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Partial IL-10 Inhibition of the Cell-Mediated Immune Response in Chronic Beryllium Disease[1]

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Chronic beryllium disease (CBD) provides a human disorder in which to study the delayed type IV hypersensitivity response to persistent Ag that leads to noncaseating pulmonary granuloma formation. We hypothesized that, in CBD, failure of IL-10 to modulate the beryllium-specific, cell-mediated immune response would result in persistent, maximal cytokine production and T lymphocyte proliferation, thus contributing to the development of granulomatous lung disease. To test this hypothesis, we used bronchoalveolar lavage cells from control and CBD subjects to evaluate the beryllium salt-specific production of endogenous IL-10 and the effects of exogenous human rIL-10 (rhIL-10) on HLA expression, on the production of IL-2, IFN-γ, and TNF-α, and on T lymphocyte proliferation. Our data demonstrate that beryllium-stimulated bronchoalveolar lavage cells produce IL-10, and the neutralization of endogenous IL-10 does not increase significantly cytokine production, HLA expression, or T lymphocyte proliferation. Second, the addition of excess exogenous rhIL-10 partially inhibited the beryllium-stimulated production of IL-2, IFN-γ, and TNF-α; however, we measured no change in T lymphocyte proliferation or in the percentage of alveolar macrophages expressing HLA-DP. Interestingly, beryllium salts interfered with an IL-10-stimulated decrease in the percentage of alveolar macrophages expressing HLA-DR. We conclude that, in the CBD-derived, beryllium-stimulated cell-mediated immune response, low levels of endogenous IL-10 have no appreciable effect; exogenous rhIL-10 has a limited effect on cytokine production and no effect on T lymphocyte proliferation or HLA expression. ***The Journal of Immunology*****, 1999, 163: 2747–2753.**

Cytokines play a central role in initiation and attenuation of the Ag-stimulated, cell-mediated immune response (CMIR)[3] and, in the presence of persistent Ag, in the development of chronic inflammatory disease. During short-term Ag exposure, the concentration of proinflammatory and anti-inflammatory cytokines and the timing of their production support an immunologic response appropriate to the amount of Ag and duration of exposure. Chronic Ag exposure can cause an imbalance in the timing or production of these cytokines, resulting in tissue damage and disease. The role of immunomodulatory cytokines in the changing patterns of cytokine production and in the development of inflammatory disease is incompletely understood.

Chronic beryllium disease (CBD) provides a human disorder in which to study the delayed type hypersensitivity response to persistent Ag that leads to noncaseating pulmonary granuloma formation. We know from previous research that this granulomatous lung disease is characterized by $CD4^+$ lymphocytosis (1) and by MHC class II-restricted, beryllium salt-specific T lymphocyte proliferation (1–3). This proliferative response involves transient IL-2 and sustained IFN-γ production (4) as well as increased IL-6 and TNF-α (3–6). To investigate the interplay of proinflammatory and anti-inflammatory cytokines in human granulomatous disease, we evaluated the ability of IL-10 to modulate the beryllium-stimulated CMIR in CBD.

IL-10 is a key inhibitory cytokine in the inflammatory response to Ag (7). It is produced by monocytes/macrophages, keratinocytes, B lymphocytes, and $CD4^+$ T lymphocytes. In the inflammatory response, IL-10 has been shown to inhibit macrophage and T lymphocyte cytokine synthesis and MHC class II and B7 expression. The combination of these effects results in an inhibition of both mitogen- and Ag-induced accessory cell-dependent T lymphocyte proliferation. Furthermore, several laboratories have reported cross-inhibition between IL-10 and IFN-γ.

Changes in IL-10 production have been associated with many inflammatory diseases. Increased IL-10 production has been documented in the disease organ for optical neuritis (8), rheumatoid arthritis (9), atopic dermatitis (10), and asthma (10). Exogenous human rIL-10 (rhIL-10) has been shown to modulate the cellular response in autoimmune diseases (11, 12), parasitic infections (13, 14), septic shock (15), and fungal infection associated with HIV (16). In addition, several laboratories have reported the absence of IL-10 protein and mRNA in bronchoalveolar lavage (BAL) cells obtained from patients with active pulmonary sarcoidosis (17, 18); Ghalib et al. documented exogenous rhIL-10 modulation of *Leishmania*-induced proliferative responses in vitro (19).

Therefore, we hypothesized that, in CBD, the failure of endogenous IL-10 to modulate the beryllium-specific CMIR results in a persistent, maximal cytokine production and T lymphocyte proliferation that contribute to the development of chronic granulomatous lung disease. To test this hypothesis, we used BAL cells from control and CBD patients to evaluate the beryllium salt-specific

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[3] Abbreviations used in this paper: CMIR, cell-mediated immune response; CBD, chronic beryllium disease; BAL, bronchoalveolar lavage; IQR, interquartile range (25th and 75th percentiles); rhIL-10, human rIL-10; LFU, lipid fluidity unit.

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production of endogenous IL-10 and to test the effects of exogenous rhIL-10 on HLA expression, on the production of IL-2, IFN-γ, and TNF-α, and on T lymphocyte proliferation.

Materials and Methods

Study design

We evaluated IL-2, IL-10, IFN-γ, and TNF-α production, T lymphocyte proliferation, and alveolar macrophage expression of HLA class II molecules in BAL cells stimulated for 72 h with medium alone, beryllium salts, LPS, or tetanus toxoid in the presence or absence of rhIL-10 or in the presence or absence of anti-IL-10 Ab. Because of the complexity of the experimental paradigm and the limited number of cells obtained by BAL, all experimental conditions could not be accomplished on each set of BAL cells. Therefore, medium only and beryllium salt-stimulating conditions were repeated for each data set. The magnitude of the cellular response varied by group; however, the overall conclusions are the same for all of the data presented. The number of patients for each set of experiments is included in the text and figure legends.

Study populations

We evaluated 18 individuals who met our case definition of CBD: 1) history of occupational or environmental beryllium exposure, 2) histologic evidence of noncaseating granulomas on transbronchial or open lung biopsy, and 3) $BeSO_4$-stimulated blood or BAL lymphocyte proliferation (20). The seven control patients had no known exposure to beryllium, were free of respiratory symptoms and lung diseases, and had normal results on chest x-rays and spirometry at the time of testing. We obtained informed consent from all participants according to the protocol approved by the National Jewish Medical and Research Center Human Subjects Review Board.

Sample collection

BAL was performed according to standard methods reported previously (21). Briefly, we instilled four 60-ml aliquots of room temperature normal saline into the right middle lobe and harvested the fluid by gentle suction on the instilling syringe. The fractions were pooled and subsequently centrifuged at 800 rpm for 10 min to pellet the cells. Cell viability, which was evaluated by trypan blue exclusion, ranged from 90 to 97%.

Culture of BAL cells

Freshly isolated BAL cells were cultured at 1×10^6 cells/ml in RPMI 1640 (BioWhittaker, Walkersville, MD) containing 2 mM L-glutamine, 10% (v/v) iron-supplemented calf serum (HyClone Laboratories, Logan, UT), 100 U/ml penicillin, and 100 mg/ml streptomycin under standard mammalian tissue culture conditions. Cells were incubated in the presence or absence of 10 ng/ml or 100 ng/ml of rhIL-10 (Schering-Plough Research Institute, Kenilworth, NJ) or in the presence or absence of 170 ng/ml anti-IL-10 neutralizing Ab (R&D Systems, Minneapolis, MN) for 1 h before the addition of 1 μM, 10 μM, or 100 μM $BeSO_4$. The neutralizing concentration of the anti-IL-10 Ab was determined from IL-10 dose response curves (data not shown), and the neutralization of endogenous IL-10 in all BAL cell supernatants was evaluated by ELISA. Tetanus toxoid at 2 LFU/ml (Wyeth Ayerst Laboratories, Philadelphia, PA) and LPS at 1 mg/ml (Sigma, St. Louis, MO) were used as positive controls. Cells were plated in duplicate 96-well plates. For cytokine analysis, supernatants were collected at 72 h, aliquoted, and frozen at -20°C; the corresponding cell pellets were used immediately in fluorocytometric analysis. The duplicate plates were used in the proliferation assay.

Lymphocyte proliferation

T lymphocyte proliferation was measured by [^{3}H]TdR incorporation. Briefly, 1×10^6 cells/ml were plated in quadruplicate as described above. After 72 h in culture, cells were pulsed with 0.5 mCi [^{3}H]TdR (Amersham, Arlington Heights, IL) for 4 h, harvested onto glass fiber filters (Gelman Sciences, Ann Arbor, MI), and counted in a liquid scintillation counter. The cpm for each set of quadruplicates were averaged.

Quantification of cytokine protein

Cytokine concentrations in cell supernatants were measured with commercially available solid-phase, two-site ELISAs (R&D Systems). The IFN-γ, TNF-α, and IL-2 ELISAs employed in this study have reported sensitivities of 3.0 pg/ml, 4.4 pg/ml, and 6.0 pg/ml, respectively. IL-10 measurements were accomplished using an ELISA protocol supplied by the Schering-Plough Research Institute with a reported sensitivity of 12 pg/ml. Cytokine concentrations were measured in duplicate, averaged, and natural log transformed for statistical analysis.

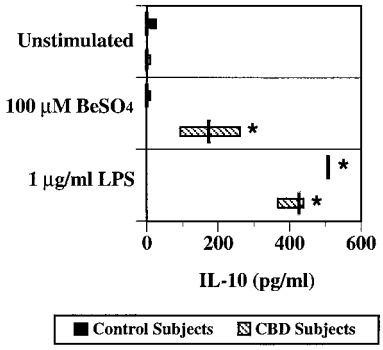

FIGURE 1. IL-10 is produced by BAL cells in vitro. BAL cells were cultured for 72 h in the presence or absence of 100 μM $BeSO_4$ (control subjects, $n = 5$; CBD patients, $n = 8$) or 1 μg/ml LPS (control subjects, $n = 2$; CBD patients, $n = 6$) as described in *Materials and Methods*. Cytokine concentrations were measured in duplicate by ELISA, averaged, and natural log transformed for statistical analysis. Data are reported as the mean natural log of the cytokine (pg/ml) $\pm$ SEM. LPS data for control subjects are reported as the mean of the natural log-transformed data. Statistically significant increases in cytokine concentration with respect to control measurements are indicated by an asterisk.

Fluorocytometric analysis

After incubation for 72 h, $\sim 1 \times 10^6$ cells/culture condition were resuspended in 50 ml of PBS staining solution containing 5% FBS, 1 mg/ml human γ globulin (Sigma), and 0.2% sodium azide (Sigma) and incubated at room temperature for 10 min. The cells were centrifuged, resuspended in 45 ml of staining solution and 5 ml of human peridinin chlorophyll protein-labeled anti-CD14 Ab (Becton Dickinson, San Jose, CA), PE-labeled anti-HLA-DR Ab (Becton Dickinson), or FITC-labeled anti-HLA-DP Ab (PharMingen, San Diego, CA), and incubated for 15 min in the dark at room temperature. FITC-labeled IgG1 and PE-labeled IgG2a (Becton Dickinson) were employed as isotype control Abs to measure background fluorescence due to nonspecific Ab binding and to control for autofluorescence. The cells were rinsed once in PBS and fixed with a 1% formaldehyde-PBS solution. The macrophage cell population was identified by size and granularity. HLA analysis was applied to this population of cells. Data were analyzed on a FACScalibur using the PC lysis program.

Statistical analysis

The data were not normally distributed, and we achieved a more Gaussian distribution by natural log transformation of the data. For cytokine measurements below the minimum detection level of the ELISA, the data points were set to the minimum detectable concentration before transformation. The data were analyzed separately for each assay using a repeated measures ANOVA. The Tukey-Kramer multiple comparison procedure was used to make all pairwise comparisons between group and treatment means. Statistical significance was defined as $p < 0.05$.

Results

Endogenous IL-10 release from $BeSO_4$-stimulated BAL cells from CBD patients

Previous studies from our laboratory documented significant increases in IL-2, IFN-γ, and TNF-α production and in T lymphocyte proliferation in $BeSO_4$-stimulated, CBD-derived BAL cells (4, 6). To determine whether endogenous IL-10 was produced under these same conditions, we measured by ELISA the concentration of IL-10 in supernatants derived from BAL cells cultured for 72 h. We measured no IL-10 production above the minimum detection level in supernatants from unstimulated BAL cells from most control and CBD individuals or in supernatants from $BeSO_4$-stimulated cells from control subjects (Fig. 1). In contrast, significant concentrations of IL-10 were measured in $BeSO_4$-stimulated, CBD-derived BAL cell cultures (median 175 pg/ml (interquartile range (IQR) 94 pg/ml, 261 pg/ml); $p < 0.001$). The ability of BAL cells to release endogenous IL-10 was also tested by stimulation

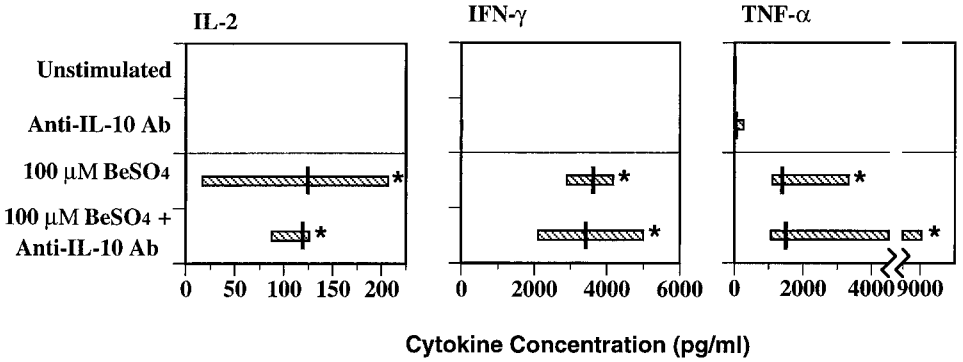

FIGURE 2. Neutralization of endogenous IL-10 does not increase beryllium-stimulated cytokine production. BAL cells from CBD patients ($n = 9$) were cultured in the presence or absence of 100 μM $BeSO_4$ and in the presence or absence of 170 ng/ml anti-IL-10 Ab as described in *Materials and Methods*. Cytokine concentrations were measured in duplicate by ELISA, averaged, and natural log transformed for statistical analysis. Data are presented as the median of the cytokine concentration (pg/ml) and as the 25th to 75th IQR. Statistically significant increases in the mean cytokine concentration with respect to measurements from unstimulated supernatants are indicated by an asterisk.

with LPS. The median concentration of IL-10 released by CBD-derived BAL cells was 427 pg/ml (IQR 368 pg/ml, 439 pg/ml; $p < 0.001$), and the mean for control subjects was 508 pg/ml.

To determine whether endogenous IL-10 modulated the beryllium-stimulated CMIR, we incubated BAL cells from CBD subjects with 1) media only, 2) 170 ng/ml anti-IL-10 Ab, 3) 100 μM $BeSO_4$, or 4) 170 ng/ml anti-IL-10 Ab for 1 h before the addition of 100 μM $BeSO_4$. After 72 h, we measured the production of IL-2, IFN-γ, and TNF-α by ELISA.

Consistent with previous findings (4), we measured no constitutive release of IL-2 or IFN-γ and low levels of TNF-α in supernatants from CBD-derived BAL cells cultured in media only or with anti-IL-10 Ab (Fig. 2). The median concentration of TNF-α in unstimulated cell supernatants (12 pg/ml (IQR 5 pg/ml, 30 pg/ml)) did not differ statistically from those measures in supernatants containing anti-IL-10 Ab (41 pg/ml (IQR 14 pg/ml, 256 pg/ml); $p > 0.05$). $BeSO_4$ stimulated significant increases in the median concentrations of IL-2, IFN-γ, and TNF-α that were not increased further by the neutralization of endogenous IL-10 (Fig. 2). The respective medians for $BeSO_4$-stimulated cytokine production were 124 pg/ml IL-2 (IQR 17 pg/ml, 207 pg/ml), 3615 pg/ml IFN-γ (IQR 2900 pg/ml, 4180 pg/ml), and 1406 pg/ml TNF-α (IQR 1104 pg/ml, 3320 pg/ml); for cells cultured in anti-IL-10 Ab and beryllium salts, those measures were 119 pg/ml IL-2 (IQR 88 pg/ml, 126 pg/ml), 3410 pg/ml IFN-γ (IQR 2121 pg/ml, 5000 pg/ml), and 1516 pg/ml TNF-α (IQR 1070 pg/ml, 9040 pg/ml).

Exogenous rhIL-10 modulation of $BeSO_4$-stimulated BAL cell cytokine production

We subsequently asked whether the concentrations of cytokines measured in the beryllium-stimulated CMIR could be modulated by the addition of exogenous rhIL-10. To evaluate this possibility, BAL cells from control and CBD subjects were preincubated in 0, 10, or 100 ng/ml rhIL-10 for 1 h before the addition of 100 μM $BeSO_4$. For most of the conditions studied, the maximum effect of rhIL-10 was observed at 10 ng/ml, and the addition of 100 ng/ml rhIL-10 did not decrease further the outcome measures. Therefore, we report only the results for culture conditions employing 10 ng/ml rhIL-10.

For control subjects, we measured no IL-2 or IFN-γ in supernatants derived from BAL cells cultured under these conditions. In contrast, the median $BeSO_4$-stimulated increase in IL-2 and IFN-γ in CBD-derived BAL cell cultures decreased by ~50% following preincubation in rhIL-10 (Fig. 3). In the presence of 10 ng/ml rhIL-10, the median concentration of IL-2 at 72 h decreased from 124 pg/ml (IQR 17 pg/ml, 207 pg/ml) to 57 pg/ml (IQR 21 pg/ml, 109 pg/ml); for IFN-γ, under the same conditions, the median concentration decreased from 3615 pg/ml (IQR 2900 pg/ml, 4180 pg/ml) to 1360 pg/ml (IQR 610 pg/ml, 2495 pg/ml). The $BeSO_4$-stimulated production of TNF-α decreased by ~20%, from a median of 1406 pg/ml (IQR 1104 pg/ml, 3320 pg/ml) to 1176 pg/ml (IQR 552 pg/ml, 2144 pg/ml).

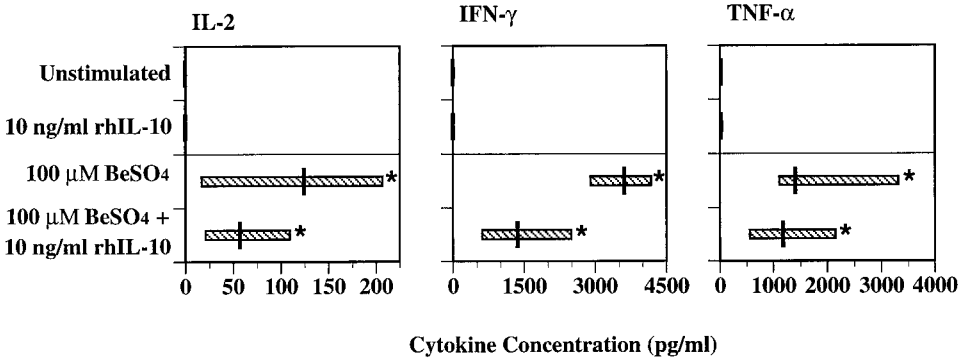

FIGURE 3. Addition of rhIL-10 to beryllium-stimulated, CBD-derived BAL cells decreases cytokine production. BAL cells from CBD patients ($n = 9$) were preincubated for 1 h in the presence or absence of 10 ng/ml rhIL-10 before the addition of 1 μM, 10 μM, or 100 μM $BeSO_4$ for 72 h as described in *Materials and Methods*. Cytokine concentrations were measured by ELISA. Duplicate wells were averaged and natural log transformed for statistical analysis. Data are presented as the median (IQR 25, 75). Statistically significant increases in the mean cytokine concentration with respect to measurements from unstimulated supernatants are indicated by an asterisk; decreases due to the addition of rhIL-10 are indicated by a dagger.

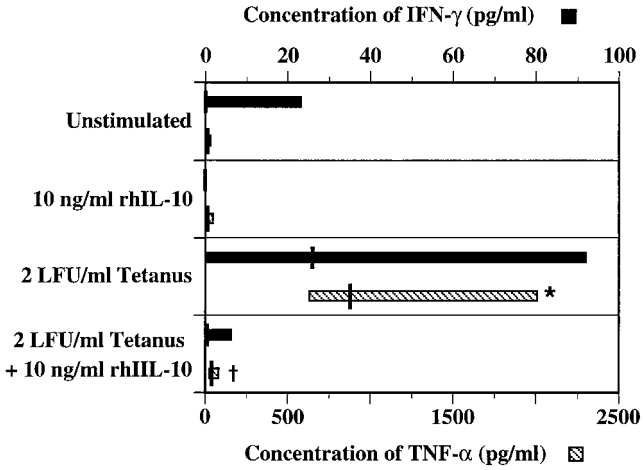

FIGURE 4. Addition of rhIL-10 to tetanus toxoid-stimulated, CBD-derived BAL cells decreases IFN-γ and TNF-α production. BAL cells from CBD patients ($n = 8$) were cultured in the presence or absence of 2 LFU/ml tetanus toxoid and in the presence or absence of 10 ng/ml rhIL-10 as described in *Materials and Methods*. Cytokine concentrations were measured by ELISA. Duplicate wells were averaged and natural log transformed for statistical analysis. Data are presented as the median (IQR 25, 75). Statistically significant increases in the mean cytokine concentration with respect to measurements from unstimulated supernatants are indicated by an asterisk; decreases due to the addition of rhIL-10 are indicated by a dagger.

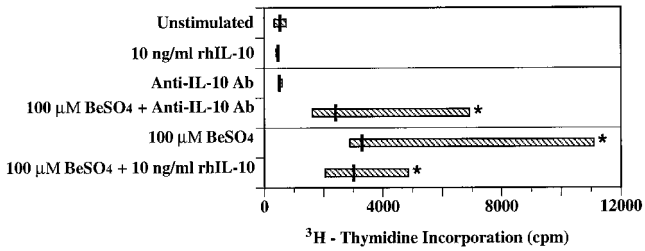

FIGURE 5. Preincubation of CBD-derived BAL cells in anti-IL-10 Ab or rhIL-10 did not alter beryllium-stimulated T lymphocyte proliferation. Following preincubation for 1 h in the presence or absence of 170 ng/ml anti-IL-10 Ab or 10 ng/ml rhIL-10, BAL cells from CBD patients were cultured for 72 h in the presence or absence of 1 μM, 10 μM, or 100 μM $BeSO_4$ ($n = 9$) or in the presence or absence of 2 LFU/ml tetanus toxoid ($n = 8$), as described in *Materials and Methods*. Cells were incubated in 0.5 mCi of [^{3}H]TdR for 4 h before harvesting. Quadruplicates were averaged and log transformed for statistical analysis. Data are presented as the median of the cpm (IQR 25, 75). Statistically significant increases in the mean T lymphocyte proliferation with respect to measurements from unstimulated cells are indicated by an asterisk.

Although BAL cells frequently respond poorly to recall Ags, we tested the ability of CBD-derived BAL cells to respond to rhIL-10 immunomodulation when stimulated by tetanus toxoid. Tetanus toxoid stimulated a modest cytokine response that was significant for TNF-α but not for IL-2 and IFN-γ. Preincubation of tetanus-stimulated cells with 10 ng/ml rhIL-10 decreased the concentration of TNF-α by 23-fold, from a median of 876 pg/ml (IQR 632 pg/ml, 2008 pg/ml) to 38 pg/ml (IQR 25 pg/ml, 82 pg/ml; $p < 0.001$). And although changes in the IFN-γ concentrations were not significant, the tetanus-stimulated median of 23 pg/ml declined to baseline for tetanus-stimulated cells preincubated in 10 ng/ml rhIL-10 (Fig. 4).

Exogenous rhIL-10 modulation of $BeSO_4$-stimulated T lymphocyte proliferation

To determine the effect of rhIL-10 on T lymphocyte proliferation, we measured [^{3}H]TdR incorporation in CBD-derived BAL cells cultured in the presence or absence of 100 μM $BeSO_4$ and in the presence of 0, 10, or 100 ng/ml rhIL-10. We measured a significant increase in [^{3}H]TdR incorporation in beryllium-stimulated cells (Fig. 5); however, the addition of either anti-IL-10 Ab or rhIL-10 to beryllium-stimulated cells did not alter significantly the median levels of [^{3}H]TdR incorporation. After 72 h in culture, [^{3}H]TdR incorporation in beryllium-stimulated, CBD-derived BAL cells measured 3,303 cpm (IQR 2,886 cpm, 11,092 cpm, $p < 0.001$), a level of [^{3}H]TdR incorporation that was not changed significantly by preincubation in 10 ng/ml rhIL-10 (3024 cpm (IQR 2057 cpm, 4850 cpm)) or 170 ng/ml anti-IL-10 Ab (median 2405 cpm (IQR 1618 cpm, 6917 cpm)).

We were unable to measure tetanus toxoid-stimulated increases in lymphocyte proliferation by [^{3}H]TdR incorporation in BAL cells.

Efficacy of rhIL-10 immunomodulation at lower concentrations of $BeSO_4$

To examine further the effect of rhIL-10 on the beryllium-stimulated CMIR, we evaluated the relationship between Ag concentration and rhIL-10 modulation of cytokine production and T lymphocyte proliferation. Previous studies have demonstrated that IL-10 has a more pronounced modulatory effect on the CMIR at lower Ag concentrations (22). To accomplish this analysis, we incubated the BAL cells from CBD patients with 1 μM or 10 μM $BeSO_4$ and 0 or 10 ng/ml rhIL-10. We measured significant 10 μM $BeSO_4$-stimulated increases in the median concentrations of IL-2 (52 pg/ml (IQR 19 pg/ml, 72 pg/ml)), IFN-γ (2880 pg/ml (IQR 665 pg/ml, 3500 pg/ml)), and TNF-α (1694 pg/ml (IQR 668 pg/ml, 3438 pg/ml); $p < 0.001$; Fig. 6*A*). Preincubation with rhIL-10 decreased the median concentrations of IL-2 and IFN-γ by ~70%, to 13 pg/ml (IQR 8 pg/ml, 41 pg/ml) for IL-2 and 846 pg/ml (IQR 335 pg/ml, 1080 pg/ml) for IFN-γ; the median concentration of TNF-α decreased by ~50% (898 pg/ml (IQR 364 pg/ml, 1554 pg/ml)). There was no statistical difference between the cytokine concentrations stimulated by 10 μM and 100 μM $BeSO_4$ in the presence or absence of rhIL-10.

Addition of 1 μM $BeSO_4$ to BAL cell cultures stimulated lower but significant increases in IFN-γ and TNF-α (respective medians 1300 pg/ml (IQR 224 pg/ml, 1990 pg/ml and 1014 pg/ml (IQR 278 pg/ml, 1552 pg/ml); $p < 0.001$) but not in IL-2 (median 10 pg/ml (IQR 4 pg/ml, 14 pg/ml); $p > 0.05$). Again, preincubation in rhIL-10 decreased the median concentration of IFN-γ by 85% (184 pg/ml (IQR 36 pg/ml, 490 pg/ml); $p < 0.03$)) and of TNF-α by 60% (median 416 pg/ml (IQR 156 pg/ml, 478 pg/ml)).

Evaluating T lymphocyte proliferation at 1 μM and 10 μM $BeSO_4$ stimulation, we measured significant [^{3}H]TdR incorporation at 10 μM $BeSO_4$ (median 1394 cpm (IQR 1015 cpm, 9857 cpm)) that was not significantly decreased by preincubation in 10 ng/ml rhIL-10 (median 1359 cpm (IQR 890 cpm, 3874 cpm); $p > 0.05$; Fig. 6*B*). Addition of 1 μM $BeSO_4$ to CBD-derived BAL cells did not significantly increase T lymphocyte proliferation above constitutive levels.

Exogenous rhIL-10 modulation of $BeSO_4$-stimulated alveolar macrophage HLA expression

IL-10 also modulates the CMIR through down-regulation of HLA class II expression on monocytes and macrophages (22, 23). Several laboratories have demonstrated the association of HLA-DPβ1 with CBD and DR3 with sarcoidosis (24–26). Therefore, we examined the effect of rhIL-10 on the beryllium-stimulated expression of HLA-DP and -DR on alveolar macrophages from control

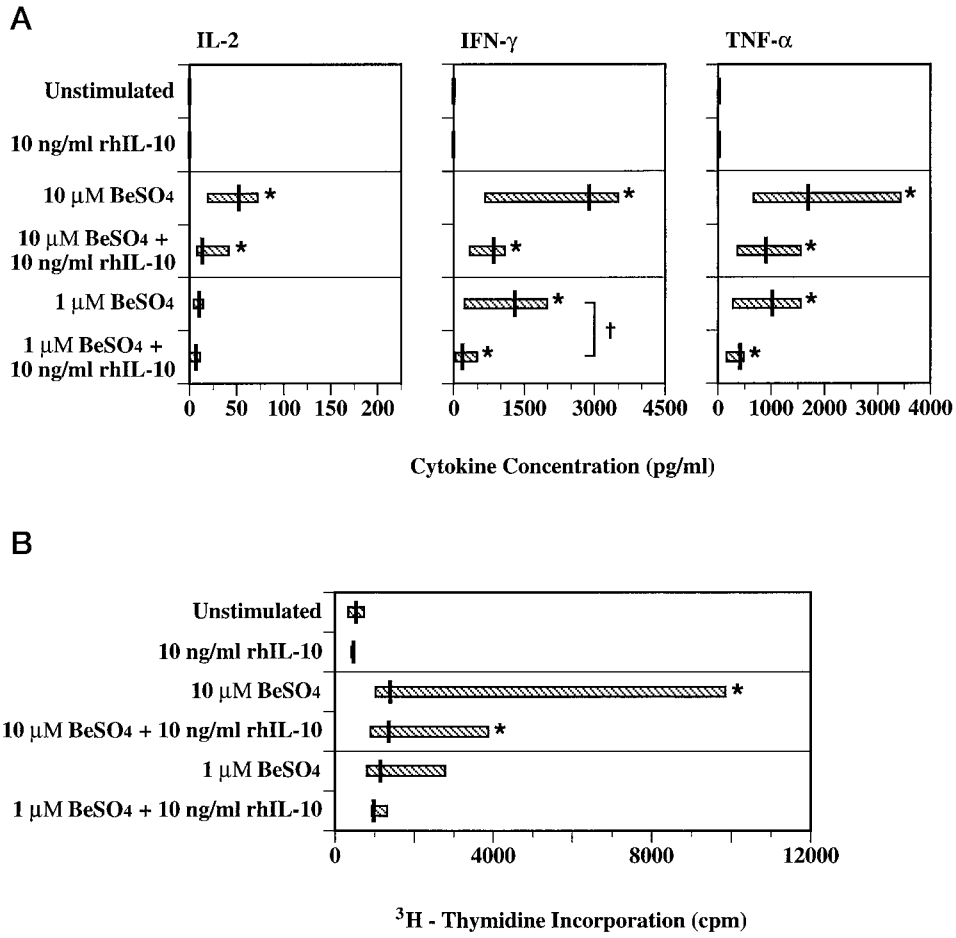

FIGURE 6. rhIL-10 modulation of the beryllium-stimulated CMIR remains limited at lower concentrations of beryllium salts. BAL cells from CBD patients ($n = 9$) were preincubated in the presence or absence of 10 ng/ml rhIL-10 and cultured in the presence or absence 1 μM or 10 μM $BeSO_4$ for 72 h. *A*, Cytokine concentrations were measured by ELISA; *B*, T lymphocyte proliferation was measured by [^{3}H]TdR incorporation (both as described in *Materials and Methods*). Both sets of results are presented as the median of the cpm (IQR 25, 75). We have repeated cytokine data and T lymphocyte proliferation data for unstimulated and 10 ng/ml rhIL-10 treatment conditions to maximize clarity. Statistically significant increases in the mean cytokine concentration or [^{3}H]TdR incorporation with respect to measurements from unstimulated supernatants are indicated by and asterisk; significant decreases due to the addition of rhIL-10 are indicated by a dagger.

and CBD subjects. Following BAL cell stimulation, three-color fluorocytometric analysis was used to identify the alveolar macrophage population and the percentages of these cells expressing HLA-DP and HLA-DR.

We measured no significant differences in the mean percentage of alveolar macrophages expressing HLA-DP for either the control or CBD group for any of the treatment pairs tested, nor any differences between the control and CBD subject groups (Fig. 7).

Significant changes in the percentage of alveolar macrophage expression of HLA-DR were observed for CBD patients for two sets of treatment conditions. First, the addition of 10 ng/ml rhIL-10 decreased the mean percentage of expression of HLA-DR by ~3-fold with respect to unstimulated conditions ($p < 0.001$); however, there was no difference in the percentage of expression of HLA-DR on beryllium-stimulated cells compared with cells incubated in rhIL-10 before the addition of beryllium salts. Second, a 10 ng/ml rhIL-10 preincubation of tetanus toxoid-stimulated cells decreased the mean percentage of expression of HLA-DR by ~5-fold ($p < 0.001$).

The mean percentage of alveolar macrophages expressing HLA-DR in the control group did not change significantly for any of the culture conditions we employed. However, preincubation of unstimulated and tetanus toxoid-stimulated cells in 10 ng/ml rhIL-10 decreased the mean expression by 16% and 24%, respectively. Preincubation of beryllium-stimulated alveolar macrophages from control subjects in 10 ng/ml rhIL-10 decreased the mean HLA-DR expression by 8%.

Discussion

In this study, we have demonstrated partial inhibition of the beryllium-stimulated CMIR by IL-10 in vitro. In particular, we have shown that exogenous rhIL-10 has a limited modulatory effect on beryllium-stimulated proinflammatory and Th1 cytokine production, on T lymphocyte proliferation, and on the percentage of alveolar macrophages expressing of HLA-DR and -DP. The failure of endogenous IL-10 to modulate a persistent inflammatory response to Ag could contribute to a pulmonary microenvironment that supports the development of chronic granulomatous disease. Previous studies have shown that failure to produce endogenous IL-10 enhances the Th1 response to Ag by overriding macrophage antimicrobial signals, thus promoting the development of chronic mycobacterial disease (27, 28); these studies have also shown that exogenous IL-10 has no effect on the size of mycobacterial, schistosomal, or *Leishmania* Ag-elicited pulmonary granulomas in animal models and is unable to halt or reverse granuloma formation (29, 30).

Our initial experiments documented the production of IL-10 by LPS-stimulated BAL cells from control subjects and from LPS and beryllium salt-stimulated BAL cells from CBD patients. These findings are consistent with previous studies showing LPS- and PHA-stimulated release of IL-10 from PBMCs (31) and from mitogen and purified protein derivative-stimulated T lymphocytes (32, 33) as well as constitutive and LPS-stimulated IL-10 production by macrophages isolated from PBMCs (34). However, two studies found no LPS-stimulated increase in IL-10 protein and mRNA production in alveolar macrophages from control subjects

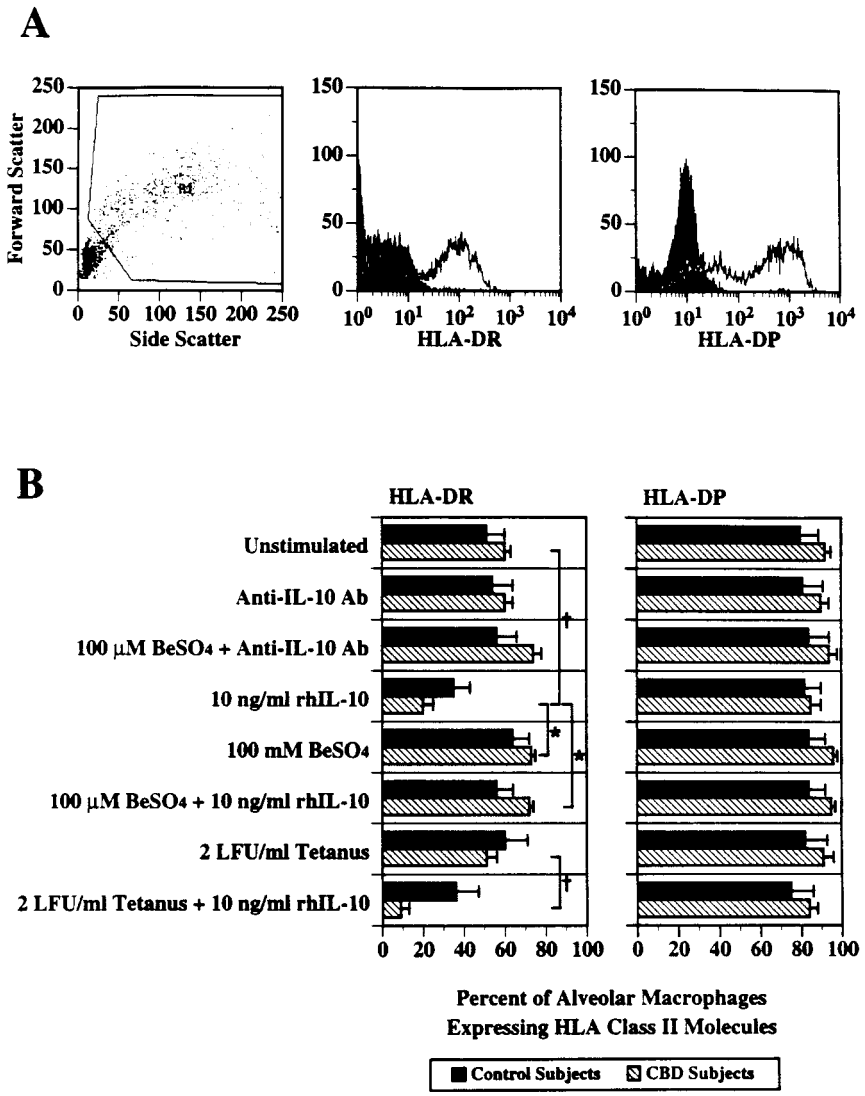

FIGURE 7. Beryllium salts interfere with rhIL-10 down-regulation of the percentage of alveolar macrophages expressing HLA-DR, but not HLA-DP. BAL cells from CBD patients were preincubated in the presence or absence of 170 ng/ml anti-IL-10 Ab or 10 ng/ml rhIL-10 and cultured in the presence or absence of 1 μM, 10 μM, or 100 μM $BeSO_4$ ($n = 9$) or in the presence or absence of 2 LFU/ml tetanus toxoid ($n = 8$) for 72 h. A three-color fluorocytometric analysis was performed as described in *Materials and Methods*. Data are reported as the mean ± SEM. Statistically significant increases in the mean percentage of alveolar macrophages expressing HLA are indicated by an asterisk; decreases due to the addition of rhIL-10 are indicated by a dagger.

(35, 36); other studies documented the absence of IL-10 in cells or BAL fluid from patients with active sarcoidosis, a pulmonary disease with an immunopathology similar to CBD (17, 18). Our data demonstrate that BAL cells from CBD subjects can be stimulated by beryllium salts to produce IL-10 in vitro; however, additional studies will be necessary to determine the identity of the BAL cell subset producing the endogenous IL-10 we measured.

Our data are also in contrast to reports that changes in the concentration of IL-10, either by Ab neutralization of endogenous IL-10 or by the addition of exogenous IL-10, result in significant changes in macrophage and T lymphocyte cytokine production and T lymphocyte proliferation, even in the presence of IFN-γ (7, 22, 32) and at 10-fold lower concentrations of Ag. However, it is possible that the residual cytokine concentrations are sufficient to maintain T lymphocyte proliferation (32, 37).

Persistent Ag-MHC class II expression on APCs may also contribute to sustained T lymphocyte proliferation. Many laboratories have demonstrated that IL-10 down-regulates constitutive, IFN-γ- and IL-4-stimulated expression of MHC class II molecules on monocytes, with a corresponding decrease in Ag-presentation capabilities (22, 38). Our findings demonstrate no change in the percentage of alveolar macrophages expressing HLA-DP for control or CBD subjects for any pair of treatment conditions employed in this study. In contrast, we found that rhIL-10 significantly decreased constitutive HLA-DR expression when added to unstimulated or to tetanus toxoid-stimulated BAL cell cultures. However, in the presence of beryllium salts, exogenous rhIL-10 did not decrease HLA-DR expression. These data underscore previous findings that HLA-DR and -DP expression can be differentially regulated within the same cell (39, 40). However, they are in contrast to studies documenting a significant induction of HLA expression by IFN-γ and inhibition by IL-10 (38, 41, 42). The inability of IL-10 to down-regulate the percentage of alveolar macrophages expressing HLA-DR in the presence of beryllium salts, coupled with the partial inhibition of beryllium-stimulated cytokine production and T lymphocyte proliferation by rhIL-10, suggests a potential pathway through which beryllium may disrupt the regulatory balance of the CMIR and promote granuloma formation.

Although our data do not address directly the mechanism underlying the partial inhibitory effect of IL-10 in CBD BAL cells, it is likely that our observations are due to a combination of the actions of beryllium as a hapten and as a divalent cation. Previous studies have shown that beryllium, probably in combination with an endogenous peptide, is able to induce Ag-specific T lymphocyte proliferation (2, 3), act as an adjuvant by increasing the synthesis of IL-5 and IL-6 mRNA in Ag-stimulated murine spleen cells, and influence isotype switching in B cells (43–45). In addition, beryllium salts inactivate cytoplasmic, but not nuclear, kinases (46),

inhibit the phosphorylation of several phosphatases (47), and bind to nonhistone nuclear proteins (48). This multiplicity of cellular effects provides many points at which the homeostatic balance of the CMIR could be disrupted. Future studies will determine the combination of haptenic and cationic effects of beryllium that promotes the pulmonary microenvironment favorable to the development of noncaseating granulomas.

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