Beryllium-Induced TNF- α Production Is Transcription-Dependent in Chronic Beryllium Disease

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Beryllium (Be)-antigen presentation to Be-specific CD4⁺ T cells from the lungs of patients with chronic beryllium disease (CBD) results in T cell proliferation and TNF- α secretion. We tested the hypothesis that Be-induced, CBD bronchoalveolar lavage (BAL) T cell, transcription-dependent, TNF- α secretion was accompanied by specific transcription factor upregulation. After 6 h of Be stimulation, CBD BAL cells produced a median of 883 pg/ml TNF- α (range, 608–1,275 pg/ml) versus 198 pg/ml (range, 116–245 pg/ml) by unstimulated cells. After 12 h CBD BAL cells produced a median of 2,963 pg/ml (range, 99–9,424 pg/ml) TNF- α versus 55 pg/ml (range, 0–454) by unstimulated cells. Using real-time RT-PCR, Be-stimulated TNF- α production at 6 h was preceded by a 5-fold increase in TNF- α pre-mRNA copy number: β -actin copy number (Be median ratio 0.21; unstimulated median ratio 0.04). The median ratio of mature TNF- α mRNA: β -actin mRNA was upregulated 1.4-fold (Be median ratio 0.17; unstimulated median ratio 0.12). Be exposure in the presence of the transcription inhibitor pentoxifylline (PTX) decreased CBD BAL cell TNF- α pre-mRNA levels > 60%, whereas treatment with the mRNA splicing inhibitor 2-aminopurine (2AP) decreased levels 40% relative to Be exposure alone. PTX treatment decreased mature TNF- α mRNA levels 50% while 2AP decreased levels > 80%, relative to Be exposure alone. Beryllium exposure specifically upregulated transcription factors AP-1 and NF- κ B. The data suggest that Be exposure induces transcription-dependent TNF- α production, potentially due to upregulation of specific transcription factors.

Keywords: granuloma; T lymphocytes; cytokines; gene regulation; lung

Chronic beryllium disease (CBD) is a CD4⁺ T cell-mediated disorder resulting in the formation of noncaseating granulomas in the lungs of beryllium (Be)-exposed workers (1). Although the precise chemical nature of the Be-antigen is unknown, studies determined that Be-antigen presentation is restricted by major histocompatibility complex (MHC) Class II molecules (2–4). In culture, Be exposure induces the proliferation of Be-specific CD4⁺ T cells and upregulates genes encoding IFN- γ , IL-2, and TNF- α expression (2, 5–7). The ability to produce high levels of TNF- α protein in response to Be exposure is a unique property of Be-specific CD4⁺ T cells. Beryllium exposure does not induce the production of TNF- α by lung CD4⁺ T cells from Be-sensitized (BeS) patients or patients with a similar granulomatous lung disorder, sarcoidosis (7). Important to the formation

CLINICAL RELEVANCE

In chronic beryllium disease, beryllium-antigen-induced TNF- α production is transcription-dependent, with up-regulated levels of AP-1 and NF- κ B. Future strategies aimed at treating this antigen-specific human lung disorder should take this into account.

of lung granulomas in CBD, Maier and coworkers (6) showed that high levels of Be-stimulated TNF- α protein are associated with more severe lung disease in CBD.

We have previously demonstrated that human leukocyte antigen (HLA)-DP molecules could mediate Be-stimulated TNF- α mRNA and protein production (7). Treatment of BAL CD4⁺ T cells from patients with CBD with an anti-HLA-DP, but not anti-HLA-DR, monoclonal antibody (mAb) inhibited the intracellular expression of Be-stimulated TNF- α . This mAb also blocked Be-specific T cell proliferation and the production of both mature TNF- α mRNA and TNF- α protein from Be-stimulated blood and bronchoalveolar lavage (BAL) T cells from subjects with CBD (5, 7). Thus, activation of these Be-specific CD4⁺ T cells requires antigen-presenting cells expressing MHC Class II molecules bearing a Be-antigen that forms a trimolecular complex with Be-specific T cell receptors for antigen.

Lymphocyte activation signals drive proinflammatory protein up-regulation in a transcription-dependent or transcription-independent manner. A clear understanding of the various activation signals inducing Be-specific TNF- α mRNA and protein synthesis and their potential therapeutic role in targeting specific pathways in the proinflammatory cascade in CBD is lacking. Transcription-independent activation signals increase protein levels by inducing the direct splicing of pre-existing pre-mRNA transcripts into mature transcripts (8). Transcription-independent protein production also occurs when the activation signal induces the nuclear export and translation of a pre-existing pool of mature mRNA (9). Transcription-dependent TNF- α gene activation occurs when the activation signal upregulates specific transcription factors that bind the TNF- α gene promoter to initiate gene transcription and the formation of TNF- α pre-mRNA (10). In the present study, we tested the hypothesis that the upregulation of Be-stimulated CBD BAL cell TNF- α mRNA and protein was transcription-dependent. To do this, we used pentoxifylline (PTX) to inhibit transcription of the TNF- α gene and 2-aminopurine (2AP) to inhibit the splicing of TNF- α pre-mRNA into mature mRNA transcripts (11–13). If Be-induced CBD BAL T cell TNF- α production is transcription-dependent, a second goal of our study was determining whether Be exposure up-regulates nuclear transcription factors associated with activation

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of the TNF- α gene promoter. We found that Be-exposed CBD BAL cells upregulated TNF- α protein production in a transcription-dependent manner, with a Be-specific increase in nuclear levels of AP-1 and NF- κ B transcription factors.

MATERIALS AND METHODS

Chemicals and Reagents

A stock solution of 0.2 M BeSO₄ (Brush Wellman Inc., Cleveland, OH) was diluted to final concentrations of 10 μ M or 100 μ M BeSO₄ (Be) used to treat cells in culture. The concentrations of Be were selected based on previous studies that establish optimal stimulatory concentrations (2, 7). A stock solution of lipopolysaccharide (LPS, *Escherichia coli* serotype 0111:B4; Sigma Chemical Co., St. Louis, MO) at a final concentration of 1 μ g/ml was diluted 1:10 to treat cells in culture. PTX and 2AP were purchased from Sigma. Staphylococcal enterotoxin B (SEB; Sigma), was used at a final concentration of 10 ng/ml. Brefeldin A (Golgi Plug; BD Biosciences, San Diego, CA) was used in intracellular TNF- α staining experiments at a final concentration of 10 μ g/ml. Primary labeled anti-CD3 and anti-CD4 antibodies and their isotype controls were purchased from BD Biosciences. Primary labeled anti-TNF- α and the corresponding isotype control antibodies were purchased from Caltag Laboratories, Inc. (Burlingame, CA). Antibodies were used according to the manufacturer's instructions.

Subjects

Twelve subjects with CBD were consecutively enrolled from the National Jewish Medical and Research Center (NJMRC) Occupational and Environmental Health Clinic. The diagnosis of CBD was established using previously defined criteria, including a history of Be exposure, a positive proliferative response of peripheral blood mononuclear cells (PBMC) and/or BAL T cells to BeSO₄ in the beryllium lymphocyte proliferation test (BeLPT), and the presence of granulomatous inflammation and/or mononuclear cell infiltration on lung biopsy (5, 14, 15). All subjects provided written informed consent according to the protocol approved by the NJMRC Institutional Review Board, and completed a modified version of the American Thoracic Society (ATS) respiratory questionnaire (16).

Demographic and clinical characteristics are shown in Table 1. Due to the hiring practices of the beryllium industry, 11 subjects with CBD enrolled in this study were white, with one Hispanic individual, and there were 9 male and 3 female subjects (17). The median age of the study subjects was 51 yr (range, 36–61 yr). None of the subjects with CBD enrolled in this study were current smokers; five were former smokers and seven were never-smokers. Six of the subjects with CBD were current steroid users, and six had never used steroids.

The BAL consists of a complex, mixed cell population. On a per-milliliter basis, the BAL cells from the twelve subjects with CBD are representative of a chronic T cell alveolitis. The CBD BAL cellularity showed an elevation in the total number of white blood cells (WBC)

in BAL fluid, with $112 \pm 31 \times 10^6$ total WBC (mean $\pm$ SEM; median 66×10^6 total WBC, minimum 37×10^6 , maximum 421×10^6). The amount of BAL fluid recovered was 312 ± 11 ml (mean $\pm$ SEM; median 319 ml, minimum 224 ml, maximum 365 ml). Differential cell counting demonstrated a median of 31.4% lymphocytes (range, 9–83%) and a median of 66.9% macrophages (range, 16–89%). These findings are consistent with the presence of lung disease in the CBD population (2, 6, 7, 14, 18). The peak stimulation index (S.I.) for PBMC from patients with CBD was elevated to a median of 4.6 (6–120.2) (range), indicating a significant level of proliferation in response to BeSO₄ exposure *in vitro* and consistent with the presence of Be-specific blood T cells in CBD (5). The peak S.I. for CBD BAL cells was elevated to a median of 33 (1–389). These findings are consistent with the presence of granulomatous inflammation in the lungs of the subject with CBD as previously reported (2, 7, 14, 15).

Preparation of Peripheral Blood and BAL Cells and Cell Culture

At bronchoscopy, BAL was performed as previously reported (19). PBMC were obtained by venipuncture. Recovered PBMC were separated using density gradient centrifugation (Ficoll-Hypaque). CBD BAL cell concentration was adjusted to 1×10^6 cells/ml of complete medium; RPMI 1640 supplemented with 10% heat-inactivated, iron-supplemented calf serum (Cambrex Bioproducts, Walkersville, MD), 20 mM HEPES, 1 mM sodium pyruvate, 100 U/ml penicillin G, 100 μ g/ml streptomycin sulfate, and 2 mM L-glutamine (Life Technologies, Gaithersburg, MD), and for BeLPT analysis, 200- μ l aliquots were then cultured in triplicate wells per treatment condition, in 96-well round bottom plates (Costar #3799; Corning Inc., Corning, NY) at 37°C in an humidified atmosphere containing 5% CO₂. For TNF- α mRNA determinations (*see below*), BAL cells were cultured, in five wells per treatment condition.

Immunofluorescence Analysis for Intracellular TNF- α

BAL cells (5×10^5 cells) were placed in polypropylene tubes (12 $\times$ 75 mm; Fisher Scientific Co., Denver, CO) containing 1 ml of complete culture media plus either medium alone, 10 ng/ml SEB, or 10 μ M BeSO₄. SEB was used as a positive control for the activation of BAL CD4⁺ T cells for TNF- α production as previously demonstrated (2, 7, 20). We previously demonstrated that BAL macrophages from patients with CBD do not produce Be-stimulated TNF- α (7), and our unpublished observations suggest that LPS stimulation does not induce TNF- α production by CD4⁺ CBD BAL T cells. Cells were incubated for a total of 6 h at 37°C in a humidified 5% CO₂ atmosphere with 10 μ g/ml Brefeldin A added after the first hour of stimulation, as previously described (2, 7, 20). After stimulation, cells were washed and stained with mAbs directed against CD3 and CD4 (BD Biosciences) as described (2, 7, 20). Cells were washed with PBS containing 1% BSA and placed in fixation medium (Caltag Laboratories, Inc.) for 15 min at room temperature. Subsequently, cells were treated with permeabilization medium (Caltag) and stained with mAbs directed against TNF- α (Caltag) for 30 min at 4°C. The lymphocyte population was identified using forward and 90° light scatter patterns, and gating was performed on CD3⁺-expressing lymphocytes, to differentiate lymphocytes from macrophage subpopulations. Fluorescence intensity was analyzed using a FACScaliber cytometer (BD Biosciences) as previously described (2, 7, 20).

Beryllium-Induced Lymphocyte Proliferation

The blood and BAL Be lymphocyte proliferation tests (BeLPT) were performed according to the methods of Mroz and colleagues (5). PBMC and BAL cell concentrations were adjusted to 1×10^6 /ml of complete culture medium and 200- μ l aliquots were then cultured in triplicate, in the absence or presence of 10 μ M BeSO₄. Untreated and treated cells were cultured for 4, 5, and 6 d. During the last 4 h of culture, cells were pulsed with DNA specific precursor tritiated thymidine deoxyriboside (³HTdR, S.A. 5 Ci/mM; Amersham, Piscataway, NJ). ³H-DNA was harvested onto glass filters, and counted in a Packard TopCount NTX scintillation counter (Packard Instruments Co., Meriden, CT). Thymidine uptake for the unstimulated controls on Days 4, 5 and 6 are normally in the range of 150–500 counts per minute (cpm). For the clinical evaluation of PBMC and BAL T cell proliferation in response to Be stimulation shown in Table 1, we report the median and range

TABLE 1. CLINICAL CHARACTERISTICS OF CBD SUBJECTS

Characteristics	n = 12
Age, yr*	51 (31–61)
Sex, M/F	9/3
Smoking status, CS/FS/NS	0/5/7
Race, C/AF	12/0
Ethnicity, NHisp/Hisp	11/1
Corticosteroid treatment	6/6
BAL cells	
Total BAL WBC cells, $\times 10^6$	66 (37–421)
Macrophages, %	66.9 (16–69)
Lymphocytes, %	31.4 (9–83)
Peak blood BeLPT stimulation index	4.6 (0.6–120.2)
Peak BAL BeLPT stimulation index	33 (1–389)

Definition of abbreviations: AF, African-American; C, Caucasian; CS, current smoker; FS, former smoker; Hisp, Hispanic; NS, never smoker; NHIS, non-Hispanic.

* Data expressed as median (minimum–maximum).

for the peak S.I. as the ratio of the median test sample cpm to the median cpm in the unstimulated (medium alone) control (5).

TNF- α Determinations

BAL cells from patients with CBD were adjusted to a final concentration of 1×10^6 cells/ml, and 200- μ l aliquots were cultured in triplicate for each treatment condition. Using culture supernatants harvested at 6 h ($n = 3$ patients with CBD) and 24 h ($n = 10$ patients with CBD) after stimulation, TNF- α levels (pg/ml) were determined using ELISA kits (R&D Systems, Minneapolis, MN) with a sensitivity of 7.8 pg/ml (6, 7). BAL cells were stimulated with either medium alone (unstimulated), 100 μ M BeSO₄, or 1 μ g/ml LPS. LPS stimulation was used as a positive control for the activation of BAL macrophages for TNF- α production as previously described (7).

Real-time RT-PCR was used to determine TNF- α pre-mRNA and mature mRNA levels using BAL cells from four subjects with CBD. Cell concentration was adjusted to 1×10^6 cells/ml of complete medium, and 200- μ l aliquots were cultured in five wells per treatment with either medium alone or 100 μ M BeSO₄. Cells were harvested at 0 time and after 0.5 and 1 h of Be stimulation, and real-time RT-PCR analysis was performed.

Real-time RT-PCR was also used to determine TNF- α pre- and mature mRNA levels, using a subset of BAL cells from four subjects with CBD, in the absence and presence of 2AP, a splicing inhibitor (11), or PTX, a transcription inhibitor (12, 13). For these experiments, cell concentration was adjusted to 1×10^6 cells/ml of complete medium, and 200- μ l aliquots were then cultured in five wells per treatment. One set of cells was cultured in medium alone or stimulated with either 100 μ M BeSO₄ or 1 μ g/ml of LPS. A second set of cells were treated in the same manner in the presence of PTX, and the third set of cells were treated in the presence of 2AP. In these experiments, LPS stimulation served as a positive activation signal for CBD BAL macrophage production of TNF- α by a transcription-dependent mechanism (7). Cells were harvested after 6 h and real-time RT-PCR analysis performed.

For real-time RT-PCR analysis, total RNA was isolated from cell pellets using the RNeasy kit (Qiagen Inc., Valencia, CA). Total RNA samples were treated with RQ1 RNase-free DNase (Promega, Madison, WI) for 30 min at 37°C and compared by electrophoresis against the untreated samples to control for the presence of DNA contamination in the RNA samples. Gel electrophoresis was performed on a 1% Agarose-TAE gel by electrophoresis for 1 h at 110 V and the gels stained with Vistra Green (Amersham). The stained bands were scanned using a FMBIO II Phosphorimager (Hitachi Inc., Alameda, CA) and fluorescent bands were quantified (counts) using FMBIO Analysis 8.0 software. All samples were run against the untreated samples to control for the presence of DNA contamination in RNA samples. Those reactions that showed DNA contamination were discarded. The RT step was performed separately in a Perkin Elmer 9,700 thermocycler, using 1 μ g of RNA (DNA-free) with the RNA PCR Core Kit (Applied Biosystems, Foster City, CA). A total of 5 μ l of reverse-transcribed cDNA and 3 μ M of each primer were added to Applied Biosystems SYBR Green Master Mix to a total volume of 50 μ l. TNF- α pre-mRNA and mature mRNA were detected via real-time PCR analysis using the MJ Research Opticon 2 Detection System (MJ Research, Cambridge, MA). Per reaction, the reaction mix employed 10 μ l DyNamo HS Master Mix (Finnzymes Espoo, Finland), 4 μ l forward and reverse primers (to a final concentration of 0.3 μ M each), 0.4 ROX reference dye, 4 μ l of reverse transcribed cDNA, and 1.6 μ L H₂O. Primers for TNF- α pre-mRNA were as follows: 5' CTTAGTGGGATACTCAGAACG (spanning the +1446 to +1468 region of the TNF- α gene) and 3' GGCGGTTTCAGCCACTGAGCT (spanning the +1534 to +1556 region of the TNF- α gene), yielding a 202-bp product. Primers for mature TNF- α mRNA were as follows: 5' TGTAGCCCATGTTG TAGCAA (spanning the +1109 to +1125 and +1410 to +1412 of the TNF- α gene) and 3' TGGGAGTAGATGAGGTACAGG (spanning the +1534 to +1556 region) resulting in amplification of a 161-bp product. The 5' primer was designed to overlap both the third and fourth exons to control against erroneous amplification of TNF- α pre-mRNA or DNA. β -actin primers were as follows: 5' GATGACC CAGATCATGTTTGA and 3' ATGAGGTAGTCAGTCAGGTCC, resulting in amplification of a β -actin mature-mRNA 200-bp product.

PCR cycling conditions were as follows: 95° for 5 min followed by 40 cycles of 60° 30 s and 95° 15 s. After the amplification cycles, a dissociation curve was run from 80° to 90°, holding for 3 s every 0.1°. Real-time analysis of known TNF- α and β -actin copy number standards allowed for quantification of test samples. A dissociation curve was run and analyzed for all samples to confirm specificity of amplification. TNF- α pre-mRNA and mature mRNA copy numbers were compared as ratios, to the levels of β -actin mature mRNA copy number. β -actin mature mRNA copy number is maintained at a constant level in Be-stimulated cells, as previously published (7).

Detection of Transcription Factors

The electrophoretic mobility shift assay (EMSA) was performed using standard procedures described previously (21). In brief, CBD BAL cells from a subset of three patients, 10×10^6 per condition, were plated in wells of a 96-well plate as described above. Cells were unstimulated or exposed to 100 μ M BeSO₄ for 0, 3, 6, or 24 h. Adherent cells and nonadherent cells were separated as previously described (22), to yield macrophage- and lymphocyte-enriched cell populations (7). Nuclear extracts were prepared using the Nuclear Extract Kit (Active Motif, Carlsbad, CA) according to manufacturer's recommendations. Transcription factor consensus oligonucleotides were obtained from Promega and labeled with ³²P-ATP (3,000 Ci/mmol, Amersham) using T4 Polynucleotide Kinase (USB Corporation, Cleveland, OH) according to manufacturer's recommendations. The nuclear extracts were pre-equilibrated in Gel Shift Binding Buffer (Promega) for 10 min at room temperature. The labeled consensus oligonucleotides were added to the extract solution and the room temperature incubation continued for an additional 20 min. Nuclear extract samples were suspended in 100 mM Tris, pH 7.5 and 8% glycerol. Samples were immediately loaded onto a 6% acrylamide gel containing 0.5X Tris-boric acid-EDTA (TBE) and 2.5% glycerol that had been pre-electrophoresed for 10 min at 200 V at room temperature. The samples were electrophoresed at room temperature at 200 V for 2 h in a buffer containing 0.5X TBE. Following fixation for 30 min in 10% methanol + 10% acetic acid, gels were dried onto filter paper and visualized by overnight exposure to X-ray film. The X-ray film was scanned using a FMBIO II Phosphorimager (Hitachi Inc., Alameda, CA) and radioactive bands were quantified (counts) using FMBIO Analysis 8.0 software. We performed our initial survey of nuclear extracts for transcription factors, know to either bind the TNF- α promoter or to be upregulated during the activation of CD4⁺ T cells, using consensus oligonucleotide probes for: AP-1, AP-2, NFATc, NF-Y, Oct-1, SIE, SP1, STAT-1 through STAT-6, C/EBP, CREB, Egr, Ets, GAS, IRF-1, NF- κ B, T-bet, TFIID and YY-1. We report a pattern of increased nuclear levels for two of these transcription factors that were > 50% after Be-stimulation relative to the unstimulated controls.

Cold oligonucleotides (Promega) competition control assays were performed by addition of non-labeled oligonucleotide at 100 times ³²P-labeled oligonucleotide concentrations, for both AP-1 and NF- κ B. Samples were prepared as before where nuclear extracts were pre-equilibrated in Gel Shift Binding Buffer (Promega) for 10 min at room temperature. Labeled and unlabeled consensus oligonucleotides were added to extracts and room temperature incubations continued for an additional 20 min. Samples were immediately loaded onto a 6% acrylamide gel containing 0.5X Tris-boric acid-EDTA (TBE) and 2.5% glycerol that had been pre-electrophoresed for at least 10 min at 200 V at room temperature. Samples were then electrophoresed at room temperature at 200 V for 2 h in a buffer containing 0.5X TBE. After fixation for 30 min in 10% methanol + 10% acetic acid, gels were dried onto filter paper and visualized by exposure to X-ray film. EMSA super shift antibody control assays were performed using an identical protocol, but with the addition of antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) specific for NF- κ B (p50 [D-17]) or AP-1 (cFOS [K-25]X), added at 1 μ g for NF- κ B super shift controls and 10 μ g for AP-1 super shift controls.

Statistical Analysis

Wilcoxon matched pairs rank sum tests were performed on all data in which there were comparisons made between treatments performed on subjects' cells to take into account the variability of subjects. All other comparisons were made using Wilcoxon rank sum tests. All tests

were two sided and a $P \leq 0.05$ was used to determine statistical significance.

RESULTS

Be Exposure Upregulates CBD BAL CD4⁺ T Cell TNF- α Protein

A representative example of increased expression of intracellular TNF- α by a BAL CD4⁺ T cells from a patients with CBD after 6 h of Be-stimulation is shown in Figure 1a. Consistent with the presence of noncaseating interstitial granulomas on lung biopsy, this individual's blood BeLPT S.I. was 85.1 (normal ≤ 2.5), and the BAL BeLPT S.I. was 58.8 (normal ≤ 2.5). The BAL contained 83% lymphocytes, indicating profound lymphocytic alveolitis. Minimal background TNF- α production was observed after culture in medium alone, whereas SEB (10 ng/ml) stimulation induced increased TNF- α levels in 38% of the CD4⁺ T cells. Compared with medium alone (0.1% CD4⁺ T cells containing TNF- α), 16% of the CD4⁺ T cells from this patient had increased TNF- α levels after Be exposure (10 μ M BeSO₄). Intracellular TNF- α staining was performed using BAL cells from 4 of the 12 subjects CBD enrolled in this study, and the percentage of CD4⁺ T cells expressing TNF- α is shown in Figure 1b. Increased levels of intracellular TNF- α were observed in the CD4⁺ CBD BAL T cells of all four subjects after both Be stimulation, with a median of 14% TNF- α ⁺ CD4⁺ T cells (range, 7.5–18%), and SEB stimulation, with a median of 41% TNF- α ⁺ CD4⁺ T cells (range, 28–60%), versus a median of 0.1% TNF- α ⁺ CD4⁺ T cells after culture in medium alone. Our previous studies demonstrated that Be-exposed CBD BAL macrophages did not upregulate TNF- α expression (7). SEB stimulation induced increased TNF- α levels in 8.6% of the CD4-negative cells (likely CD8⁺ T cells) that co-localize in the CD3⁺ lymphocyte gate (Figure 1a) (7, 20).

We used ELISA to determine TNF- α levels (pg/ml) produced by unstimulated CBD BAL cells as compared with cells that were stimulated with either 100 μ M BeSO₄ or 1 μ g/ml LPS for 6 h and 24 h (Table 2). In these experiments, the 6-h TNF- α levels were determined using BAL cells from 3 of the 10 CBD subjects that were used for the 24 h determinations, due to the availability of excess numbers of BAL cells from these subjects. LPS stimulation served as a positive control for the production of TNF- α by CBD BAL macrophages as described previously

(7). Be-stimulated CBD BAL T cells produced significantly increased levels of TNF- α after 6 h and 24 h compared with the unstimulated controls. LPS-stimulated CBD BAL macrophages likewise produced significant levels of TNF- α at 6 h and 24 h compared with the unstimulated controls.

Be Exposure Upregulates Early TNF- α mRNA and Protein Levels in CBD

We tested the hypothesis that upregulation of Be-induced TNF- α mRNA levels preceded the production of TNF- α protein. Real-time PCR was used to quantitate TNF- α pre-mRNA and mature mRNA at 0, 0.5, and 1 h in unstimulated and 100 μ M BeSO₄-exposed CBD BAL cells ($n = 4$). TNF- α mRNA copy number was expressed as a ratio of the corresponding β -actin mature mRNA copy number (Figures 2a and 2b). Amplification of the 200-bp β -actin mature mRNA transcript copy number was chosen for the purpose of comparisons based on our previous observation that Be stimulation does not alter transcript levels from this housekeeping gene over time (7). Beryllium exposure upregulated the levels of TNF- α pre-mRNA from a median ratio of 0.03 (range, 0.00–0.08) at 0 time to a median ratio 0.21 (range, 0.07–0.39), an 86% increase in levels in comparison to 0 time levels, after 1 h of Be exposure (Figure 2a). The levels of TNF- α pre-mRNA from unstimulated CBD BAL cells did not significantly change during the assay period of 1 h.

Levels of mature TNF- α mRNA, following exposure to Be, were a median ratio of 0.02 (range, 0.01–0.06) at 0 time, a median ratio of 0.12 (range, 0.02–0.31) detected at 0.5 h and a median ratio of 0.17 (range, 0.03–0.54) detected at 1 h. This represents an increase of 89% in TNF- α mature mRNA transcript levels after 1 h of Be exposure (Figure 2b). The ratio of mature TNF- α mRNA expressed by Be-stimulated and unstimulated CBD BAL cells were not significantly different at any time. This point was due in part to an increase in TNF- α mature-mRNA levels in unstimulated cells from a median ratio of 0.01 (range, 0.00–0.05) at 0 time to a median ratio of 0.12 (range, 0.01–0.36) at 1 h, representing a > 90% increase in transcript levels.

Be Induces TNF- α Gene Transcription

We used BAL cells from a subset of four patients with CBD to test the hypothesis that the Be-induced upregulation of TNF- α

Figure 1. (a) A representative example of intracellular TNF- α expression by CD4⁺ BAL T cells from a patient with CBD after short-term culture with medium alone, 10 μ M BeSO₄, or 10 ng/ml SEB. The percentage of CD4⁺ CBD BAL T cells expressing intracellular TNF- α is shown in the upper right quadrant of each density plot. (b) The percentage (%) of CD4⁺ BAL T cells expressing intracellular TNF- α in response to either medium alone, 10 μ M BeSO₄, or SEB is shown. The bar indicates the median value for the four subjects with CBD tested.

TABLE 2. PRODUCTION OF TNF- α BY BRONCHOALVEOLAR LAVAGE CELLS IN RESPONSE TO 100 μ M BeSO₄ OR 1 μ g/ml LPS

Treatment	TNF- α (pg/ml)	
	6 h (n = 3)	24 h (n = 10)
Media alone	198 (116–245)	55 (0–454)
BeSO ₄	883 (608–1,278)*	2963 (99–9,424)*
LPS	24,060 (20,163–24,565)*	36,611 (2,896–142,294)*

Data are expressed as median (range).

* $P < 0.05$ versus Media alone, Wilcoxon rank sum test.

mRNA was due to TNF- α gene transcription. PTX inhibited the formation of TNF- α pre-mRNA transcripts and 2AP inhibited the splicing of these TNF- α pre-mRNA transcripts into mature TNF- α mRNA transcripts (11–13). Real-time PCR analysis was performed on treated and untreated control cells after 6 h of Be or LPS exposure. Previous study (7) shows that CBD BAL macrophages do not upregulate TNF- α mRNA or protein production in response to Be stimulation (7). Thus, changes in TNF- α mRNA levels in unstimulated control CBD BAL cells are due to the activation of BAL macrophages as they adhere to the plastic culture medium, whereas changes in TNF- α mRNA levels in Be-stimulated CBD BAL cells result most likely from a similar level of BAL macrophage activation due to plastic adherence, but result largely from the MHC class II restricted activation of Be-specific CD4⁺ T cells as previously demonstrated (7). LPS stimulation activates BAL macrophages for TNF- α mRNA and protein production and in this experiment served as a positive stimulation control showing that the patient's BAL sample macrophages were able to respond to a disparate activation signal not mediated by MHC class II.

After exposure to 100 μ M BeSO₄, BAL cells of patients with CBD expressed high levels of TNF- α pre-mRNA copy number with a median ratio of 0.18 (range, 0.04–0.42) relative to β -actin mature mRNA copy number (Figure 3a). Treatment with PTX decreased the levels of Be-induced TNF- α pre-mRNA to a median ratio of 0.06 (range, 0.00–0.11), a 66% reduction. Treatment with 2AP resulted in an $\sim$ 40% reduction in the levels of TNF- α pre-mRNA levels (median ratio 0.10; range, 0.1–0.14); however, this was not significantly different compared with Be-exposed cells (median ratio 0.18). After exposure to 100 μ M BeSO₄ the levels of TNF- α mature-mRNA were increased to a median ratio of 0.14 (range, 0.05–0.40) (Figure 3b). Treatment with PTX decreased the levels of mature TNF- α mRNA to approximately half the levels observed in Be-stimulated cells, or a median ratio of 0.07 (range, 0.03–0.13). Mature TNF- α mRNA levels in the presence of 2AP were reduced $> 80\%$ of those observed after Be exposure alone, to a median ratio of 0.02 (range, 0.00–0.12).

PTX treatment reduced unstimulated CBD BAL cell TNF- α pre-mRNA levels to a median ratio of 0.11 (range, 0.04–0.15) from a median ratio of 0.15 (range, 0.04–0.42) (Figure 3c) and unstimulated mature-mRNA levels from a median ratio of 0.13 (range, 0.05–0.21) to a median ratio of 0.05 (range, 0.00–0.16) (Figure 3d). After 2AP treatment TNF- α pre-mRNA levels (median ratio, 0.26; range, 0.16–0.38) were increased relative to levels in unstimulated control cells (median ratio, 0.16), while the levels of mature TNF- α mRNA were reduced to a median ratio of 0.01 (range, 0.00–0.04).

LPS is a well-described TNF- α gene transcription-dependent activation signal for macrophages (10). LPS-stimulated CBD BAL cell TNF- α pre-mRNA levels to a median ratio of 0.10 (range, 0.01–1.04) (Figure 3e), while mature TNF- α mRNA levels exhibited a median ratio of 3.9 (range, 0.15–13.64) (Figure 3f). After PTX treatment, TNF- α pre-mRNA levels were unchanged (median ratio of 0.15; range, 0.04–0.26); however, mature mRNA levels were significantly reduced to a median ratio of 0.72 (range, 0.2–2.87). After 2AP treatment, TNF- α pre-mRNA levels (median ratio, 0.68; range, 0.13–3.61) increased significantly. After 2AP treatment, the levels of TNF- α mature mRNA were reduced to a median ratio of 0.06 (range, 0.02–4.26).

Beryllium-Specific Upregulation of Nuclear Transcription Factors

Sufficient amounts of nuclear protein from isolated BAL T cells were available to perform EMSA analysis after 24 h of Be exposure, for AP-1 and NF- κ B, from three subjects with CBD (Table 3). For two of these subjects with CBD, represented in Figure 4, we were able to perform cold oligonucleotides competition controls and specific antibody supershift analysis; however, the isolated T cells of the third subject with CBD did not yield nuclear extract amounts sufficient to perform these two control analyses. The BAL from these three subjects contained $264 \pm 78 \times 10^6$ total WBC (mean $\pm$ SEM; median 192×10^6 total cells, minimum 180×10^6 , maximum 421×10^6) (BAL fluid collected was 308 ± 28 ml, mean $\pm$ SEM; median 285 ml, minimum 275 ml, maximum 365 ml) with a medium (range) of 76.5% (51–83%) lymphocytes. At 0 time, T cells and macrophages were isolated from CBD BAL mixed cell populations by adherence to plastic, as previously described (7, 22). To determine constitutive nuclear levels of transcription factors in these cell populations, EMSA was performed on these 0 time, isolated T cells and macrophages. Cultures of isolated T cells were either unstimulated or Be-stimulated (100 μ M BeSO₄) for 24 h and EMSA performed using nuclear extracts from these isolated T cells. The macrophages from the CBD BAL mixed cell population, which provided T cell nuclear extracts for EMSA analysis, were not used in this experiment since a previous study showed that Be stimulation triggers CBD BAL macrophage apoptosis, making the

Figure 2. The kinetics of TNF- α mRNA upregulation in Be-stimulated CBD BAL cells (n = 4). (a) The median ratio of TNF- α pre-mRNA copy number to β -actin mature mRNA copy number at 0, 0.5, and 1 h in unstimulated (open bars) and Be-stimulated (filled bars; 100 μ M BeSO₄) CBD BAL cells. (b) The median ratio of TNF- α mature-mRNA copy number to β -actin mature mRNA copy number at 0, 0.5, and 1 h in unstimulated (open bars) and Be-stimulated (filled bars; 100 μ M BeSO₄) CBD BAL cells. * $P < 0.05$, Wilcoxon matched pairs.

Figure 3. Transcription-mediated TNF- α pre-mRNA copy number and mature mRNA copy number: β -actin mature mRNA copy number in CBD BAL cells ($n = 4$) after 6 h of Be stimulation ($100 \mu\text{M BeSO}_4$). (a) The median ratio of TNF- α pre-mRNA to β -actin after exposure to $100 \mu\text{M BeSO}_4$ alone (solid bars) versus Be + PTX (open bars) or Be + 2AP (striped bars). (b) The median ratio of TNF- α mature mRNA to β -actin after exposure to $100 \mu\text{M BeSO}_4$ alone (solid bars) versus Be + PTX (open bars) or Be + 2AP (striped bars). (c) The median ratio of TNF- α pre-mRNA to β -actin after culture in medium alone (solid bars) or medium alone + PTX (open bars) or + 2AP (striped bars). (d) The median levels of TNF- α mature mRNA to β -actin after culture in medium alone (solid bars) or medium alone + PTX (open bars) or + 2AP (striped bars). (e) The median levels of TNF- α pre-mRNA to β -actin after exposure to $1 \mu\text{g/ml LPS}$ alone (solid bars) versus LPS + PTX (open bars) or LPS + 2AP (striped bars). (f) The median levels of TNF- α mature mRNA to β -actin after exposure to $1 \mu\text{g/ml LPS}$ alone (solid bars) versus LPS + PTX (open bars) or LPS + 2AP (striped bars).

isolation of nuclei from this cellular fraction problematic (23). However, we acknowledge this limitation to our study.

For the three subjects with CBD, quantitative measurement of nuclear transcription factor levels (counts) in isolated CBD BAL T cells (Table 3) demonstrated that after 24 h of Be stimulation, nuclear levels of both AP-1 and NF- κ B were increased relative to the levels observed in isolated CBD BAL T cells at

TABLE 3. NUCLEAR TRANSCRIPTION FACTOR LEVELS (COUNTS) OF ISOLATED BRONCHOALVEOLAR LAVAGE T CELLS FROM PATIENTS WITH CHRONIC BERYLLIUM DISEASE AT TIME 0 AND AFTER CULTURE FOR 24 h WITH AND WITHOUT $100 \mu\text{M BE}$

	Transcription Factor	
	Activator Protein-1	NF- κ B
Time 0	32 (29–92)	162 (94–574)
Unstimulated, 24 h	259 (85–350)	503 (385–733)
Beryllium-stimulated, 24 h	1,181 (728–1,258)*	1,404 (1,184–1,768)

Data are expressed as median (range).

* $P < 0.05$ compared to Time 0, Wilcoxon rank sum test.

0 time, or after 24 h in culture. A representative example of EMSA results, including the cold oligonucleotides competition and antibody supershift controls, for two of the subjects with CBD (Figure 4) demonstrates that CBD BAL isolated macrophages (MO at 0 time) contained constitutive levels of NF- κ B. In comparison, isolated T cells (0 time) contained constitutive levels of both AP-1 and NF- κ B. After 24 h of Be stimulation, isolated T cell nuclei contained levels of AP-1 and NF- κ B that were increased relative to the levels observed in isolated T cell nuclei from unstimulated cells at 0 time and at 24 h, supporting the quantitative measurements shown in Table 3. For these two subjects with CBD subjects, cold oligonucleotides competition and EMSA supershift controls for NF- κ B and AP-1 confirmed the identity of these two transcription factors and their ability to bind consensus oligonucleotides in our assays.

DISCUSSION

In CBD, the granulomatous lung disease is associated with a lymphocytic alveolitis that is dominated by the presence of Be-specific CD4⁺ T cells. The BAL of our subjects with CBD contained > 100 million total WBC with $\sim 31\%$ lymphocytes, consistent with our previous observations (2, 6, 7, 14, 18). Moreover,

Figure 4. The levels of transcription factors NF-κB and AP-1 as determined by EMSA, in the nuclei of isolated CBD BAL cells from two subjects, that were unstimulated (–) or exposed to 100 μM BeSO₄ (+). Constitutive levels of nuclear transcription factors were determined for NF-κB and AP-1 by EMSA in unstimulated, isolated CBD BAL macrophages (MO), and isolated CBD BAL T cells (T cell) at 0 time and are compared with the changes in transcription factor levels after 24 h in unstimulated isolated T cells (–) or isolated T cells exposed to 100 μM BeSO₄ (+). Cold oligonucleotide competition (CC) controls (absence of a band), and EMSA antibody supershift (SS) assay controls (arrows on right) for isolated T cells after 24 h of exposure to 100 μM BeSO₄ (+) indicated the specificity of the transcription factor bands.

Be exposure activates these T cells, resulting in proliferation and clonal expansion into an effector-memory T cell population (2), and studies show that these T cells upregulate proinflammatory cytokines skewed toward a Th1-type phenotype (24). The signal driving this Be-specific T cell activation is MHC class II restricted, and an interesting aspect of these activated CD4⁺ T cells is their ability to also produce high levels of TNF-α (7). Previous studies show that Be-stimulated CBD BAL cells produce high levels of TNF-α, IFN-γ, IL-2, IL-6, and IL-10 mRNA and protein (25–28), but do not upregulate production of IL-4 or IL-7 (25). The addition of recombinant human IL-10 (26) to Be-stimulated CBD BAL cells, but not the addition of IL-4 (27), downregulates the production of TNF-α, IFN-γ, and IL-2. Maier and coworkers (27) and Amicosante and colleagues (28) extended these observations, demonstrating that Be-stimulated CBD PBMCs also upregulate production of TNF-α and IFN-γ. Hong-Geller and associates (29) recently showed that PBMCs and dendritic cells from normal subjects upregulate mRNA and protein levels of MIP-1α, MIP-1β, GRO1, GRO3, and RANTES. To date, studies demonstrating the precise cellular source of these various cytokines and chemokines in PBMC and BAL cells from patients with CBD have been limited. Fontenot and his coworkers used intracellular cytokine staining (2, 20) and ELISPOT analysis (24) to demonstrate that CD4⁺ T cells from blood and BAL of subjects with CBD upregulate TNF-α and IFN-γ. Sawyer and colleagues (7) demonstrated that Be stimulation does not upregulate TNF-α production by CBD BAL macrophages. The ability of Be to induce high levels of TNF-α protein is augmented in subjects with CBD with the TNF-α promoter region -308 A variant (30). In turn, high TNF-α levels have been associated with more severe pulmonary disease in CBD (6). Together, these studies show the importance of Be-antigen-specific CD4⁺ T cells and Be-stimulated TNF-α production in the pathogenesis of CBD (1). The role of TNF-α, IL-6, and IFN-γ in regulating cytokine production by lung T cells as well as other bystander cells such as alveolar macrophages, dendritic cells, and lung epithelial cells has not been explored. We speculate that as yet unidentified T cell mediators such as IL-12, IL-15, or RANTES may prove to be as, or more, important than TNF-α in the pathogenesis of CBD. However, the focus of the present study was to determine if Be-stimulated CBD BAL cells TNF-α upregulation was transcription dependent and

accompanied by the upregulation of Be-stimulated transcription factors.

In the present study, we observed that BAL CD4⁺ T cells of subjects with CBD were Be-specific, upregulating the levels of intracellular TNF-α after Be stimulation. Our previous studies show that the percentage of Be-specific CD4⁺ T cells are found in the range of 1.4–29% in CBD BAL cells (2). Be-induced TNF-α pre-mRNA upregulation was inhibited by PTX, a transcription inhibitor (12, 13), and the splicing of TNF-α pre-mRNA into mature mRNA transcripts was inhibited by 2AP, a splicing inhibitor (11). In addition, the data indicate that this Be-induced TNF-α production was associated with Be-specific upregulation of AP-1 and NF-κB transcription factors in the nuclei of CBD BAL cells. Based on these observations, we conclude that Be exposure induces the production of TNF-α mRNA and protein by CBD BAL CD4⁺ T cells in a transcription-dependent manner.

Before understanding this study, we considered three possible ways in which Be exposure could upregulate CBD BAL cell production of TNF-α mRNA and protein: by (1) increasing gene transcription, (2) directly activating the splicing of pre-mRNA into mature mRNA transcripts (8), or (3) bypassing mRNA splicing and driving the nuclear export of pre-existing mature mRNA transcripts and their translation (9).

Our data show that Be exposure increased the levels of TNF-α pre-mRNA and mature mRNA transcripts followed by an increase in the production of TNF-α protein. The slight but measurable increase in mature TNF-α mRNA observed in the unstimulated cells was likely a response by CBD BAL macrophages as they adhere to the plastic culture medium and was not, therefore, Be-antigen-specific. This is illustrated in our previous study showing the intracellular expression of Be-induced TNF-α is specific to CD3⁺ CD4⁺ CBD BAL T cells and not CBD BAL macrophages (7). LPS, a well-characterized activation signal mediated by TNF-α gene transcription (31, 32), also upregulated TNF-α mRNA levels. Similar to adherence to plastic, LPS likely induces TNF-α expression in CBD BAL macrophages; however, LPS-induced TNF-α upregulation is mediated by the Toll-like receptors. By comparison, Be-antigen-specific TNF-α gene activation is MHC-restricted and dependent on the ligation of T cell antigen receptors, and possibly accessory molecules, on Be-specific CD4⁺ CBD BAL T cells (2, 7). Regardless of cell type, the goal of this study was to determine whether the

Be-induced signal, like adherence and LPS, results in transcription-dependent TNF- α gene expression.

Critical to resolving this question, we first observed that CBD BAL cells upregulated the expression of TNF- α pre-mRNA transcripts after 1 h of Be exposure, and there was an up-regulated expression of both TNF- α pre-mRNA and mature mRNA transcripts after 6 h T cell activation signals that bypass transcription-dependent mRNA synthesis, such as activation-induced pre-mRNA splicing (8) and stimulation of pre-existing mature mRNA nuclear export and translation into protein (9), result in a decrease in the levels of pre-existing pre-mRNA and mature mRNA in the nucleus, unlike the observations made in our study. In the case of activation-induced splicing, pre-mRNA transcript levels decrease as they are spliced into mature mRNA and exported for translation. Gene transcription is not up-regulated during activation-induced splicing. Therefore, new pre-mRNA transcripts are not synthesized at greater rates, resulting in decreased levels measured by quantitative PCR. During direct stimulation of mature mRNA export from the nucleus, the levels of pre-existing mature mRNA begin to fall as transcripts are exported to the cytoplasm for translation. We can envisage that for key pro-inflammatory cytokines, especially those involved in innate immune responses, there could be a significant survival advantage to the rapid upregulation of protein synthesis in the absence of gene transcription.

We tested this hypothesis by including PTX, a transcription inhibitor, or 2AP, a mRNA splicing inhibitor in Be-stimulated CBD BAL cell cultures. In keeping with a transcription-dependent mechanism, PTX treatment (which should block the formation of Be-induced pre-mRNA transcripts) resulted in a significant reduction in the levels of pre-mRNA. As the levels of pre-mRNA are reduced in the presence of PTX, mature mRNA continues to be exported to the cytoplasm, but new pre-mRNA is not available for splicing and thus the levels of mature mRNA decrease. 2AP blocks the splicing of pre-mRNA transcripts. But, as shown previously, TNF- α pre-mRNA transcripts are unstable (11). As Be stimulation continues to drive the synthesis of pre-mRNA transcripts, we observed a slight increase in the levels relative to the profound decrease in levels in the presence of PTX. As these new pre-mRNA transcripts are synthesized, they cannot be spliced in the face of the 2AP splicing block, and due to the instability of the transcripts their levels fall relative to the very high levels of pre-mRNA induced by Be alone. As the levels of Be-induced pre-mRNA transcripts accumulate and are degraded behind the 2AP block, the levels of mature mRNA fall significantly due to the loss of this precursor pool. These observations support our findings that Be-specific TNF- α production occurs in a transcription-dependent manner.

Previous study (7) shows that BAL macrophages from patients with CBD do not upregulate TNF- α mRNA or protein production in response to Be stimulation (7). Thus, changes in TNF- α mRNA levels in unstimulated control CBD BAL cells are likely due to the activation of BAL macrophages as they adhere to the plastic culture dish, whereas changes in TNF- α mRNA levels in Be-stimulated CBD BAL cells result most likely from some level of BAL macrophage activation due to plastic adherence but largely from the MHC class II restricted activation of Be-specific CD4⁺ T cells as previously demonstrated (7). LPS stimulation activates BAL macrophages for TNF- α mRNA and protein production and serves as a positive stimulation control, showing that the patient's BAL sample was able to respond to a disparate activation signal not mediated through the MHC class II molecule.

In comparing the levels of TNF- α pre-mRNA in LPS-stimulated versus Be-stimulated CBD BAL cells, in the presence of

the splicing inhibitor 2AP, TNF- α pre-mRNA levels were greatly increased in the LPS-stimulated cells (median 0.68) versus the Be-stimulated cells (median 0.11). This suggests the possibility that behind the 2AP splicing block, the newly transcribed Be-induced pre-mRNA transcripts were less able to accumulate, whereas the LPS-induced pre-mRNA transcripts made a very large accumulation behind the 2AP block. Based on this observation we hypothesize that in Be-specific T cells, the Be activation signal induces transcription of unstable pre-mRNA transcripts that once formed in the presence of 2AP are quickly degraded and do not accumulate, whereas LPS induces the transcription of stable pre-mRNA transcripts that are not degraded and hence accumulate behind the 2AP block. It is unknown if this represents a fundamental difference in how CBD BAL macrophages and Be-specific T cells synthesize new pre-mRNA transcripts, or alternatively represents differences in how LPS versus Be activation is mediated in these disparate cells. Studies have shown that pre-mRNA transcript stability can be mediated by factors that interact with the 3'UTR in the newly formed transcript (32), an attractive idea that could explain our present observation.

This is the first study to show Be-specific upregulation of nuclear transcription factors that are believed to play a role in TNF- α gene transcription (10). We used consensus oligonucleotides transcription factor probes in our initial survey to determine if Be stimulation would upregulate nuclear levels of a variety of transcription factors demonstrated to be associated with the activation of T cells and known to bind the TNF- α gene promoter. We observed Be-specific increases in the nuclear levels of NF- κ B and AP-1 transcription factors in the isolated BAL T cells from three subjects with CBD who had a lymphocytic alveolitis, increased Be-induced BAL T cell proliferation, and increased BAL cell TNF- α protein production. These observations support our findings that Be-specific TNF- α production occurs in a transcription-dependent manner. NF- κ B has been identified as an important early enhancer of TNF- α gene transcription, as have the AP-1 family members Fos and Jun (33). Due to the limited number of patients with CBD available for study and the requirements of large numbers of cells, our preliminary study has some limitations. We were not able to perform complete kinetic studies of Be-induced transcription factor upregulation, and we had nuclear extract sufficient to perform cold oligonucleotide competition and antibody supershift assay controls on only two of the three subjects with CBD tested. Moreover, we were not able to directly test the involvement of these transcription factors in Be-induced T cell TNF- α gene activation or protein synthesis. Future studies will include sufficient patient numbers to perform a more detailed analysis using nuclear run-on transcription assays or RNA Pol II ChIP assays to provide formal biochemical proof that Be-stimulation induces TNF- α gene transcription. Due to this limitation, it is reasonable to suggest that some, or all, of these transcription factors could mediate the activation of genes encoding other Be-stimulated proteins produced by CBD BAL CD4⁺ T cells—for example, IFN- γ , IL-2, IL-6, and IL-10 (7, 24–28).

We observed that at 0 time, isolated CBD BAL T cells expressed constitutive levels of NF- κ B and AP-1 transcription factors. Twenty-four hours after Be stimulation, we were able to demonstrate the up-regulation in nuclear levels of both transcription factors, an observation that supports our hypothesis that Be stimulation induces TNF- α gene transcription. However, limited by the availability of patient BAL cells from patients with CBD, we were unable to measure the kinetics of Be-induced transcription factor upregulation in isolated T cells. It therefore, remains unclear as to why we were able to detect increased TNF- α mRNA and protein levels as early as 1 h. We speculate

on the possibility that the constitutive levels of these two transcription factors already present in the nuclei of Be-specific CD4⁺ T cells, were able to activate the TNF- α gene for mRNA transcription immediately following Be-stimulation. It is also possible that this early TNF- α protein itself induced an autocrine response upregulating its own expression by enhancing the levels of these transcription factors. Last, CBD is a chronic lung disease characterized by Be-antigen induced amplification of cytokines (1). Bystander T cell activation and the recruitment of activated T cells to the lung in a nonspecific manner could contribute to the observed upregulation of TNF- α protein in the absence of the upregulation of transcription factors to high levels. Our study does not exclude the possibility that there are other cellular sources, in addition to Be-specific CD4⁺ CBD BAL T cells, of Be-induced TNF- α within the complex CBD BAL mixed cell population. For example, studies show that peripheral blood monocytes that newly emigrate into sites of inflammation produce TNF- α (34), and that a dendritic cell subset derived from emigrant blood monocytes are also a potent source of TNF- α (35). Our own studies show that Be-stimulated H36.12j cells produce TNF- α mRNA and protein (36). The H36.12j mouse hybrid macrophage cell line, derived from the fusion of P388D.1 macrophages and protease peptone elicited exudate macrophages from C57Bl/6N mice, has an inflammatory phenotype (37). Beryllium metal has been demonstrated inside the lung granulomas of patients with CBD who had ceased Be exposure an average of 9 yr previously (38). We envisage that newly emigrant blood monocytes, likely destined to differentiate into antigen-presenting dendritic cells, might produce TNF- α in response to Be metal trapped within the CBD granuloma. The present study can only hint at these possibilities, and the temporal relationships between Be exposures and TNF- α protein production by a variety of cells in the granulomatous CBD lung remain unknown. However, our study demonstrates the Be-specific nature of the increase in nuclear levels of TNF- α mRNA and transcription factor upregulation, we believe consistent with the interpretation that Be induces CBD BAL cell TNF- α production in a transcription-dependent manner.

Our study illustrates the importance of conducting translational research using cells obtained from human volunteers. The formation of granulomas in the lungs of patients with CBD is a complex and debilitating process. Our data show that Be-antigen-induced TNF- α production is transcription-dependent, and future strategies aimed at treating this antigen-specific human lung disorder should take this into account. Our data unexpectedly show that CBD BAL cells may synthesize TNF- α pre-mRNA in disparate ways depending on the responding cell class and the activation signal, also an important consideration in therapy for this disease. These studies also demonstrate that Be upregulates nuclear levels of the transcription factors AP-1 and NF- κ B in CBD. Further studies will be required to determine the role of these transcription factors in Be-stimulated TNF- α gene transcription by CD4⁺ CBD BAL T cells.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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