Epidemiology of beryllium sensitization and disease in nuclear workers. *Am Rev Respir Dis* 1993;148:985-91.
4. Kreiss K, Wasserman S, Mroz MM, Newman LS. Beryllium disease screening in the ceramics industry: blood test performance and exposure-disease relations. *J Occup Med* 1993;35:267-74.
5. Richeldi L, Sorrentino R, Saltini C. HLA-DPB1 glutamate 69: a genetic marker of beryllium disease. *Science* 1993;262:242-4.
6. Rossman MD, Kern JA, Elias JA, Cullen MR, Epstein PE, Preuss OP, et al. Proliferative response of bronchoalveolar lymphocytes to beryllium. *Ann Intern Med* 1988;108:687-93.
7. Mroz MM, Kreiss K, Lezotte DC, Campbell PA, Newman LS. Reexamination of the blood lymphocyte transformation test in the diagnosis of chronic beryllium disease. *J Allergy Clin Immunol* 1991;88:54-60.
8. Newman LS, Mroz MM, Balkissoon R, Maier LA. Beryllium sensitization progresses to chronic beryllium disease: a longitudinal study of disease risk. *Am J Respir Crit Care Med* 2004;171:54-60.
9. Pott GB, Palmer BE, Sullivan AK, Silviera L, Maier LA, Newman LS, et al. Frequency of beryllium-specific, T_H1-type cytokine-expressing CD4⁺ T cells in patients with beryllium-induced disease. *J Allergy Clin Immunol* 2005;115:1036-42.
10. Fontenot AP, Palmer BE, Sullivan AK, Joslin FG, Wilson CC, Maier LA, et al. Frequency of beryllium-specific, central memory CD4⁺ T cells in blood determines proliferative response. *J Clin Invest* 2005;115:2886-93.
11. Palmer BE, Mack DG, Martin AK, Gillespie M, Mroz MM, Maier LA, et al. Up-regulation of programmed death-1 expression on beryllium-specific CD4⁺ T cells in chronic beryllium disease. *J Immunol* 2008;180:2704-12.
12. Kelleher PC, Martyny JW, Mroz MM, Maier LA, Rutenber AJ, Young DA, et al. Beryllium particulate exposure and disease relations in a beryllium machining plant. *J Occup Environ Med* 2001;43:238-49.
13. Newman LS, Mroz MM, Maier LA, Daniloff EM, Balkissoon R. Efficacy of serial medical surveillance for chronic beryllium disease in a beryllium machining plant. *J Occup Environ Med* 2001;43:231-7.

14. Newman LS, Kreiss K, King TE Jr, Seay S, Campbell PA. Pathologic and immunologic alterations in early stages of beryllium disease: re-examination of disease definition and natural history. *Am Rev Respir Dis* 1989;139:1479-86.
15. Newman LS. Significance of the blood beryllium lymphocyte proliferation test. *Environ Health Perspect* 1996;104:953-6.
16. Mack DG, Lanham AM, Falta MT, Palmer BE, Maier LA, Fontenot AP. Deficient and dysfunctional regulatory T cells in the lungs of chronic beryllium disease subjects. *Am J Respir Crit Care Med* 2010;181:1241-9.
17. Fontenot AP, Kotzin BL, Comment CE, Newman LS. Expansions of T-cell subsets expressing particular T cell receptor variable regions in chronic beryllium disease. *Am J Respir Cell Mol Biol* 1998;18:581-9.
18. Fontenot AP, Falta MT, Freed BM, Newman LS, Kotzin BL. Identification of pathogenic T cells in patients with beryllium-induced lung disease. *J Immunol* 1999;163:1019-26.
19. Fontenot AP, Canavera SJ, Gharavi L, Newman LS, Kotzin BL. Target organ localization of memory CD4+ T cells in patients with chronic beryllium disease. *J Clin Invest* 2002;110:1473-82.
20. Fontenot AP, Gharavi L, Bennett SR, Canavera SJ, Newman LS, Kotzin BL. CD28 costimulation independence of target organ versus circulating memory antigen-specific CD4+ T cells. *J Clin Invest* 2003;112:776-84.
21. Rossman MD, Stubbs J, Lee CW, Argyris E, Magira E, Monos D. Human leukocyte antigen class II amino acid epitopes: susceptibility and progression markers for beryllium hypersensitivity. *Am J Respir Crit Care Med* 2002;165:788-94.
22. Maier LA, McGrath DS, Sato H, Lympny P, Welsh K, Du Bois R, et al. Influence of MHC class II in susceptibility to beryllium sensitization and chronic beryllium disease. *J Immunol* 2003;171:6910-8.
23. Palmer BE, Mack DG, Martin AK, Maier LA, Fontenot AP. CD57 expression correlates with alveolitis severity in subjects with beryllium-induced disease. *J Allergy Clin Immunol* 2007;120:184-91.
24. Shearer GM. HIV-induced immunopathogenesis. *Immunity* 1998;9:587-93.
25. Henneberger PK, Cumro D, Deubner DD, Kent MS, McCawley M, Kreiss K. Beryllium sensitization and disease among long-term and short-term workers in a beryllium ceramics plant. *Int Arch Occup Environ Health* 2001;74:167-76.

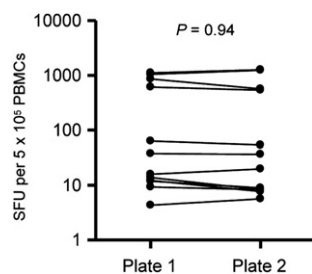


FIG E1. Interassay variability in the IFN- γ ELISpot assay. PBMCs from 13 subjects were split, and IFN- γ ELISpot assays were performed on separate plates to assess interassay variability. Statistical comparisons were made with the paired t test.

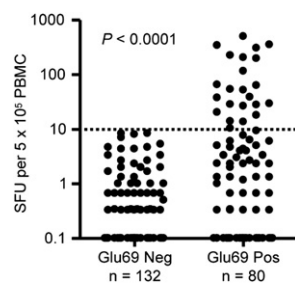


FIG E2. Frequency of Be-specific T cells in the blood of subjects with and without expression of the genetic susceptibility marker HLA-DPB1 Glu69. Subjects were divided based on the presence ($n = 80$) or absence ($n = 132$) of HLA-DPB1 Glu69, and the number of IFN- γ SFUs was compared between the 2 groups. Values above the *dotted line* represent a positive cytokine response. Significance was determined with the Mann-Whitney U test.

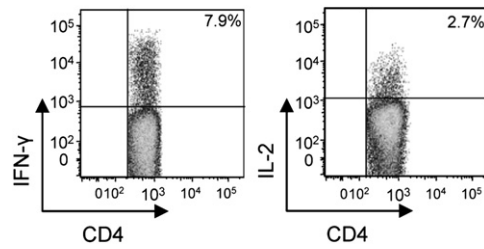


FIG E3. Representative examples of intracellular IFN- γ and IL-2 staining in BAL CD4⁺ T cells from a patient with CBD after 100 μ mol/L BeSO₄ stimulation.

TABLE E1. Demographics of BeS subjects and patients with CBD*

Characteristics	Healthy subjects (n = 27)	BeS subjects (n = 125)	Progressors (n = 14)	Patients with CBD (n = 31)
Age (y)	43 (21-73)	60 (35-80)	63 (47-78)	57 (31-79)
Sex (M/F)	16/11	89/36	14/0	27/4
Race (% white)	81	88	100	84
Smoking status (CS/FS/NS)	0/9/18	0/47/63	0/7/7	0/17/14
Industry (nuclear/ceramic/other)		110/0/15	12/0/2	17/2/12
Treatment (none/prednisone)		125/0	14/0	27/4
BeLPT (SI)				
Blood		1.7 (0.7-61)	7.4 (0.6-63)	3.3 (0.9-90)
BAL		1.3 (0.8-17)	2.7 (1-43)	20 (0.9-111) [†]
BAL cells				
White blood cells ($\times 10^6$)		22 (4.3-152)	22 (11-58)	34 (4.5-141)
Lymphocytes ($\times 10^6$)		1.5 (0.2-60)	3.4 (0.6-18) [‡]	6.1 (1.3-85) [†]

CS, Current smoker; F, female; FS, former smoker; M, male; NS, never smoker.

*Data are expressed as medians (ranges).

[†] $P < .01$ between BeS subjects and patients with CBD.[‡] $P < .05$ between BeS subjects and progressors.

TABLE E2. Statistical analysis of SFU cut-points for clinical decision making

SFUs	Sensitivity	Specificity	Positive predictive value	Negative predictive value
>10	85%	77%	57%	93%
>14	78%	86%	67%	91%
>20	72%	88%	69%	89%
>40	46%	96%	81%	83%
>50	43%	98%	87%	82%