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IL-4 fails to regulate *in vitro* beryllium-induced cytokines in berylliosis

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IL-4 fails to regulate in vitro *beryllium-induced cytokines in berylliosis. L.A. Maier, R.T. Sawyer, S.S. Tinkle, L.A. Kittle, E.A. Barker, R. Balkissoon, C. Rose, L.S. Newman. ©ERS Journals Ltd 2001.*

ABSTRACT: Bronchoalveolar lavage (BAL) cells from patients with chronic beryllium disease (CBD) have been used to evaluate the beryllium-specific immune response and potential immunotherapeutics. Beryllium induces interferon-γ (IFN-γ), interleukin-2 (IL-2), tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-10 (IL-10) from BAL cells. An antibody to IL-2 and recombinant human (rHu) IL-10 is able to partially suppress the beryllium-stimulated immune response. To obtain BAL cells, bronchoscopy is required, providing risk to the patient and a limited number of cells to study the immune response. As a result, the objectives of the study were to determine 1) whether CBD peripheral blood mononuclear cells (PBMNs) stimulated with beryllium would produce a similar cytokine pattern as BAL cells, and 2) whether this response could be modulated by interleukin-4 (IL-4), an immunomodulatory cytokine.

CBD and normal individuals' PBMN and BAL cells were stimulated with and without beryllium sulfate. To modulate this antigen-stimulated response, we added rHu IL-4 to the unstimulated and beryllium-stimulated cells. IFN-γ, IL-2, TNF-α, IL-6 and IL-10 cytokine concentrations were determined from cell supernatants by enzyme-linked immunosorbent assays (ELISA), while IL-4 messenger ribonucleic acid (mRNA) was assessed using polymerase chain reaction (PCR).

Beryllium did not stimulate any of these cytokines from normal PBMNs. Increasing levels of IL-6 and TNF-α were produced constituitively by CBD PBMNs over time. Compared to the unstimulated CBD PBMNs, beryllium stimulated significant IFN-γ, TNF-α, IL-2, IL-6 and IL-10 production. This response was similar to that stimulated from CBD BAL cells, although of a much lower magnitude. Low levels of IL-4 mRNA were found in CBD and control PBMNs, which were not increased with beryllium stimulation. The beryllium-stimulated cytokine levels were not decreased by the addition of IL-4. IL-4 was unable to downregulate any of these beryllium-stimulated cytokines from CBD BAL cells or increase IL-4 mRNA from either CBD PBMN or BAL cells, and thus is an unlikely immunomodulatory agent in CBD.

From the data, it was concluded that chronic beryllium disease peripheral blood mononuclear cells provide a model to study the beryllium-stimulated immune response. Interleukin-4's inability to downregulate any of the beryllium-stimulated cytokines makes it an unlikely therapeutic candidate in chronic beryllium disease.

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Chronic beryllium disease (CBD) is a granulomatous lung disease that results from exposure to beryllium in the workplace [1]. The current understanding of the disease leads to the belief that individuals with CBD first develop a sensitization to beryllium, demonstrated by a positive peripheral blood beryllium lymphocyte proliferation test (BeLPT) [1, 2]. Some of these individuals eventually develop CBD, which is defined by the presence of a positive blood or bronchoalveolar lavage (BAL) BeLPT, plus granulomatous inflammation on lung biopsy [1, 2].

In CBD and other granulomatous lung diseases such as sarcoidosis, mixed bronchoalveolar lavage cells have been used as a model to evaluate the cellular immune response [3–5]. It appears that the pathogenesis of CBD begins with alterations in alveolar/capillary permeability [6] and lung recruitment of beryllium-specific $CD4^+$ T cells [7–9] and activated macrophages [10]. These beryllium antigen-specific T cell clones proliferate in response to beryllium [11, 12], while cells from other granulomatous diseases do not [2, 8]. CBD BAL cells produce a number of cytokines, which probably act as key regulators in granulomatous inflammation [3, 13, 14]. Specifically, beryllium induces high BAL cell production of interferon-γ (IFN-γ), and lower levels of interleukin-2 (IL-2) [3]. Interleukin-4 (IL-4), which is important in determining the Th1 *versus* Th2 cytokine pattern [15], is notably absent from

CBD BAL cells and is not induced by beryllium stimulation [3, 16]. While low levels of interleukin-10 (IL-10) are stimulated by beryllium, this cytokine may play a counter regulatory role in CBD, as exogenous IL-10 is able to partially downregulate the production of beryllium-induced cytokines [13]. Finally, the proinflammatory macrophage product tumour necrosis factor-α (TNF-α), which is associated with the development of florid granulomas [17] and interleukin-6 (IL-6), which is important in T cell activation and commitment to a Th2 cytokine profile [18], are highly upregulated in CBD beryllium-stimulated BAL cells [10, 14].

Although the use of BAL has improved understanding of the immune response in this granulomatous disease, it is an invasive procedure and provides only a limited number of cells with which to study the CBD immune response. While BAL is critical in the diagnostic evaluation of a patient with suspected CBD, a less invasive model is desired to study the cellular immune response in CBD. In CBD, beryllium-responsive cells are present in the peripheral blood as evidenced by incorporation of tritiated thymidine upon stimulation with beryllium in the BeLPT [19] and a positive beryllium skin patch test [20, 21]. Thus, it may be possible to dissect the cellular immune response to beryllium using peripheral blood cells as a model. It is hypothesized that beryllium stimulates a cytokine response from peripheral blood mononuclear cells (PBMNs), in a manner analogous to that found in BAL cells.

Furthermore, it is hypothesized that the beryllium-induced cytokine response can be modulated by cross-regulatory cytokines. IL-4 has been used *in vivo* in animal models of Th1-mediated diseases, including autoimmune encephalitis [22] and inflammatory bowel disease [23], to incite a Th2 predominant cytokine immune response and disease amelioration. It has also been found to reduce inflammation, cellular infiltration, and TNF-α production in murine arthritis [24] and human rheumatoid arthritis PBMNs [25]. In numerous cell models, IL-4 has been found to downregulate proinflammatory cytokines such as TNF-α [26, 27], along with the T cell cytokines IFN-γ and IL-2 [28, 29]. Based on these observations, it was hypothesized that absence or low levels of IL-4 present in CBD may help explain the predominant Th1 cytokine response, with high levels of IFN-γ and low levels of IL-10. In addition, exogenous recombinant IL-4 would result in a switch to a prominent Th2 cytokine response *in vitro*.

This study evaluated beryllium-stimulated product of IFN-γ, IL-2, IL-6, IL-10 and TNF-α in PBMNs from normal and CBD patients and the ability of recombinant human IL-4 to modulate this antigen-specific cell-mediated immune response in CBD PBMNs and BAL cells.

Materials and methods

Study populations

Chronic beryllium disease subjects. CBD was defined by the presence of 1) an abnormal blood beryllium sulphate. ($BeSO_4$) lymphocyte proliferation test (BeLPT) and/or BAL BeLPT, indicative of a $BeSO_4$-specific immune response and 2) histological evidence of granulomas or mononuclear cell infiltrates on lung biopsy, usually obtained *via* transbronchial biopsy [1]. Twenty-two subjects were enrolled into this study who met this case definition and were evaluated in the Occupational and Environmental Medicine Clinic at National Jewish Medical and Research Center (Denver, CO, USA). Thirteen were recruited for the peripheral blood experiments and five for the BAL experiments alone, while four were enrolled in both the peripheral blood and BAL experiments.

Control subjects. Ten control subjects were enrolled into the peripheral blood study, who had no known exposure to $BeSO_4$ and no respiratory symptoms. One individual had mild asthma and was using inhaled corticosteroids.

Informed consent was obtained from all subjects according to our institution′s Human Subjects Review Board. Demographic characteristics, including age and sex, smoking status, and use of immunosuppressants, were obtained from all of the CBD cases. A "never smoker" was defined as someone who had smoked fewer than 20 packs of cigarettes in their lifetime (table 1).

Study design

The cytokine protein response, including IFN-γ, TNF-α, IL-2, IL-10 and IL-6, were assessed over time in unstimulated, beryllium ($BeSO_4$)-stimulated, *Salmonella typhimurium* lipolysaccharide (LPS) (Sigma, St. Louis, MO, USA) and phytohaemagglutanin-stimulated (PHA) (Sigma) PBMN and BAL cell supernatants. PHA was used as a positive control of T cell cytokine stimulation, while LPS was used as a positive control of monocyte cytokine stimulation. Recombinant human IL-4 (rHu IL-4) was added for 30–60 min before stimulation with the above agents. Interleukin-4 mRNA was evaluated in the rHu IL-4 treated unstimulated and $BeSO_4$-stimulated PBMNs and BAL cells. Timepoints resulting in maximal cytokine levels were used to evaluate the cytokine production in the PBMN and BAL supernatants, because of the complexity of the experimental design and necessity for large cell numbers. Because of limited number and availability of BAL cells, four CBD subjects′ cells were used in both the PBMN and BAL experiments; ideally, paired data would have been available on all subjects and controls for both BAL and PBMN cells. The number and type of subjects included in each experiment is indicated in the text and figure legends.

Reagents

Cell culture reagents used in this study included complete media consisting of Roswell Park Memorial Institute (RPMI) 1640 (Biowittaker, Walkersville, MD, USA), 10% heat inactivated iron supplemented calf serum (Hyclone, Logan, UT, USA), 0.29 $mg \cdot mL^{-1}$

Table 1. – Demographic characteristics, smoking status, and use of corticosteroids for the subjects enrolled in the peripheral blood and bronchoalveolar lavage studies

	Peripheral blood		Bronchoalveolar lavage
	Control subjects (n = 10)	CBD subjects* (n = 17)	CBD subjects (n = 9)
Mean ± SD age, yr	34.6 ± 9.0	52.0 ± 2.0	47 ± 7.0
Sex male/female	4/6	13/4	7/2
Smoking status n			
Never	9	7	6
Former	1	9	2
Current	0	1	1
Prednisone use			
Yes/no	0/10	8/9	2/7
Inhaled steroids			
Yes/no	1/10	4/13	4/5
Other Immunosuppressive therapy			
Yes/no	0/10	1/16: Methotrexate	2/7: Methotrexate pentoxifylline

*: 4 subjects were included in the peripheral blood and bronchoalveolar lavage experiments.

L-glutamine, 100 U·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin (Biowittaker, Walkersville, MD). Beryllium sulphate was maintained as stock solutions of 100 and 1000 μM $BeSO_4$ in water, and diluted 1:10 during cell culture for a final concentration of 10 and 100 μM. rHu IL-4 was purchased from R & D Systems (Minneapolis, MN, USA). The undiluted protein preparation contained <0.1 ng LPS per μg of undiluted cytokine, as measured by the supplier. PHA and LPS were used as positive controls for T cell and macrophage cytokine production. All reagents and plasticware used in this study were free of LPS contamination as measured using the Limulus Amebocyte Lysate Assay (Associates of Cape Cod, Woods Hole, MA, USA). The limit of detection of this assay is 0.025 μg·mL^{-1} LPS.

Sample collection

Peripheral blood mononuclear cells. Venous blood was obtained in heparinized tubes from CBD and control subjects. Mononuclear cells were isolated using Ficoll-Paque® (Pharmacia Biotech AB, Uppsala, Sweden) density centrifugation, washed three times in balanced salts solution, and resuspended in complete media as previously described [7, 19]. The mixed cell population was counted and evaluated for cell viability by trypan blue dye exclusion. The cells were resuspended at 2.5×10^6·mL^{-1}. An automated complete blood cell count and differential were obtained and evaluated by standard commercial laboratory methods for polymorphonuclear leukocytes, lymphocytes, and monocytes. This cell count was used to approximate the cell differential of the PBMNs. These cells provided a simplified model of the mixed BAL cell population (see table 2).

Bronchoalveolar lavage cells. BAL was performed on 9 CBD subjects as previously described [7]. Briefly, aliquots of 60 mL sterile normal saline, at room temperature, were instilled into a segment of the right middle or lower lobe, for a total of 240 mL of saline. The fluid was retrieved by gentle hand suctioning and the aliquots of recovered fluid were combined and mixed gently. The BAL fluid and cells were centrifuged at $5000 \times g$ for 10 min at 4°C to form a pellet. The BAL fluid was removed and the mixed cell pellet was resuspended in media. The mixed BAL cells were counted, evaluated for cell viability by trypan blue dye exclusion, and resuspended at 1×10^6 cells·mL^{-1}. Cell differential counts were obtained for lymphocytes, macrophages, eosinophils and neutrophils, and reported as total cell number per mL and percentages of the total cell count (table 2).

Culture of bronchoalveolar lavage and peripheral blood mononuclear cells. The PBMNs were suspended at 2.5×10^6 cells·mL^{-1}. The BAL cells were suspended at 1×10^6 cells·mL^{-1}. The cells were then cultured in 96 well flat bottom plates (Becton Dickinson, Lincoln Park, NJ, USA) in the presence or absence of 10 or 100 μM $BeSO_4$ for 0, 24, 72, or 120 h at 37°C, in 5% CO_2. In some experiments, cells were treated with 10 or 50 ng·mL^{-1} of rHu IL-4 for 30 – 60 min at 37°C, in 5% CO_2 before the addition of the $BeSO_4$. The concentration of rHu IL-4 [30, 31] and preincubation [27] time used were based on those found to be effective in altering the Th1 cytokine response in previous studies. Following rHu IL-4 treatment, PBMNs were unstimulated, PHA-stimulated (10 μg·mL^{-1}), LPS-stimulated (1 μg·mL^{-1}) or $BeSO_4$-stimulated (10 or 100 μM). Culture supernatants were harvested at the indicated intervals and the cells removed by centrifugation at 1,500 rpm, at room temperature for 5 min. The harvested, cell-free culture supernatants were stored at -20°C until use. The harvested cells were suspended and lysed in 500 μL·L $\times 10^6$ cells of 4 M guanidine isothiocyanate (Gibco/BRL, Grand Island, NY, USA) plus 8 per cent (volume per volume) 2-mercaptoethanol (Sigma) (GN-2ME). The cell lysates were stored at -80°C until use.

Table 2. – Blood and bronchoalveolar lavage cell counts, and beryllium lymphocyte proliferation test in chronic beryllium disease (CBD) subjects participating in the peripheral blood and bronchoalveolar lavage studies. Data presented are median (interquartile range)

	CBD PBMN group (n = 17)	CBD BAL group (n = 9)
Blood peak BeLPT, SI	5.8 (3.2 – 14.5)	12.6 (4.2 – 91.6)
Blood WBC count ($\times 10^6 \cdot mL^{-1}$)	6.5 (4.9 – 8.0)	6.5 (5 – 7.9)
Polymorphonuclear leukocyte % of total	63.5 (57.5 – 69.5)	65 (62 – 80)
Lymphocytes % of total	27 (19.5 – 29)	22 (9 – 28)
Monocyte % of total	5 (3 – 7)	7 (3 – 9)
BAL peak BeLPT, SI	44.6 (26.3 – 174.6)	58.4 (7.6 – 177.1)
BAL WBC, absolute ($\times 10^6$)	56.0 (32.3 – 74.1)	54.6 (37.85 – 88.5)
BAL macrophages % of total	53 (34 – 62)	53 (36 – 64.5)
BAL lymphocytes % of total	46 (37.5 – 65)	46 (33 – 60)

BeLPT: beryllium lymphocyte proliferation test; SI: stimulation index; WBC: white blood cell; BAL: bronchoalveolar lavage; PBMN: peripheral blood mononuclear cell.

Cytokine measurements in cell culture supernatants

Quantitative levels of IFN-γ, IL-2, IL-6, TNF-α and IL-10 cytokines were determined using two-site sandwich enzyme linked immunosorbent assays (ELISA). Quantikine Kits (R & D Systems) or matched antibody pairs kits (R & D Systems) were used to measure IFN-γ, IL-2, IL-6 and TNF-α per the manufacturers′ instructions. The minimum detection limits for these assays were 3.0 $pg \cdot mL^{-1}$, 7.0 $pg \cdot mL^{-1}$, 4.4 $pg \cdot mL^{-1}$ and 7.8 $pg \cdot mL^{-1}$ for IFN-γ, IL-2, TNF-α, and IL-6, respectively. The IL-10 ELISA was prepared as an ELISA supplied by Schering-Plough (Kenilworth, NJ, USA). Briefly, a 96 well plate was coated with 1 $\mu g \cdot mL^{-1}$ protein G purified rat monoclonal IL-10 antibody (Schering-Plough), incubated at 4°C overnight, washed three times with 0.05% Tween 20 in phosphate buffered saline (PBS), and blocked overnight at 4°C in 1% bovine serum albumin (BSA) with 0.05% sodium Azide in PBS. One hundred microlitres of sample or standard (625 $pg \cdot mL^{-1}$ diluted 1:2 to 9.76 $pg \cdot mL^{-1}$) was added and incubated overnight at 4°C. The plate was washed four times as above, and a 1:2,500 dilution of immunoglobulin-G (IgG) purified rabbit polyclonal antibody (Schering-Plough) was added for 2 h. The plate was washed four times and 100 μL of 1:10,000 dilution of peroxidase-conjugated goat anti-rabbit IgG was added for 2 h. The plate was washed four times and 100 μL 3′,5,5′ Tetramethylbenzadine (TMB) substrate solution (BioRad, Herculies, CA, USA) was added for 15 min. The reaction was stopped by adding 50 μL of 2 M H_2SO_4. The ELISA was read using a dual wavelength of 450 nm/590 nm. The minimum detection limit of the IL-10 ELISA was 10.0 $pg \cdot mL^{-1}$. Results are presented as the mean of duplicates, expressed in $pg \cdot mL^{-1}$ of cytokine.

Quantification of cytokine messenger ribonucleic acid transcripts using reverse transcriptase polymerase chain reaction

Cellular RNA was isolated from the cell lysates in GN-2ME using the Glass MAX RNA microisolation spin cartridge system (Life Technologies, Inc., Gaithersburg, MD, USA) as previously described [3]. Reverse transcription was initiated using an oligo dT primer and the avian myoblastosis virus reverse transcriptase in a 20 μL reaction (Promega, Madison, WI, USA).

Primers for β-actin and IL-4 were obtained from Clontech (Palo Alto, CA, USA). The PCR reactions for β-actin and IL-4 were performed simultaneously for each condition and timepoint, to minimize variability. Positive controls (Clontech) were used for each primer to ensure the specificity of the PCR reaction. Complementary deoxyribonucleic acid (cDNA) was amplified for 35 cycles with 10 s at 94°C for denaturation, 45 s at 60°C for re-annealing, 2 min at 72°C for extension per cycle, and a 7 min final extension phase for both β-actin and IL-4, as optimized by the manufacturer. For all PCR amplification reactions, the amount of cDNA per reaction was standardized to 2 μg as determined using a Beckman DU-64 spectrophotometer (Beckman Instruments, Fullerton, CA, USA). A 50 μL PCR reaction, containing 2 μg cDNA, 1 mM $MgCl_2$, 1 mM deoxyribonucleoside triphosphate (dNTP), 5 μL 10 × Taq PCR buffer (Perkin Elmer, Norwalk, CT, USA), 0.4 μM primers, and 2 units of Taq (Perkin Elmer) was used for semi-quantitative PCR. For the BAL samples, 2 μL of ^{32}P-α-d cytosine triphosphate (CTP) (New England Nuclear Research Products, Boston, MA, USA) was added to the PCR reactions. Twenty-five μL of the PCR product and 10 μL of a 100 base pair ladder (Promega, Madison, WI, USA) was electrophoresed on a 2.0% (weight/volume) agarose gel and visualized by ethidium bromide (EtBr) staining. The area corresponding to the IL-4 and β-actin positive control bands at 344 and 838 base pairs were respectively excised and ^{32}P-α-d CTP incorporation was measured by scintillation counting. The advent of new methodology employing the fluorescent dye Vistra Green (Amersham Life Science, Buckinghamshire, England) permitted us to substitute a more sensitive detection method to measure IL-4 mRNA in CBD PBMNs. Therefore, the data presented for PBMNs employs Vistra Green staining as described by Hamada *et al.* [32]. PBMN cDNA was electrophoresed as above, and the gel incubated at 4°C for 1 h in 0.01% Vistra

Green. Fluorescence was detected using a Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA) and semi-quantitative analysis of the cDNA was performed using Image Quant software (Molecular Dynamics). Ratios of the IL-4 *versus* β-actin mRNA using CPM for ^{32}P-α-d CTP incorporation or densitometry counts for Vistra Green staining provide semi-quantitative analysis. mRNA methods using ethidium bromide staining detects approximately 1–5 ng of DNA [33], Vistra Green detects approximately 10–20 pg while ^{32}P-α-d CTP incorporation is of intermediate sensitivity. These methods have provided comparable results in the authors' laboratory.

Lymphocyte proliferation

The blood and BAL BeLPTs were performed according to the methods of Mroz *et al.* [19]. The counts per minute (cpm) from each set of quadruplicates is meaned and expressed as a ratio of the cpm of the beryllium-stimulated cells to the unstimulated cells (stimulation index, (SI)) [19]. The peak SI is reported for PBMN and BAL cells. All test results used in this study were obtained within 12 months of enrollment.

Statistical analysis

Cytokine responses measured below the minimum detection level of the ELISA were assigned the minimum detectable concentration value and used in the statistical analysis. Because the data were not normally distributed, for purposes of statistical analysis the cytokine levels were log transformed, which provided a more normal distribution. Repeated measures analysis of variance (ANOVA) was used to measure each cytokine response and lymphocyte proliferation separately in each study, across time and treatment condition. When differences across time or condition were found to be significant, Tukey-Kramer multiple comparisons were used to make each pairwise contrast between groups, timepoints and treatment conditions. Cytokine, lymphocyte proliferation and BAL and PBMN cell values are expressed as medians, with interquartile ranges (IQR). Wilcoxon Rank-Sum and Spearman's correlation were used to compare demographic and clinical variables, cell counts and cytokine levels at one time. Statistical significance was defined as $p < 0.05$.

Results

Beryllium stimulated peripheral blood mononuclear cell proliferation and cytokine responses

We measured the cytokine levels in the culture supernatants of PBMNs from 17 CBD patients and 10 control subjects after stimulation (figs. 1–5) with and without $BeSO_4$. The one control with asthma displayed a similar cytokine response to the remainder of the controls.

Unstimulated PBMNs from CBD and control subjects produced levels of IFN-γ and IL-2 at or below the detection limit of the assay over time (figs. 1 and 2). Beryllium sulphate did not stimulate the production of IFN-γ or IL-2 from control PBMN cells. However, CBD PBMNs stimulated with 100 μM $BeSO_4$ produced elevated levels of IFN-γ detected by 24 h, which remained elevated for up to 120 h of culture (fig. 1). The levels of IFN-γ produced at 24 (median 14.0 pg·mL^{-1} IQR [3.0–45.5]), 72 (150 pg·mL^{-1} [94.2–262.3]), and 120 h (325.0 pg·mL^{-1} [101.6–650.0]), differed significantly over time from that produced by the unstimulated CBD cells ($p < 0.01$). In CBD PBMNs, $BeSO_4$ stimulated low levels of IL-2, which peaked at 24 h (23.2 pg·mL^{-1} [8.5–46.0]), and began to decline by 72 h (13.0 pg·mL^{-1} [7.0–73.45]), returning to baseline levels by 120 h (7.0 pg·mL^{-1}

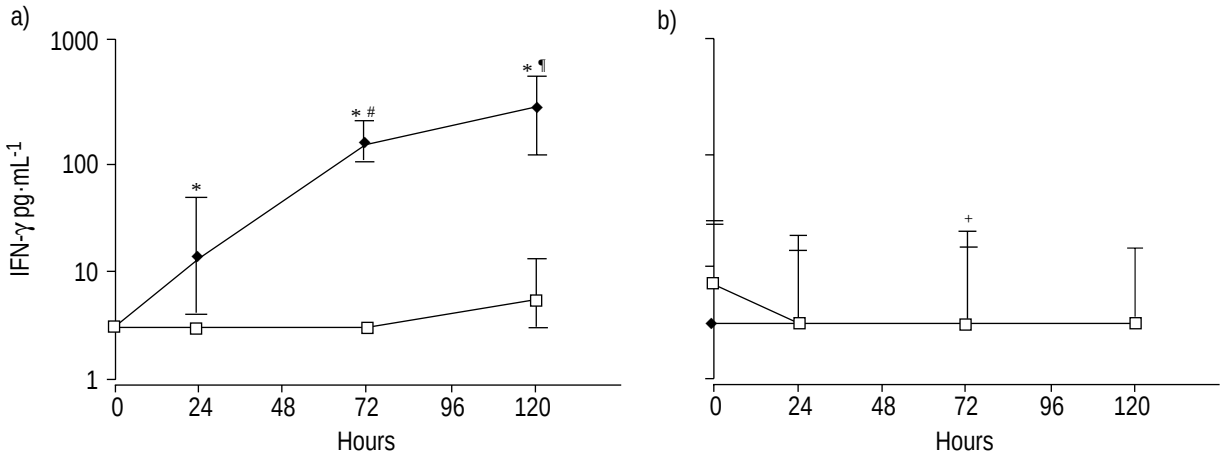

Fig. 1. – Peripheral blood mononuclear cells (PBMNs) were cultured in the absence (□) or presence (◆) of 100 μM beryllium sulphate for up to 120 h. Data presented are medians with interquartile range. a) Beryllium sulphate stimulates significant interferon-γ (IFN-γ) from chronic beryllium disease (CBD) PBMNs (n = 17). b) Beryllium sulphate does not stimulate IFN-γ from control PBMNs (n = 10). *: denotes a significant difference comparing the unstimulated and beryllium-stimulated PBMNs at the indicated time; #: indicates a difference between the 24 and 72 h beryllium-stimulated times; ¶: indicates a difference between the 24 and 120 h times. +: denotes a significant difference comparing the beryllium-stimulated CBD and control PBMNs ($p < 0.05$).

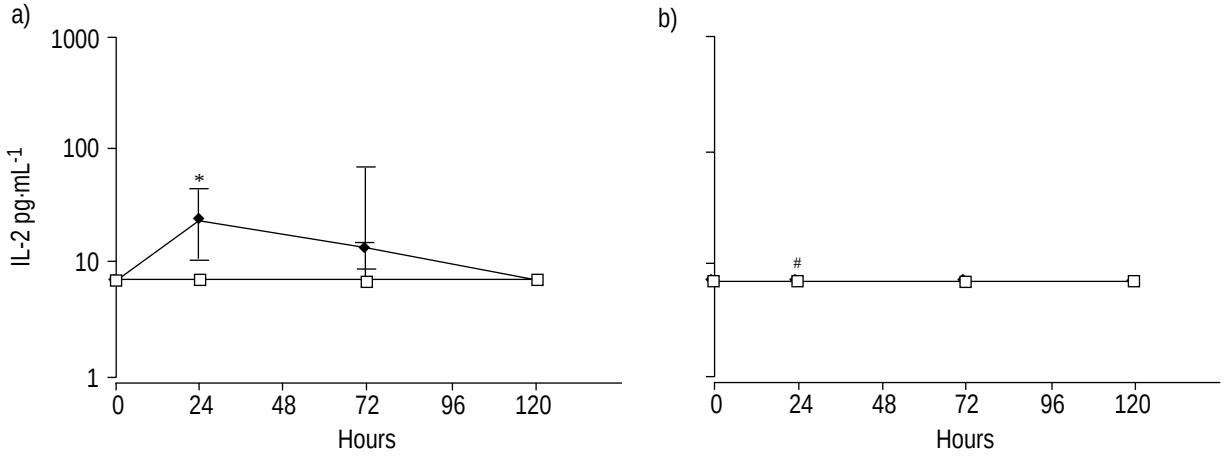

Fig. 2. – Peripheral blood mononuclear cells (PBMNs) were cultured in the absence (□) or presence (◆) of 100 μM beryllium sulphate for up to 120 h. Data presented are medians with interquartile range. a) Beryllium sulphate stimulates significant interleukin-2 (IL-2) from chronic beryllium disease (CBD) PBMNs (n = 17). b) Beryllium does not stimulate IL-2 from control PBMNs (n = 10). *: denotes a significant difference comparing the unstimulated and beryllium-stimulated PBMNs at the indicated time. #: denotes a significant difference comparing beryllium-stimulated control and CBD PBMNs at the indicated time ($p < 0.05$).

[7.0 – 7.0]) (fig. 2). The low median levels partially reflect the lack of IL-2 production by two individuals, as some individuals produced up to 350 pg·mL^{-1}. The levels of IL-2 produced at 24 h by $BeSO_4$-stimulated CBD PBMNs differed significantly from unstimulated CBD PBMNs ($p < 0.01$).

Constitutive levels of IL-6 were produced by unstimulated CBD PBMNs by 72 – 120 h of culture (33.0 pg·mL^{-1} [21.0 – 184.0]). This differed significantly from unstimulated control subjects′ cells, which produced negligible IL-6 (7.8 pg·mL^{-1} [7.8 – 17.0], $p = 0.0002$) (fig. 3). Control subjects′ PBMNs did not produce significant levels of IL-6 following $BeSO_4$ stimulation. Beryllium sulphate-stimulated release of IL-6 peaked at 24 h (30.5 pg·mL^{-1} [13.0 – 54.7]) from CBD PBMNs, and remained elevated up to 120 h (46.2 pg·mL^{-1} [25.1 – 107.42]). However, beryllium stimulation only significantly increased the production of IL-6 above constitutive levels in unstimulated CBD PBMNs at 24 h (7.8 pg·mL^{-1} [7.8 – 12.0], $p = 0.045$).

As noted with IL-6, unstimulated CBD PBMNs produced constitutive levels of TNF-α by 72 h (30.0 [10.0 – 91.9]), which increased further by 120 h (302.5 pg·mL^{-1} [114.8 – 652.0]) compared to 24 h (8.0 pg·mL^{-1} [4.4 – 19.45], $p < 0.0001$) (fig. 4). This constitutive production of TNF-α was not a result of endotoxin contamination in culture as determined by the Limulus Amebocyte Lysate Assay. TNF-α was not apparent in unstimulated or stimulated control cells over time. Beryllium sulphate-stimulated TNF-α production from CBD PBMNs increased by 24 h (59.2 pg·mL^{-1} [12.5 – 99.5]), and continued to increase slowly up to 72 h (166.0 pg·mL^{-1} [70.9 – 273.0]) and 120 h of culture (270.0 pg·mL^{-1} [180.0 – 383.9], $p < 0.0001$). Because of the spontaneous TNF production, the TNF-α expression achieved statistical

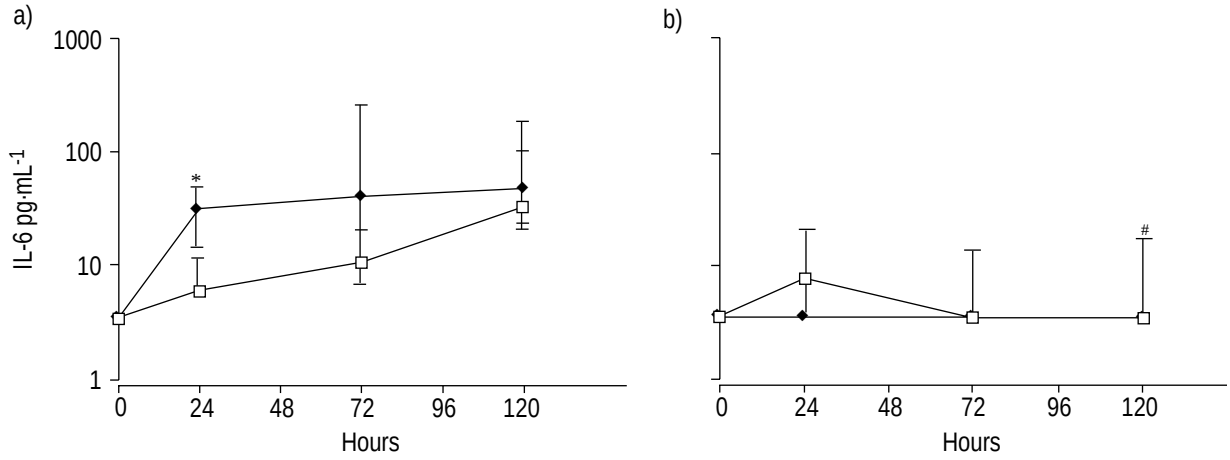

Fig. 3. – Peripheral blood mononuclear cells (PBMNs) were cultured in the absence (□) or presence (◆) of 100 μM beryllium sulphate for up to 120 h. Data presented are medians with interquartile range. a) Beryllium sulphate stimulates significant interleukin-6 (IL-6) from chronic beryllium disease (CBD) PBMNs (n = 9). b) Beryllium does not stimulate IL-6 from control PBMNs (n = 10). *: denotes a significant difference comparing the unstimulated and beryllium-stimulated PBMNs at the indicated time. #: denotes a significant difference comparing beryllium-stimulated control and CBD PBMNs at the indicated time ($p < 0.05$).

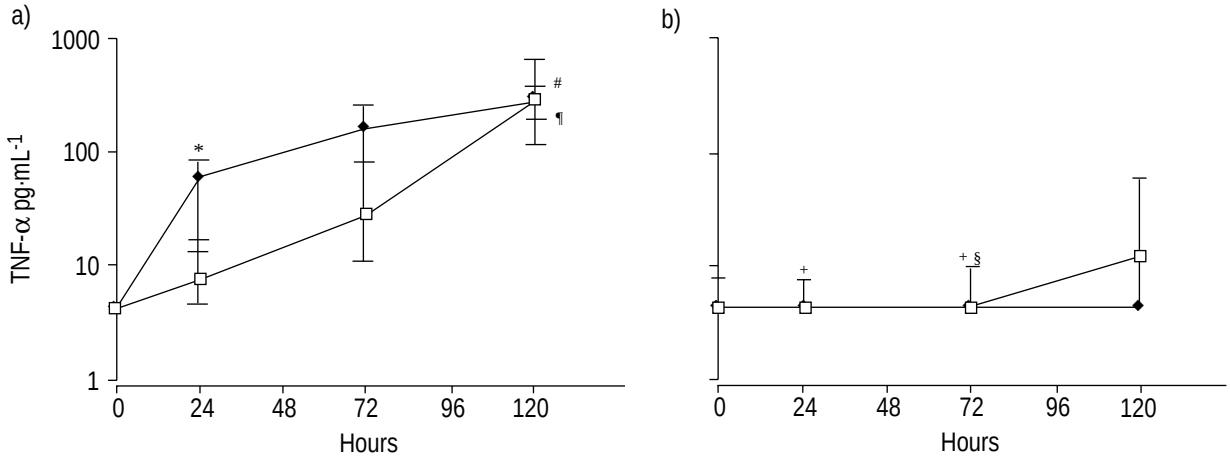

Fig. 4. – Peripheral blood mononuclear cells (PBMNs) were cultured in the absence (□) or presence (⧫) of 100 μM beryllium sulphate for up to 120 h. Data presented are medians with interquartile range. a) Beryllium sulphate stimulates significant tumour necrosis factor-α (TNF-α) from chronic beryllium disease (CBD) PBMNs (n = 17). b) Beryllium does not stimulate TNF-α from control PBMNs (n = 10). *: denotes a significant difference comparing the unstimulated and beryllium-stimulated PBMNs at the indicated time; #: indicates a difference between the 24 and 120 h beryllium-stimulated times; ¶: indicates a difference between the 24 and 120 h unstimulated times. +: denotes a significant difference comparing beryllium-stimulated control and CBD PBMNs at the indicated time; §: indicates a significant difference between unstimulated control and CBD PBMNs ($p < 0.05$).

significance between unstimulated and $BeSO_4$-stimulated CBD PBMNs at 24 h ($p < 0.01$) of culture.

By 120 h of culture, the unstimulated CBD subjects' PBMNs produced low but elevated levels of IL-10 in culture (25.3 pg·mL^{-1} [12.0 – 40.2], $p < 0.01$) compared to the unstimulated control PBMNs (fig. 5). Beryllium sulphate stimulated low but statistically significant IL-10 production from CBD PBMNs at 72 h only (35.33 pg·mL^{-1} [10.00 – 77.0], compared to the unstimulated CBD cells (10.0 pg·mL^{-1} [10.0 – 39.21], $p = 0.02$). The control subjects did not express any IL-10 above the minimum detection of the ELISA in either unstimulated or $BeSO_4$-stimulated cultures.

Messenger-RNA levels were determined for IL-4. Low levels of IL-4 mRNA were detected in some CBD (n = 5) and control PBMNs (n = 6), difficult to appreciate on ethidium bromide staining (fig. 6), while no IL-4 was found in others. The IL-4 mRNA, expressed as a ratio of β-actin production, did not differ over time, with or without beryllium stimulation or between CBD and control PBMNs ($p > 0.05$).

Relation of peripheral blood mononuclear cell cytokine production to demographics, beryllium lymphocyte proliferation test and blood cell count

To determine whether the peak beryllium-stimulated cytokine levels were affected by, or associated with, steroid use, tobacco smoking, peripheral blood cell count or BeLPT (table 2), these characteristics in our CBD cases were compared to IL-2 and TNF-α production at 24 h, and to IFN-γ and IL-10 production

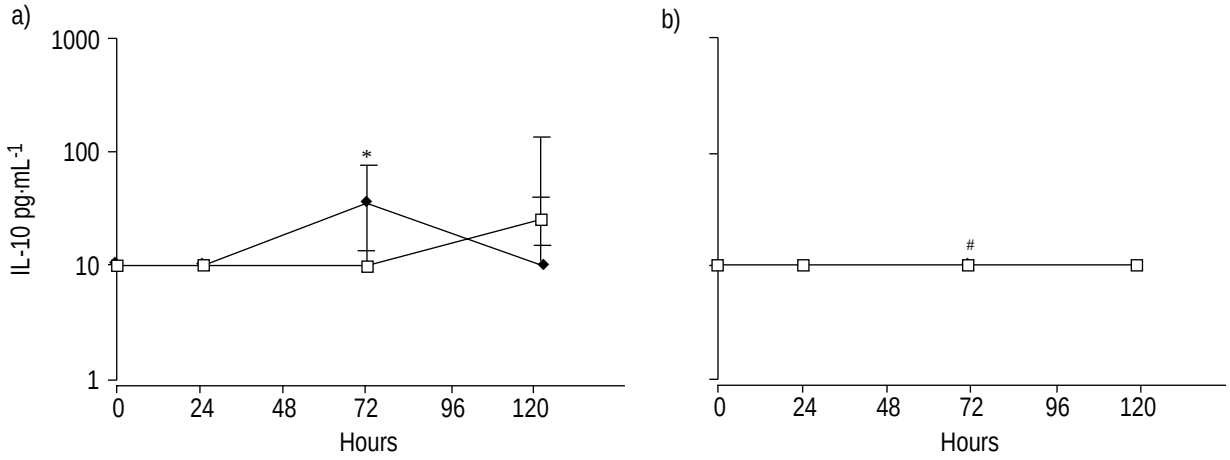

Fig. 5. – Peripheral blood mononuclear cells (PBMNs) were cultured in the absence (□) or presence (⧫) of 100 μM beryllium sulphate for up to 120 h. Data presented are medians with interquartile range. a) Beryllium sulphate stimulates significant interleukin-10 (IL-10) from chronic beryllium disease (CBD) PBMNs (n = 10). b) Beryllium does not stimulate IL-10 from control PBMNs (n = 10). *: denotes a significant difference comparing the unstimulated and beryllium-stimulated PBMNs at the indicated time. #: denotes a significant difference comparing beryllium-stimulated control and CBD PBMNs at the indicated time ($p < 0.05$).

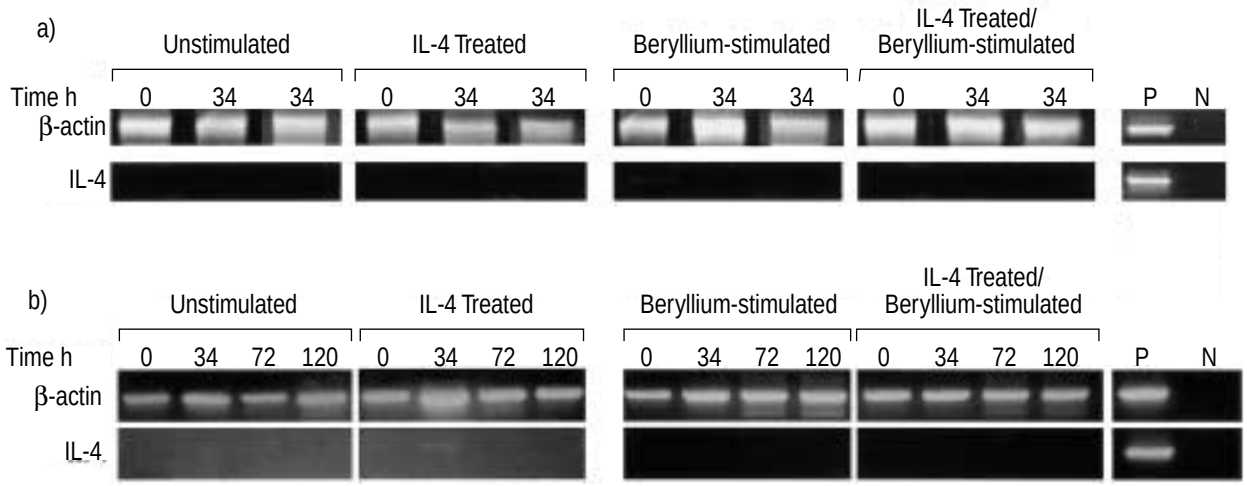

Fig. 6. – Interleukin-4 (IL-4) messenger ribonucleic acid (mRNA) production from chronic beryllium disease (CBD) and control peripheral blood mononuclear cells (PBMNs) and CBD bronchoalveolar lavage (BAL) cells, expressed as a ratio of β-actin mRNA. Cells were cultured in the presence or absence of beryllium sulphate (100 μM) with or without IL-4 (10 ng·mL^{-1}) pretreatment for up to 120 h of culture. Total ribonucleic acid was extracted, reverse transcribed and subjected to polymerase chain reaction (PCR). PCR products were analysed on a 2% agarose gel. a) A representative time course for IL-4 and β-actin mRNA from a CBD subject's PBMN cells. Similar results were apparent from other CBD and normal subjects. b) A representative time course for IL-4 and β-actin mRNA from one CBD subject's BAL cells. Similar results were apparent from the other CBDs' BAL cells. P: positive control; N: negative control.

at 72 h of culture. Neither steroid use nor smoking were statistically significantly associated with the level of cytokine production. However, a trend was apparent in the levels of TNF-α and steroid use, such that those individuals treated with steroids had lower PBMN TNF-α levels (14.0 pg·mL^{-1} [6.8 – 37.2] in steroid users *versus* 94.5 pg·mL^{-1} [15.0 – 200] in nonsteroid users, p = 0.07). A similar trend was apparent for IFN-γ (122.1 pg·mL^{-1} [6.0 – 198.8] in steroid users *versus* 200 pg·mL^{-1} [99.9 – 900] for nonsteroid users, p = 0.269), IL-2 (14.3 [6.4 – 25.0] in steroid users *versus* 38.9 pg·mL^{-1} [7.0 – 162.2] for nonsteroid users, p = 0.144), and IL-6 (13.0 [7.0 – 30.5] in steroid users *versus* 52.2 pg·mL^{-1} [21.0 – 158.9] for nonsteroid users, p = 0.156), although none met our criterion for statistical significance. The TNF-α production correlated with the peripheral blood BeLPT (Spearman's correlation coefficient r = 0.54, p = 0.03) and with the peripheral blood monocyte count (r = 0.58, p = 0.03).

Interleukin-4 is unable to downregulate beryllium-stimulated cytokines from chronic beryllium disease peripheral blood mononuclear cells

To determine whether IL-4 is able to modulate the $BeSO_4$-stimulated PBMN cytokine response, CBD (n = 10) and control PBMNs (n = 10) were preincubated in the presence or absence of 10 or 50 ng·mL^{-1} of rHu IL-4 before stimulation with or without 1) $BeSO_4$ (100 μM), 2) PHA (10 μg·mL^{-1}), or 3) LPS (1 μg·mL^{-1}). Because of the kinetics noted above, IL-2 and TNF-α production were evaluated after 24 h and IFN-γ and IL-10 after 72 h of culture. Since similar results were found with both rHu IL-4 at 10 and 50 ng·mL^{-1}, data were presented using the 10 ng·mL^{-1} rHu IL-4 concentration. Stimulation of CBD PBMNs with PHA resulted in significant IL-2 (1,087 pg·mL^{-1} [170 – 2,863] at 24 h, $p < 0.01$) and IFN-γ production (8.927 pg·mL^{-1} [6,228 – 12,548] at 72 h, $p < 0.01$) compared to unstimulated cells. Lipolysaccarhide stimulation produced significant TNF-α (1,420 pg·mL^{-1} [1,108 – 3,671] at 24 h, $p < 0.01$) and IL-10 (704 pg·mL^{-1} [499 – 1,165] at 72 h, $p < 0.01$) from CBD PBMNs compared to the unstimulated condition. Treatment of PHA-stimulated CBD PBMNs with rHu IL-4 did not decrease IFN-γ production (for PHA alone *versus* 12,184 pg·mL^{-1} [9,706 – 14,044] for PHA + rHu IL-4). rHu IL-4 increased PHA-stimulated CBD IL-2 production (2,270 pg·mL^{-1} [944 – 3,922], $p < 0.01$) and reduced LPS-stimulated TNF-α from CBD PBMNs (463 pg·mL^{-1} [402 – 1,941], p = 0.01). Levels of IL-10 stimulated by LPS were not significantly affected by the addition of rHu IL-4 ($p > 0.05$). No difference was observed in the production of any of the cytokines by CBD (table 3) or control PBMNs with the addition of rHu IL-4 alone, at a concentration of 10 ng·mL^{-1}. Furthermore, pretreating the $BeSO_4$-stimulated CBD and control PBMNs with rHu IL-4 did not affect the production of IL-2 or TNF-α at 24 h of culture, or IFN-γ at 72 h of culture. The median IL-10 production from CBD PBMNs increased by 100% after pretreatment with rHu IL-4, although this was not statistically significant ($p > 0.05$). Finally, no significant increase in IL-4 mRNA was found with rHu IL-4 pretreatment of unstimulated or $BeSO_4$-stimulated control or CBD PBMNs over time ($p > 0.05$, table 4, fig. 6).

Because previous studies have shown that immunomodulation by cytokines may be dependent on the antigen concentration [34, 35], we examined the dose response to $BeSO_4$ in PBMN cells from CBD patients (n = 10). The PBMNs were pretreated with rHu IL-4 at 0, 10 or 50 ng·mL^{-1} and then stimulated in the presence or absence of 10 and 100 μM $BeSO_4$.

Table 3. – Effect of treatment with recombinant human (rHu) interleukin-4 (IL-4) at 10 ng·mL^{-1} on chronic beryllium disease (CBD) peripheral blood (n = 10) and bronchoalveolar lavage (BAL) cells (n = 9) cytokine production. Data presented are median (IQR) at 24 or 72 h of culture

Cytokine (pg·mL^{-1})	Condition			
Blood	Unstimulated	rHu IL-4 (10 ng·mL^{-1})	$BeSO_4$ (100 μM)	rHu IL-4 (10 ng·mL^{-1})/$BeSO_4$ (100 μM)
IFN-γ#	4.7 (3.0 – 14.1)	6.8 (3.0 – 15.1)	215.8 (94.2 – 886.8)	235.5 (80.9 – 880.1)
IL-2*	7.0 (7.0 – 7.0)	7.0 (7.0 – 7.0)	19.9 (25.9 – 38.9)	32.4 (8.5 – 58.8)
TNF-α*	8.8 (4.4 – 19.5)	6.6 (4.4 – 14.0)	30.7 (14.7 – 177.0)	18.8 (13.0 – 139.7)
IL-10#	22.2 (10.0 – 39.2)	28.7 (10.0 – 56.9)	42.7 (26.5 – 97.6)	92.4 (71.9 – 129.6)
BAL				
IFN-γ#	13.9 (3.7 – 34.9)	7.8 (3.9 – 35.4)	2472 (1754.1 – 8488.5)	3524 (1135.7 – 7813.3)
IL-2*	7.0 (7.0 – 7.0)	7.0 (7.0 – 7.0)	485 (267.0 – 1060.5)	543 (217.0 – 1039.5)
TNF-α*	85.7 (50.5 – 495.7)	96.0 (28.0 – 330.7)	3195.0 (1312.5 – 6513.0)	2256 (1457.5 – 7505.3)
IL-10#	25.1 (10.0 – 10.0)	28.7 (10.0 – 56.9)	200 (18.8 – 1202.0)	475 (31.6 – 1474.0)

*: At 24 h of culture. #: At 72 h of culture. $BeSO_4$: beryllium sulphate; IFN-γ: interferon-γ; IL-2: interleukin-2; TNF-α: tumour necrosis factor-α; IL-10: interleukin-10.

Although not statistically significant, the median level of IFN-γ stimulated from CBD PBMNs by 10 μM $BeSO_4$ alone (57.4 pg·mL^{-1} [20.7 – 231.0]) was 75% lower than those stimulated by 100 μM $BeSO_4$ (215.8 pg·mL^{-1} [94.2 – 886.8]) at 72 h. Beryllium sulfate at 10 μM did not stimulate TNF-α from CBD PBMNs above that produced by unstimulated PBMNs. Similar supernatant levels of IL-2 (10.0 pg·mL^{-1} [7.1 – 23.9] with 10 μM $BeSO_4$ stimulation) and IL-10 (43.3 pg·mL^{-1} [8.1 – 100.8] with 10 μM $BeSO_4$ stimulation) were induced by both concentrations of $BeSO_4$. At 10 μM $BeSO_4$, the IFN-γ production was unaffected by the addition of rHu IL-4 10 ng·mL^{-1} (180.8 pg·mL^{-1} [40.8 – 219.9]). A similar increase in IL-10 was seen with rHu IL-4 plus 10 μM $BeSO_4$ treatment (88.9 pg·mL^{-1} [73.7 – 108.9]), as with rHuIL-4 and 100 μM $BeSO_4$. The addition of rHu IL-4 did not affect the production of IL-2 or TNF-α in the presence of 10 μM $BeSO_4$.

Interleukin-4 is unable to downregulate beryllium-stimulated cytokines from chronic beryllium disease bronchoalveolar lavage cells

It was questioned whether rHu IL-4′s inability to modulate the cytokine response in CBD PBMNs was a result of the 10 – 100 fold lower cytokine response from PBMN compared to BAL cells [3, 13, 14]. To examine these issues, CBD BAL cells were pretreated with 10 or 50 ng·mL^{-1} of rHu IL-4 for 30 – 60 min before stimulation with or without 100 μM $BeSO_4$, as outlined above. The data derived from pretreatment with 10 or 50 ng·mL^{-1} rHu IL-4 were similar. Therefore, the data are presented for the cultures using rHu IL-4 at 10 ng·mL^{-1} (table 3).

As has been previously reported and similar to the CBD PBMN response, negligible levels IFN-γ or IL-2 were found, and only low levels of TNF-α and IL-10 produced in the media-only supernatants from CBD BAL cells (see table 3) [3, 13, 14]. Our previous findings that $BeSO_4$ stimulates IFN-γ production 10 times higher than that from CBD PBMNs were also confirmed. Similar to the PBMN response, the IFN-γ production peaked at 72 h of culture ($p < 0.0001$ compared to the media-only condition) and began to decline by 120 h. Beryllium stimulated low IL-2 levels, 20 times higher than that from CBD PBMNs, which peaked at 24 h ($p < 0.001$ compared to media-only condition) and then declined over 72 and 120 h of culture. TNF-α levels, 50 – 100 fold higher than those from CBD PBMNs, peaked at 24 h ($p < 0.0001$ compared to unstimulated condition) and

Table 4. – Effect of treatment with recombinant human (rHu) interleukin-4 (IL-4) (10 ng·mL^{-1}) on chronic beryllium disease (CBD) (n = 5) and control peripheral blood cells′ IL-4 messenger ribonucleic acid (mRNA) production. IL-4 mRNA is expressed as a ratio of IL-4/β-actin mRNA. Data are presented as median (IQR) over time

IL-4/β-actin mRNA ratio	Condition			
CBD	Unstimulated	rHu IL-4 10 ng·mL^{-1}	$BeSO_4$ 100 μM	rHu IL-4 10 ng·mL^{-1}/$BeSO_4$ (100 μM)
0 h	0.03 (0.01 – 0.13)	0.14 (0.01 – 0.35)	0.08 (0.01 – 0.17)	0.02 (0.01 – 0.18)
24 h	0.01 (0 – 0.04)	0 (0 – 0.08)	0 (0.03 – 0.10)	0.02 (0.01 – 0.04)
72 h	0.01 (0 – 0.05)	0.03 (0.01 – 0.05)	0.05 (0.02 – 0.07)	0.04 (0.01 – 0.11)
Control*				
24 h	0.01 (0 – 0.15)	0 (0 – 0.08)	0 (0 – 0.03)	0.01 (0 – 0.09)
72 h	0.02 (0 – 0.17)	0.01 (0 – 0.12)	0.01 (0 – 0.17)	0.01 (0 – 0.13)

*: Data from the 0 timepoint is not available. $BeSO_4$: beryllium sulphate.

Table 5. – Effect of treatment with recombinant human (rHu) interleukin-4 (IL-4) (10 ng·mL^{-1}) on chronic beryllium disease (CBD) (n = 5) bronchoalveolar lavage (BAL) cells' IL-4 messenger ribonucleic acid (mRNA) production. IL-4 mRNA is expressed as a ratio of IL-4/β-actin mRNA. Data presented as median (IQR) over time

IL-4/β-actin mRNA ratio	Condition			
Time	Unstimulated	rHu IL-4 (10 ng·mL^{-1})	$BeSO_4$ (100 μM)	rHu IL-4 (10 ng·mL^{-1})/$BeSO_4$ (100 μM)
0 h	0 (0 – 0.01)	0 (0 – 0.01)	0.01 (0 – 0.02)	0 (0 – 0.02)
24 h	0 (0 – 0.01)	0 (0 – 0.01)	0 (0 – 0)	0 (0 – 0.01)
72 h	0 (0 – 0.02)	0 (0 – 0.01)	0 (0 – 0)	0 (0 – 0.01)
120 h	0 (0 – 0.01)	0 (0 – 0.01)	0 (0 – 0.01)	0 (0 – 0.01)

$BeSO_4$: Beryllium sulphate.

remained elevated at 72 and 120 h of culture. Beryllium-stimulated IL-10 production peaked at 72 h ($p = 0.009$ compared to media-only culture) and declined by 120 h with a similar time response to CBD PBMNs, but with a 5-fold higher level. Part of the difference in the beryllium-stimulated BAL cytokine response compared with PBMN response may be the different number and types of cells present in the BAL cells (53% monocytes and 46% lymphocytes) compared to the PBMN cells (5 – 7% macrophages and 22 – 27% lymphocytes) (table 2). We found no appreciable IL-4 mRNA in unstimulated or $BeSO_4$-stimulated BAL cells (fig. 6, table 5).

The production of these cytokines at peak timepoints was not associated with steroid use or smoking status. The percentage of BAL lymphocytes was positively correlated with the production of IFN-γ (Spearman's correlation coefficient $r = 0.78$, $p = 0.01$), TNF-α ($r = 0.70$, $p = 0.04$), and IL-10 ($r = 0.81$, $p < 0.011$). The peak BAL BeLPT was also positively associated with IL-10 production ($r = 0.85$, $p < 0.01$), while a trend was apparent with IFN-γ production ($r = 0.65$, $p = 0.06$). The addition of rHu IL-4 to the unstimulated BAL cell cultures did not affect the production of any of these four cytokines at any timepoint. The results for IFN-γ, IL-2, TNF-α, and IL-10 are shown at the peak level in table 3. Furthermore, rHu IL-4 failed to significantly downregulate the production of beryllium-stimulated IFN-γ, IL-2, and TNF-α or to upregulate beryllium-stimulated IL-10 production or IL-4 mRNA (fig. 6, table 5) at any time.

Discussion

The current findings indicate that $BeSO_4$-stimulated PBMNs from individuals with CBD produce a cytokine profile similar in pattern, but not magnitude of response, to that observed from $BeSO_4$-stimulated CBD BAL cells [3, 13, 14]. The emerging pattern is consistent with the presence of cell populations in the blood and in BAL that are compelled to produce IFN-γ and IL-2, along with TNF-α, IL-6 and IL-10. Although IL-4 mRNA can be detected in some CBD PBMNs, these cells lack the ability to respond to beryllium with an increase in IL-4, and the levels of IL-4 mRNA are much less than that seen in other antigen stimulated models. Furthermore, high levels of exogenous IL-4 are incapable of limiting the $BeSO_4$-stimulated cytokine production, even when the concentration of antigen is reduced significantly. A major goal in dissecting the beryllium-induced cytokine profile in CBD is to allow us to identify possible immunomodulatory agents *in vitro*. In the granulomatous disease CBD, IL-4 is unable to alter the cytokine response to beryllium.

Previously, it has been possible to partially modify the cytokine response and T cell proliferation to beryllium by neutralizing IL-2 with anti-IL-2 antibody [3] and by the addition of exogenous rHu IL-10 to CBD BAL cells [13]. In the current study, in order to maximize the ability to inhibit the beryllium-stimulated cytokine response, cells were pretreated with two concentrations of rHu IL-4 [30, 31] before antigen stimulation [27], both of which were effective in altering the cytokine production in LPS-stimulated cells [27, 36] and other cells systems in previous studies [25, 30, 31, 35]. Interestingly, rHu IL-4 (10 ng·mL^{-1}) was capable of downregulating LPS-stimulated TNF-α production from CBD cells, which would indicate that a sufficient IL-4 concentration had been used to modulate cytokine response. However, contrary to our expectation from previous *in vitro* studies, rHu IL-4 did not reduce beryllium-stimulated IFN-γ, IL-2 [28, 29] or TNF-α production [26, 27] in either CBD BAL or PBMN cells. Furthermore, CBD BAL and PBMN IL-4 mRNA was not increased by treatment with rHu IL-4. Of note, the addition of IL-4 did increase albeit low levels of beryllium-stimulated IL-10 from blood cells as has been found in other studies [37]. This might indicate recruitment of a naïve Th0 cell population. No increase in the production of IL-10 in CBD BAL cells treated with rHu IL-4 was found, potentially indicating that a naïve T cell component is not present, or present at a lower level in BAL cells.

Antigen concentration influences the cytokine response stimulated by antigen [34, 35]. To enhance IL-4 immunomodulatory capabilities, a lower dose of beryllium was tested, but still could not alter the IL-4 response. The findings in BAL and PBMN cells would indicate that once beryllium hypersensitivity is established, it cannot be readily reversed by IL-4. This has been found in other cell [30] and animal models [22]. Although IL-4 ameliorated a Th1 autoimmune encephalitis in mice, a reduction in IL-2, IFN-γ or TNF-α was not apparent in the periphery [22]. It is likely that CBD BAL cells and PBMNs have been exposed to beryllium in some form *in vivo*, prior to restimulation *in vitro*. Thus, CBD blood and BAL cells are probably already

primed and committed to this cytokine profile, which may explain IL-4′s inability to modify cytokine response in PBMN and BAL cells. It cannot be excluded, that longer pretreatment, or a higher dose of IL-4, might have immunomodulatory capabilities. However, the present data does not suggest that IL-4 is likely to be useful as an immunotherapeutic.

The relationship between clinical parameters such as tobacco use, steroid use, and peripheral blood cell count and blood cytokine levels was evaluated. Although not statistically significant, a decrease in the blood cytokine levels (except IL-10) was noted in the individuals treated with steroids. Similar results have been found from sarcoidosis BAL cells for TNF-α [5] and IFN-γ [38], indicating that corticosteroids are able to modify the immune response in these disease processes. Furthermore, these findings substantiate the use of blood cells to evaluate future immunotherapeutics. No association between the peripheral white blood cell counts and beryllium-stimulated blood cytokines was found, probably because most of our CBD cases had normal blood cell counts. It is speculated that blood T cells stimulated with beryllium produce IFN-γ, IL-2, IL-6 and IL-10, while the blood macrophage or monocyte is responsible for TNF-α. It is possible that monocytes produce IL-10 and IL-6 in CBD. Future studies involving separation of T cell and monocyte populations will be necessary to address which cell type(s) produce which cytokines. Finally, although BAL cell IL-2 is partially responsible for CBD BAL cell proliferation [3], no correlation between cytokine levels and blood BeLPT was found. This may be because cellular proliferation does not result from a single cytokine, but is instead multifactorial. The association between other clinical parameters, such as pulmonary function testing, and cytokine production was not evaluated because of limited power with a small sample size. However, use of PBMNs to evaluate cytokine response to beryllium in the future will allow study of a larger number of CBD subjects and comparisons between cytokine production and clinical disease parameters.

Despite general concordance in CBD blood and BAL cell cytokine response to beryllium, several potentially important differences were noted. Most importantly, the magnitude of response was much lower in CBD PBMNs than in CBD BAL cells. There are several possible explanations for this observation. Different types and numbers of cytokine-producing cells are present in the periphery compared to the lung compartment, as evidenced by the difference in lymphocyte and macrophage/monocyte percentages in table 2. The antigen presenting cells in blood, such as peripheral monocytes or dendritic cells, probably differ from the alveolar macrophages and dendritic cells present in BAL, in their ability to stimulate T cells and produce cytokines themselves [31, 39, 40]. The number of cells previously "exposed" or committed to beryllium are likely to be fewer in peripheral blood cells, as suggested by the lower proliferative response from PBMNs compared to BAL cells [9]. To further optimize PBMN cytokine production in the future, consideration may be given to increasing the number of monocytes and lymphocytes to approximate that in the BAL. Interestingly, a higher spontaneous production of IL-6 and TNF-α from CBD PBMNs than controls was found. Thus, the production of these cytokines was not a cell culture phenomenon. Previously, a comparable spontaneous TNF-α production from CBD and control BAL cells [13, 14] was found. Sarcoidosis blood and BAL cells produce spontaneous TNF-α [4, 5]. This might indicate that CBD blood mononuclear cells are already primed to produce these cytokines before they are even stimulated with beryllium *in vitro*.

In summary, chronic beryllium disease blood cells produce a cytokine pattern when stimulated with beryllium that is similar to that observed in chronic beryllium disease bronchoalveolar lavage. The bronchoalveolar lavage response is of higher magnitude, and further study may be required to optimize the peripheral blood mononuclear cell response. However, chronic beryllium disease peripheral blood mononuclear cells provide a good model of the beryllium-stimulated immune response without the risks and disadvantages of bronchoalveolar lavage. Specifically, the production of the cytokines interferon-γ, tumour necrosis factor-α, interleukin-6, and low levels of interleukin-2 and interleukin-10 was found to be similar to that from bronchoalveolar lavage cells. From the above data, the absence or low levels of interleukin-4 messenger ribonucleic acid present in chronic beryllium disease peripheral blood mononuclear cell bronchoalveolar lavage cells does not appear to be sufficient to result in this cytokine profile, as the addition of interleukin-4 is unable to modify this response. It is unlikely that interleukin-4 will provide specific immunotherapy for chronic beryllium disease. This peripheral blood mononuclear cell model can be used to investigate other potential immunotherapeutics, which are needed clinically to treat these patients. Using this model may allow continued evaluation of the role of beryllium as an antigen or hapten in the development of beryllium sensitization and chronic beryllium disease, and to understand better how beryllium is able to indirectly or directly affect regulation of these cytokines.

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