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Andrew P. Fontenot, … , Lee S. Newman, Brian L. Kotzin


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Frequency of beryllium-specific, central memory CD4\(^+\) T cells in blood determines proliferative response

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Beryllium exposure can lead to the development of beryllium-specific CD4\(^+\) T cells and chronic beryllium disease (CBD), which is characterized by the presence of lung granulomas and a CD4\(^+\) T cell alveolitis. Studies have documented the presence of proliferating and cytokine-secreting CD4\(^+\) T cells in blood of CBD patients after beryllium stimulation. However, some patients were noted to have cytokine-secreting CD4\(^+\) T cells in blood in the absence of beryllium-induced proliferation, and overall, the correlation between the 2 types of responses was poor. We hypothesized that the relative proportion of memory T cell subsets determined antigen-specific proliferation. In most CBD patients, the majority of beryllium-specific CD4\(^+\) T cells in blood expressed an effector memory T cell maturation phenotype. However, the ability of blood cells to proliferate in the presence of beryllium strongly correlated with the fraction expressing a central memory T cell phenotype. In addition, we found a direct correlation between the percentage of beryllium-specific CD4\(^+\) T\(_{EM}\) cells in blood and T cell lymphocytosis in the lung. Together, these findings indicate that the functional capability of antigen-specific CD4\(^+\) T cells is determined by the relative proportion of memory T cell subsets, which may reflect internal organ involvement.

Introduction

With a known antigen and an accessible target organ, chronic beryllium disease (CBD) serves as an important model of an organ-specific immune-mediated disease. This disorder occurs in susceptible individuals exposed to beryllium in the workplace, and more than 1 million workers are at risk for the development of CBD (1–5). Beryllium sensitization is associated with the presence of circulating beryllium-specific CD4\(^+\) T cells, which proliferate in the presence of beryllium salts in culture in an MHC class II–restricted manner (6–10). The intravenous response forms the basis of the beryllium lymphocyte proliferation test (BeLPT), which is currently used to detect beryllium sensitization in the workplace (11–13). Progression from sensitization to CBD occurs at a rate of 6–8% per year (14) and is characterized by the accumulation of large numbers of beryllium-specific, Th1-type cytokine–secreting CD4\(^+\) T cells in the lung and granulomatous inflammation. Important differences exist between antigen-specific T cells that reside in the lung and those present in the circulation. For example, beryllium-specific memory CD4\(^+\) T cells in blood remain dependent on CD28 engagement for optimal T cell activation, while the memory CD4\(^+\) T cells present in bronchoalveolar lavage (BAL) no longer require CD28 costimulation for either proliferation or Th1-type cytokine secretion; this suggests a maturation within the memory CD4\(^+\) T cell population (15).

Using ELISPOT analysis, we found significant numbers of IFN-\(\gamma\)-secreting CD4\(^+\) T cells when peripheral blood cells from CBD patients were stimulated with beryllium salts in culture (16). The correlation between cytokine secretion and proliferative responses was poor. Furthermore, we identified 13 subjects with beryllium-specific, IFN-\(\gamma\)-secreting T cells in blood yet no beryllium-induced lymphoproliferation. These findings suggested that low beryllium-induced proliferation with cells from some CBD patients is due to a different proliferative capacity rather than the absence of beryllium-specific CD4\(^+\) T cells. We hypothesized that this reduced proliferative response might parallel differences in memory T cell maturation. Recent studies suggest that memory T cells of different maturation states possess different migratory and functional capabilities (17, 18). Based on the expression of the lymph node homing receptor CCR7 and the CD45RA isoform, at least 2 subsets of memory cells exist (17–21). Central memory T (T\(_{CM}\)) cells have lost the expression of CD45RA while retaining CCR7 expression. Retention of CCR7 expression renders cells capable of entering lymph nodes and recirculating between blood and lymph (22). In contrast, effector memory T (T\(_{EM}\)) cells have downregulated the expression of both CD45RA and CCR7 and migrate to and reside in target organs such as the lung. T\(_{CM}\) cells appear capable of differentiating into T\(_{EM}\) cells upon restimulation. Based on this paradigm, T\(_{EM}\) and T\(_{CM}\) cells are thought to play complementary roles when reexposed to antigen. The T\(_{EM}\) cells provide rapid effector function within the target organ, while the T\(_{CM}\) cells proliferate in the lymph node, generating increased numbers of effector cells (17, 18).

In this study, we analyzed the Th1-type cytokine profile, proliferative capacity, and maturation phenotype of beryllium-specific CD4\(^+\) T cells in blood and BAL of CBD and beryllium-sensitized (BeS) subjects. No correlation between the frequencies of beryllium-specific, Th1-type cytokine–expressing CD4\(^+\) T cells in blood and beryllium-induced T cell proliferation in culture was seen. The majority of beryllium-specific CD4\(^+\) T cells in blood and BAL expressed a
**Results**

**Lack of correlation between proliferating and cytokine-secreting responses after stimulation of blood CD4+ T cells with beryllium.** Our previous studies have detected the presence of beryllium-specific, cytokine-producing CD4+ T cells in the blood of some CBD patients in the absence of a beryllium-induced proliferative response (16). These findings raise the possibility that patients may have different subsets of beryllium-specific memory T cells in blood. To further assess this possibility, fresh PBMCs from 13 CBD patients were CFSE-labeled and cultured in the presence of medium alone or 1 × 10−4 M beryllium sulfate (BeSO₄) for 7 days. At the same time point, the frequency of beryllium-specific, Th1-type cytokine-expressing CD4+ T cells in blood was determined. Two representative examples of patient responses are shown in Figure 1A. The frequency of beryllium-specific, IFN-γ-secreting CD4+ T cells in the blood of patients 10 and 11 was 0.66% and 1.1%, respectively (Figure 1A). Marked differences in the ability of the beryllium-specific CD4+ T cells to proliferate (i.e., percentage CFSE-low cells) and their ability to generate IFN-γ (r = 0.20; P = 0.51) or IL-2 (r = 0.36; P = 0.23) when stimulated with BeSO₄ (Figure 1B). Consistent with the lack of correlation, 3 subjects with substantial populations of beryllium-responsive, Th1-type cytokine-expressing CD4+ T cells in blood (ranging from 0.14% to 0.66% of total blood CD4+ T cells) demonstrated minimal beryllium-induced T cell proliferation (Figure 1B). On the other hand, 2 subjects had vigorous beryllium-induced proliferation despite low numbers of beryllium-induced, cytokine-secreting CD4+ T cells in blood. For example, in 1 CBD patient, 0.2% of ex vivo CD4+ T cells expressed IFN-γ after beryllium exposure, while 55% of CD4+ T cells from this subject exhibited beryllium-induced proliferation after 7 days of culture (Figure 1B).

**Characterization of memory CD4+ T cell populations in the blood of CBD and BeS individuals.** Surface staining of blood CD4+ T cells for CD45RA and CCR7 has identified 3 distinct T cell populations: naive (CCR7⁺CD45RA⁺), central memory (Tcm; CCR7⁺CD45RA⁻), and effector memory (Tem; CCR7⁻CD45RA⁻) T cells. To determine whether the distribution of these memory cell subsets was altered in our study population, we examined total blood CD4+ T cell population in 20 CBD, 9 BeS, and 9 normal control subjects for these subpopulations. In Figure 2A, a representative example of immunofluorescent staining is shown, with 49% of the CD4+ T cells expressing a naive phenotype, 40% expressing a Tcm phenotype, and 11% expressing a Tem phenotype, and Tem cells accounting for the remaining 11% of blood CD4+ T cells. Unlike CD8+ T cells, very few, if any, CD4+ T cells re-express CD45RA (<0.01% in the example shown) (17). Considerable overlap was seen among the study groups in the overall percentages of CD4+ T cells expressing these phenotypes (Figure 2, B–D). Although a slightly higher median Tcm percentage was measured in the CBD group (16.7%) compared with the BeS and normal control subjects (median percentage of 10.8% and 10.5%, respectively), no significant differences were noted.

**Memory phenotype of beryllium-specific CD4+ T cells.** To determine the maturation state of beryllium-specific CD4+ T cells, we stimulated PBMCs with 1 × 10−4 M BeSO₄ for 6 hours and analyzed IFN-γ- and IL-2–secreting beryllium-specific CD4+ T cells for surface expression of CD45RA and CCR7 (Figure 3). In order to ensure that enough gated events were observed, only subjects with at least 0.04% IFN-γ- and IL-2–secreting beryllium-specific CD4+ T cells in blood were studied. For the CBD patient shown in Figure 3A, 1.0% of the CD4+ T cells expressed IFN-γ after beryllium stimulation. The majority (69%) of these cells...
expressed a T_{EM} cell phenotype, compared with 31% T_{CM} cells. As expected, the naive CD4^{+} T cell population did not include beryllium-responsive cytokine-secreting cells. Similar proportions of IFN-\gamma–expressing CD4^{+} T_{EM} and T_{CM} cells were seen after staphylococcal enterotoxin B (SEB) stimulation (Figure 3B) as compared with beryllium stimulation. Few, if any, CD8^{+} T cells stained positively for IFN-\gamma after BeSO_{4} stimulation, whereas CD8^{+} cells capable of IFN-\gamma secretion were present in the blood of this patient and expressed IFN-\gamma after SEB stimulation (data not shown).

Analysis of IL-2 secretion in the same individual showed that 0.5% of the CD4^{+} T cells expressed this cytokine following BeSO_{4} exposure (Figure 3C). A similar percentage of these cells expressed the T_{EM}, T_{CM}, and naive phenotypes compared with IFN-\gamma– cells generated under the same conditions. However, when cells were stimulated by SEB, the majority of IL-2–expressing cells had a T_{CM} phenotype (57%), and 15% expressed a naive phenotype (Figure 3D). Thus, SEB stimulation appears to reveal IL-2–producing CD4^{+} T cells that are less differentiated (i.e., more T_{CM} cells than T_{EM} cells) compared with those secreting IFN-\gamma under the same conditions, being more representative of the total CD4^{+} T cell population.

Figure 4 shows the maturation states of IFN-\gamma– and IL-2–producing beryllium-specific CD4^{+} T cells in blood for all CBD (n = 12) and BeS (n = 3) patients evaluated. Because of the low frequency of beryllium-specific cells in the blood of BeS patients, we identified only 3 BeS individuals with sufficient numbers of these cells to evaluate using intracellular cytokine staining. The median percentage of beryllium-specific, IFN-\gamma–expressing CD4^{+} T cells with a T_{EM} phenotype (76%, range 31–96%) was significantly greater than the median percentage of those with either a T_{CM} (24%, range 3.2–69%; P < 0.001) or a naive (0%, range 0–0.7%; P < 0.001) phenotype (Figure 4A, left). Similar findings were seen...
IL-2 production in the BeS subjects (47% versus a median of 23% in the CBD patients). Although only 3 BeS subjects had sufficient numbers of beryllium-specific CD4+ T cells in blood to evaluate, these findings raise the possibility that progression from sensitization to disease may be associated with a further differentiation of memory T cells to an effector memory phenotype.

Four CBD patients underwent repeat analysis at time intervals ranging from 7 to 14 months after the initial evaluation (Figure 4C). These subjects experienced no change in their disease status over the interval time period and possessed similar quantities of IFN-γ-expressing beryllium-specific CD4+ T cells at both time points (data not shown). The median percentage of beryllium-specific CD4+ T cells expressing a TEM cell phenotype was 92% (range 80–96%) at time 0 compared with 95% (range 75–99%; P < 0.01) at the subsequent evaluation. These findings suggest that in the presence of persistent antigen exposure, the circulating pool of TEM cells in beryllium disease remains stable over time.

Memory maturation phenotype determines beryllium-induced T cell proliferation in blood. To determine whether the memory cell phenotype influenced the ability of CD4+ T cells to divide after BeSO4 exposure, PBMCs from 12 CBD patients were stimulated with BeSO4 in culture for 7 days, and CD4+ T cell proliferation was assessed using the CFSE-based proliferation assay. As shown in Figure 5A (top left), 20% of the IFN-γ-expressing CD4+ T cells after BeSO4 exposure displayed a TEM cell phenotype. After culture of fresh PBMCs from this individual for 7 days in the presence of 1 x 10^-4 M BeSO4, 52% of CD4+ T cells proliferated compared with after culture with medium alone. In contrast, only 3.2% of beryllium-induced, IFN-γ-expressing CD4+ T cells from another CBD subject displayed a TEM cell phenotype, with the remaining 97% representing beryllium-specific effector memory CD4+ T cells (Figure 5A, bottom left). Only 2.3% of the CD4+ T cells from this individual proliferated in response to BeSO4 exposure. Similar findings were seen with respect to beryllium-induced IL-2 expression. Overall, in 12 CBD subjects, an inverse correlation (r = –0.92; P ≤ 0.0001) was observed between the percentage of beryllium-stimulated proliferating cells and the percentage of beryllium-specific CD4+ T cells expressing a TEM phenotype ex vivo (Figure 5B, left). Conversely, we found a significant correlation (r = 0.93; P ≤ 0.0001) between beryllium-induced T cell proliferation and the percentage of beryllium-specific CD4+ T cells expressing a TEM phenotype (Figure 5B, right).

Correlation of the memory phenotype of beryllium-specific CD4+ T cells in blood and the degree of inflammation in the lung. Based on our previous observation that higher frequencies of beryllium-specific cells in blood were associated with the extent of alveolar inflammation as measured by both BAL wbc and lymphocyte counts (16), we correlated the percentage of beryllium-specific CD4+ T cells
expressing a $T_{EM}$ phenotype in blood with these parameters of lung involvement. As shown in Figure 6, a significant positive correlation was seen for the percentage of lymphocytes in BAL ($r = 0.69, P = 0.009$), and a trend was noted with total BAL wbc count ($r = 0.49, P = 0.09$). Conversely, an inverse correlation was observed between the percentage of lymphocytes in BAL and the percentage of beryllium-specific CD4$^+$ T cells expressing a $T_{CM}$ phenotype in blood ($r = -0.71, P = 0.007$). We did not observe a correlation between percentage of beryllium-specific CD4$^+$ T cells expressing a $T_{EM}$ phenotype in blood and any pulmonary or exercise physiologic variable (data not shown). Thus, the severity of the CD4$^+$ T cell–mediated inflammation in BAL is closely tied to the maturation state of circulating beryllium-specific memory T cells.

Comparison of the memory phenotype of beryllium-specific CD4$^+$ T cells in blood and lung. Because of the inaccessibility of the target organ in most diseases, few studies have been able to compare the T cell memory phenotypes of antigen-specific cells found in blood versus the target organ. In a disease characterized by CD4$^+$ T cell alveolitis, BAL allows the direct sampling of lung lymphocytes, with up to 25% of those CD4$^+$ T cells expressing Th1-type cytokines after beryllium exposure in culture (14). Using blood and BAL from 4 of the CBD patients described above, we determined the memory phenotype of the beryllium-specific CD4$^+$ T cell populations. In a representative example shown in Figure 7A, the majority of beryllium-specific, IFN-$\gamma$–expressing CD4$^+$ T cells possessed a $T_{EM}$ cell phenotype (97% of blood and 99% of BAL beryllium-specific CD4$^+$ T cells). Overall, the percentages of beryllium-specific CD4$^+$ T cells in blood and BAL expressing a $T_{EM}$ cell phenotype were similar, with a median of 94% (range 69–96%) in blood compared with 96% (range 74–99%; $P = 0.69$) in BAL (Figure 7B). In addition, similar findings were seen with respect to beryllium-specific CD4$^+$ T cells expressing a central memory phenotype in blood (median, 6.1%, range 3.2–31%) and BAL (median, 4.4%, range 1.5–26%; $P = 0.89$). Despite the compartmentalization of the majority of beryllium-specific CD4$^+$ T cells in the target organ (14), these findings suggest that beryllium-specific $T_{EM}$ cells are either trafficking from secondary lymphoid organs to the lung or recirculating from the lung.

Discussion

Our previous studies suggest that the frequency of beryllium-specific, cytokine-secreting CD4$^+$ T cells in blood is significantly greater in CBD patients compared with individuals with beryllium sensitization (16). However, no difference in beryllium-induced T cell proliferation in these disease groups was seen. Furthermore, in CBD patients, the correlation between numbers of circulating beryllium-specific, cytokine-secreting CD4$^+$ T cells and beryllium-stimulated lymphoproliferation is poor. With a known antigenic stimulus and an accessible target organ, beryllium-induced disease serves as an ideal system in which to investigate the role of different human memory CD4$^+$ T cell subsets in disease. Using blood and BAL from patients with beryllium sensitization and CBD, the current studies show that the majority of beryllium-specific CD4$^+$ T cells in blood and lung express an effector memory phenotype, irrespective of IFN-$\gamma$ or IL-2 production. Importantly, the ability of CD4$^+$ T cells to proliferate after beryllium exposure in culture depends on the maturation state of the memory T cell, which is significantly correlated with the severity of the CD4$^+$ T cell–mediated inflammation in the lung.
Similar discordance between proliferation and cytokine-secreting circulating beryllium-specific CD4+ T cells with beryllium-induced disease is due not to the absence of beryllium-induced lymphoproliferation seen in certain individuals with beryllium-induced disease but rather to a high fraction of TEM cells that are incapable of vigorous proliferation. Similar discordance between proliferation and cytokine-secreting responses has been seen in other diseases characterized by persistent antigen exposure such as HIV (24–26).

Even in the presence of low numbers of beryllium-induced, cytokine-secreting CD4+ T cells in blood, an increased percentage of those cells expressing a TEM cell phenotype was associated with strong proliferation. The ability of CD4+ TEM cells to secrete IL-2 may be responsible for their enhanced proliferation, further improving their ability to mediate protective immunity. Despite the absence of a correlation between the frequency of IL-2–expressing beryllium-specific CD4+ T cells in blood and lymphoproliferation (r = 0.35; P = 0.26), we did observe a significant association between the frequency of beryllium-specific CD4+ T cells expressing a central memory phenotype and beryllium-induced CD4+ T cell proliferation (r = 0.92; P = 0.0001). Direct proof that beryllium-specific CD4+ TEM cells possess a proliferative advantage over TEM cells would require separation of these cells at time 0 followed by analysis of beryllium-induced proliferation. Because of limitations on the usage of peripheral blood from CBD patients and the low frequency of beryllium-specific CD4+ T cells in blood, we were unable to perform these studies. However, in preliminary experiments using blood from healthy subjects stimulated with phytohemagglutinin, CD4+ TEM cells displayed greater proliferative responses compared with TEM cells (data not shown).

In the current studies of patients with beryllium-induced disease, we noted that the overall proportions of CD4+ T cell memory subsets were similar to those in control subjects. This is not surprising, since the circulating beryllium-specific CD4+ T cell population represents only a very small fraction of the total CD4+ T cell compartment (9, 16). When the superantigen SEB was used as a polyclonal activator, CD4+ T cells that secreted both IL-2 and IFN-γ, compared with those that made IFN-γ, were enriched in the TEM population, and even a few naive CD4+ cells were capable of secreting IL-2. However, when we focused on beryllium-specific CD4+ T cells, the TEM and TCM composition of cytokine-secreting cells was similar whether or not the cells retained the capacity to express IL-2. We found that the majority of beryllium-specific CD4+ cells that secreted both IL-2 and IFN-γ, similar to the ones that expressed IFN-γ alone, were TEM cells, irrespective of their location in blood or the target organ. In addition, the memory phenotype of the beryllium-specific CD4+ T cells in blood mirrored that expressed by the beryllium-specific cells in the target organ.

We have previously shown that 2 populations of beryllium-specific CD4+ T cells exist in blood and BAL of CBD patients: those cells capable of secreting both IFN-γ and IL-2 and those only able to express IFN-γ (9, 16). Few, if any, beryllium-specific CD4+ T cells secreted only IL-2. These observations are very similar to those reported in persistent viral infections such as progressive HIV infection in which virus-specific CD4+ T cells primarily produce IFN-γ and little, if any, IL-2 (24, 26, 27). However, when viral antigen burden is reduced by the administration of highly active antiretroviral therapy, a partial recovery of IL-2–producing T cells is seen (24, 27). It has also been shown in a perforin-deficient murine model of chronic lymphocytic choriomeningitis virus infection that T cells first lose the capacity to produce IL-2, then TNF-α, and finally IFN-γ (24, 27). Our findings suggest that persistent antigen exposure drives the beryllium-specific CD4+ T cells toward a maturation state in which their cytokine and other functional characteristics are inconsistent with a vigorous proliferative response, particularly in those subjects with an intense CD4+ T cell alveolitis. One limitation of our analysis is that we were only able...
to evaluate stages of maturation in subjects with significant quantities of beryllium-specific, Th1-type cytokine–expressing CD4+ T cells in blood; this resulted in a decreased sample size. We have previously shown that greater frequencies of beryllium-specific cells in blood are associated with the extent of alveolar inflammation, as measured by both BAL wbc and lymphocyte counts (16). Despite this limitation, the association between memory maturation phenotype of the circulating beryllium-specific CD4+ T cell and T cell alveolitis is quite strong, which suggests that the degree of inflammation in the target organ determines the maturation state of the beryllium-specific T cell. In addition, our findings suggest that the combination of the frequency of circulating beryllium-specific CD4+ T cells and their memory phenotype may predict the extent of alveolitis.

Fifty percent of the CBD patients enrolled in this study were receiving immunosuppressant treatment at the time of enrollment. Of the subjects with frequencies of beryllium-specific CD4+ T cells in blood detectable by intracellular cytokine expression (see Figure 3), 5 of 13 (38%) were receiving immunosuppressant therapy. Our previous studies investigating the TCR repertoire of CD4+ T cells in BAL showed persistence of clonal expansions despite the initiation of corticosteroids (6). In addition, no significant difference in the frequency of beryllium-specific T cells in blood was seen between treated and untreated CBD subjects (16). In the present study, a significantly increased number of lymphocytes in the BAL was seen in the treated versus the untreated subjects (data not shown). This almost certainly reflects the fact that the severity of pulmonary involvement (and the need for treatment) parallels the severity of the CD4+ T cell–mediated inflammation. There was no evidence that treatment status affected the parameters being measured (e.g., memory T cell maturation state) in the current study.

Of all the subjects evaluated, the 3 BeS patients studied had the highest frequency of beryllium-specific, IL-2–expressing CD4+ TCM cells. These subjects also had the lowest BAL absolute lymphocyte counts. Only 1 of these subjects had a positive beryllium-induced proliferative response from BAL cells, and none had granulomatous inflammation in the lung. Interestingly, there was an association between the extent of lung inflammation as measured by the percentage of lymphocytes in the BAL and the percentage of beryllium-specific CD4+ T cells expressing a TCM phenotype in blood. Taken together, our findings support the hypothesis that disease progression and potentially disease severity may be associated with a further differentiation of memory T cells from a proliferation-competent beryllium-specific CD4+ TCM cell to an IFN-γ-expressing, poorly proliferating TCM cell. In the presence of continuous antigenic exposure, the redevelopment of a predominantly IL-2–expressing TCM population is thus prevented. In conclusion, these findings help explain some of the variability that has been observed with the BeLPT and extend our current understanding of beryllium-induced disease and persistent antigen exposure.

**Methods**

**Study population.** Twenty-three patients with a diagnosis of CBD and 9 BeS patients were enrolled in this study. Nine 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 BeSO4 in vitro (28, 29). The diagnosis of beryllium sensitization was established based on a history of beryllium exposure, positive proliferative response of PBMCs to BeSO4 in vitro, and the absence of granulomatous inflammation or other abnormalities on lung biopsy (30, 31). 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 Health Sciences Center and National Jewish Medical and Research Center.

The demographics of the BeS and CBD patients are shown in Table 1. 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. No difference in the estimated duration of beryllium exposure was observed in BeS and CBD subjects. Eight CBD patients were treated with oral glucocorticoids, and 5 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, 11.2, range 1.0–262, versus median, 3.1, range 1.1–6.5; *P* = 0.02). At the time of this study, BAL cells from 3 of the BeS subjects proliferated in the presence of beryllium. All clinical BeLPTs were performed in the Clinical Immunology Laboratory at National Jewish Medical and Research Center as previously described (16). CBD subjects had a statistically significant increase in the percentage of BAL lymphocytes (median, 27, range 1–87, versus median, 9.1, range 1.0–22; *P* < 0.005) compared with BeS patients.

**Preparation of peripheral blood cells and beryllium-induced CD4+ T cell proliferation.** PBMCs were isolated from heparinized blood by Ficoll-Hypaque density gradient separation, and BAL was performed as previously described (6, 7). Proliferation assays were performed using fresh PBMCs (10 × 10^6 cells per well) labeled with 1.5 μM CFSE (Invitrogen Corp.) for 20 minutes at 37°C and washed twice with PBS (27). The CFSE-labeled cells were cultured at 37°C for 7 days in a humidified 5% CO2 atmosphere in 12-well flat-bottom microtiter plates in complete culture media containing RPMI 1640 supplemented with 10% heat-inactivated human serum (Gemini Bio-Products), 20 mM HEPES, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine (all from Invitrogen Corp.) with 1 of the following stimulants added: medium alone, 2.5 μg/ml phytohemagglutinin (Sigma-Aldrich), or 1 × 10^-4 M BeSO4.

### Table 1

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>BeS patients</th>
<th>CBD patients</th>
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<tbody>
<tr>
<td><strong>Characteristics</strong></td>
<td><em>(n = 9)</em></td>
<td><em>(n = 23)</em></td>
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<tr>
<td>Age (yr)</td>
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<td>Gender (M/F)</td>
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<td>Treatment (none/prednisone/methotrexate)</td>
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**Note:** Data are expressed as median (range). *P* < 0.05. W, white; AA, African American; H, Hispanic; CS, current smoker; FS, former smoker; NS, never smoker.
BeSO₄ (Brush Wellman). Cells were stained with mAbs directed against CD3 and CD4 (all from BD Biosciences — Pharmingen). The lymphocyte population was identified using forward and 90° light scatter patterns, and fluorescence intensity was analyzed using a FACSCalibur cytometer (BD) as previously described (9, 15).

**Immunofluorescence staining and analysis for intracellular cytokine expression.** Since ex vivo intracellular cytokine staining assays are only able to detect circulating antigen-specific cells present at frequencies greater than approximately 1 in 2,500 cells, only subjects with at least 0.04% IFN-γ- and IL-2–secreting beryllium-specific CD4+ T cells in blood were studied. For cytokine expression, PBMCs (2 × 10⁶ cells) and BAL cells (5 × 10⁶ cells) were placed in polypropylene tubes (12 × 75 mm; Fisher Scientific) containing 1 ml of complete culture media and 3 µg/ml anti-CD28 and anti-CD49d (BD Biosciences — Pharmingen) with 1 of the following added: medium alone, 10 ng/ml SEB, or 1 × 10⁻⁴ M BeSO₄. Cells were incubated for a total of 6 hours at 37°C in a humidified 5% CO₂ atmosphere with 10 µg/ml brefeldin A added after the first hour of stimulation, as previously described (9, 15). After stimulation, cells were washed and stained with mAbs directed against CD4, CD45RA, and CCR7 (all from BD Biosciences — Pharmingen) as previously described (9, 15). Cells were washed with PBS containing 1% BSA and placed in fixation medium (CALTAG Laboratories) for 15 minutes at room temperature. After washing with PBS containing 1% BSA, cells were added to permeabilization medium (CALTAG Laboratories), and stained with mAbs directed against IFN-γ and/or IL-2 (both from CALTAG Laboratories) for 30 minutes at 4°C. Fluorescence intensity was analyzed as described above.

**Statistical analysis.** ANOVA analysis was used to determine whether there was a global difference between groups. After the data were checked for overall group differences, individual contrasts were calculated to compare group means of interest. A Spearman correlation was performed to analyze the associations among beryllium-induced T cell proliferation, frequency of Th1-type cytokine–producing cells, and percentage of cells expressing each maturation phenotype. A value of less than 0.05 was considered statistically significant.

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