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Nitric Oxide Attenuates Beryllium-Induced IFNγ Responses in Chronic Beryllium Disease: Evidence for Mechanisms Independent of IL-18

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In chronic beryllium disease (CBD), a granulomatous lung disease characterized by hypersensitivity to beryllium salts (BE), BE challenge of bronchoalveolar lavage cells induces IFNγ. Although nitric oxide (NO) is elevated in CBD airways, the effects of NO on CBD IFNγ responses are unknown. Here we report that BE-stimulated IFNγ production in CBD lavage cells was markedly reduced (74%) by the NO generator DETA NONOate. Investigation of IFNγ-stimulatory cytokine involvement indicated that lavage cell IL-18 was significantly increased (fourfold) by BE and reduced (64%) by DETA NONOate but IL-12 was undetectable. IL-18 production was caspase-1-dependent but caspase 1 inhibition reduced IFNγ only partially (43%). Specific antibody depletion of lavage cell IL-18 yielded marginal reduction (19%) of IFNγ. Data are the first to show that: (1) BE stimulates IL-18 as well as IFNγ in CBD; (2) BE cytokine responses are NO-sensitive; and (3) NO down-regulation of IFNγ involves other sites in addition to IL-18. © 2002 Elsevier Science (USA)

***Key Words:* beryllium; lung; granuloma; bronchoalveolar lavage cells; cytokine; nitric oxide; IL-18; IFNγ.**

INTRODUCTION

Chronic beryllium disease (CBD) is a granulomatous disease characterized by specific immunologic reactivity directed against beryllium salts (BE) (1). CBD occurs following environmental exposure, usually in the workplace, and may affect 1–6% of exposed workers, with rates increased in some high-exposure worker groups (1, 2). The hallmarks of CBD are the presence of noncaseating granulomas in the lung parenchyma. BE hypersensitivity is demonstrated by the proliferation of peripheral blood mononuclear cells (PBMC) when challenged with BE *in vitro* (3, 4). In CBD, airway immune cells obtained by bronchoalveolar lavage also proliferate to BE challenge *in vitro* (5, 6). BE-exposed individuals who demonstrate PBMC reactivity to BE but have no manifestations of lung disease are referred to as "BE-sensitized" and are considered at risk for CBD development (7).

When challenged with BE, bronchoalveolar lavage cells from CBD patients, unlike those from unexposed, healthy individuals, produce IFNγ *in vitro* (8). The lack of IL-4 production and the presence of IFNγ together with TNFα in BE-treated pulmonary immune cells have resulted in categorization of CBD as a Th1-mediated granulomatous disease (1). Synthesis of IFNγ is tightly regulated, however, and mechanisms of IFNγ control in CBD have not been defined. IL-18, formerly known as interferon γ inducing factor, is a member of the IL-1 cytokine family and is produced by many cell types, including macrophages, dendritic cells, and pulmonary epithelium (9). In both mice and humans, IL-18 promotes IFNγ production in Th1, NK, and $CD8^+$ cells (9). IL-12, predominantly a macrophage product, promotes development of and upregulates IL-18 receptor expression on Th1 cells (10, 11). IL-18 also augments IL-12 receptor expression but in addition has other complex actions that can stimulate Th2 responses (9, 12). Together, IL-18 and IL-12 act synergistically to stimulate IFNγ production (13, 14). The roles of IL-18 and IL-12 in CBD are unknown.

In previous studies, we detected elevated levels of exhaled nitric oxide (NO) in CBD airways (15). Data from experimental animal models suggest that NO may regulate size and composition of granulomas (16). Inducible nitric oxide synthase (NOS II) is constitutively expressed by human respiratory epithelium and contributes to NO synthesis in the lung (17, 18). NOS II expression is increased by IFNγ (17, 19), but NO itself reduces IFNγ production, suggesting regulatory feedback (20). NO also mediates reduction of IL-18 (as well as IL-1β) levels by antagonizing caspase 1, which is responsible for cleavage of active IL-18 and IL-1β peptides from inactive precursors (21). In view of the high NO levels in CBD airways, it was hypothesized that NO might antagonize lavage cell production of IFNγ, possibly by affecting IL-18 output. The primary aim of this study therefore was to investigate the effects of NO on the IFNγ responses of CBD lavage cells to BE challenge. A second aim of the study was to compare lavage cell responses of CBD patients to those

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of BE-sensitized individuals with no granulomatous lung disease since this comparison had not been done previously. We hypothesized that responses of CBD and BE-sensitized groups would differ and that results might have potential relevance to the diagnosis or prognosis of CBD.

MATERIALS AND METHODS

Study population and design. Subjects were diagnosed as having CBD if there were (a) a history of beryllium exposure; (b) evidence of noncaseating granulomas by histologic inspection of transbronchial lung biopsies; and (c) BE stimulation of proliferation in bronchoalveolar lavage cells (1). Non-CBD subjects had a history of beryllium exposure but no evidence of granulomatous or clinical pulmonary disease. Non-CBD subjects were considered BE-sensitized if PBMC underwent proliferation in the presence of BE in lymphocyte proliferation assays (4). Healthy, control subjects had no history of BE exposure. All subjects were provided written informed consent. Study procedures were approved by the Institutional Review Board of the Cleveland Clinic Foundation. Because of limitations in individual lavage cell yields, not all experiments could be carried out on cells of each subject. CBD subjects were designated "granuloma (+)" and BE-sensitized, non-CBD subjects as "granuloma (−)."

Specimen collection and culture. Lavage cells were obtained by fiberoptic bronchoscopy as previously described (22). Briefly, the tip of the bronchoscope was wedged into the right middle lobe or the lingula. Three aliquots of normal saline (50 ml) were instilled by gravity and gently withdrawn with the instilling syringe. Lavage fluid was filtered and cells were collected by centrifugation, washed with Hank's balanced salt solution (GIBCO, Grand Island, NY), and counted under a hemocytometer. Differential cell counts were obtained from cytospins after staining with modified Wright's stain. The mean viability of lavage cells was 93% as determined by trypan blue dye exclusion. Cells were cultured in 24-well plates on the basis of 300,000 alveolar macrophages per well in RPMI 1640 medium supplemented with 5% human AB serum (Gemini, Calabasas, CA), L-glutamine, and antibiotics. PBMC were derived by centrifugation of citrated blood collected in CPT vacutainer tubes (BD, Franklin Lakes, NJ). The PBMC band was removed, washed, and counted and differential cytospins were done. The mean viability was >98%. PBMCs were cultured at 1 million cells/ml in 48-well plates in the above medium. All cultures were terminated at 24 h except where noted.

Reagents. Salmonella typhimurium lipopolysaccharide (LPS) was obtained from Sigma Chemical (St. Louis, MO) and used at 0.5 μg/ml. The NO generator DETA NONOate (DNO) (Cayman Chemical, Ann Arbor, MI) was used at 1.0 mM in all experiments. This concentration was previously shown to optimally block LPS-stimulated cytokine production of human lavage cells without compromising viability as assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) cleavage assay (22, 23). DNO releases NO in culture with a half-life of 20 h at 37°C. Previous studies have indicated that human lavage cells do not release detectable NO (22). The interleukin 1 converting enzyme (ICE) (caspase 1) inhibitor *N*-acetyl-Tyr-Val-Ala-Asp-aldehyde (Ac-YVAD-cho) was obtained from Alexis Biochemicals (San Diego, CA) and used at 10 and 20 mM. Neutralizing monoclonal antibody to human IL-18, control irrelevant murine IgG1, and recombinant human IL-18 were obtained from R & D Systems (Minneapolis, MN). $BeSO_4$ stock solution was provided by Brush Wellman (Cleveland, OH) and was used at concentrations of 10 and 100 μM.

Cytokine assays. Cell-free conditioned media were collected from lavage cell or PBMC cultures and assayed for IL-1β, IL-12 (p70), IL-18, and IFNγ by enzyme-linked immunosorbent assay (ELISA) (Endogen, Cambridge, MA or R & D Systems). Assay sensitivity ranged from 10.2 to 25.6 pg/ml, depending upon cytokine. All samples were assayed in duplicate or triplicate and the mean coefficient of variation for ELISA assays was <10%.

Statistical analyses. Lavage cell data from CBD, BE-sensitized, and healthy control subjects were analyzed by Wilcoxon test. Comparative data from treated vs untreated cells were analyzed by the Bonferroni method and *t* test. All analyses were carried out using GraphPad Prizm software version 3.00 for Windows (GraphPad, San Diego, CA). Significance was defined as $P \leq 0.05$.

RESULTS

Lymphocytes and IFNγ responses are elevated in CBD lavage relative to BE-sensitized, non-CBD subjects. Analyses of bronchoalveolar lavage subpopulations indicated that the percentage of lymphocytes was significantly higher in CBD (granuloma positive) (median, 17%) than in BE-sensitized (granuloma negative) subjects (3.5%) ($P < 0.002$) (Fig. 1). The percentage of lavage lymphocytes from CBD but not from BE-sensitized subjects was also higher than that of healthy controls (2%, $P < 0.002$, $n = 10$) (data not shown). Challenge with BE (100 μM) induced production of IFNγ in conditioned media from CBD lavage cells within 24 h (median, 263 pg/ml) compared to that from untreated cells (<25.6, $P < 0.0004$, $n = 14$) (Fig. 2). Lavage cells from BE-sensitized subjects showed no detectable IFNγ (<25.6 pg/ml, $n = 15$) in conditioned media either with or without BE challenge (Fig. 2A).

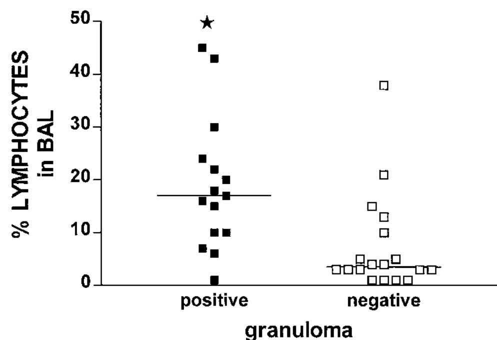

FIG. 1. Increased lymphocytes in CBD bronchoalveolar lavage. Percentages of lymphocytes in lavage preparations were determined in 15 CBD (granuloma positive) and 18 BE-sensitized non-CBD (granuloma negative) subjects. Medians are shown; $*P = 0.0004$ compared to granuloma negative.

NO attenuates BE-induced IFNγ responses. CBD lavage cells were challenged with BE in the presence or the absence of DNO (1.0 mM) (22). Results from studies of three CBD subjects indicated that DNO significantly ($P < 0.01$) reduced IFNγ levels in all cases (Fig. 2B). The mean percentage of reduction was 73.6 ± 4.2 (SEM)% ($P = 0.003$ compared to that in untreated cells; Table 1).

IL-18 production is stimulated by BE and attenuated by NO. Examination of lavage cell-conditioned media for the presence of IFNγ-stimulatory cytokines indicated that IL-18 was present and was significantly increased by BE challenge (Fig. 3A). IL-12 was below detectable levels in conditioned media either with or without BE challenge (<15.4 pg/ml; $n = 6$). In CBD lavage, BE elicited a 4-fold increase in IL-18 (median, 191 pg/ml) compared to control medium (45 pg/ml, $P = 0.03$, $n = 8$) (Fig. 3A). IL-18 levels of BE-sensitized lavage cells were also modestly increased by BE (71 pg/ml) (1.4-fold) compared to medium (49 pg/ml, $P < 0.01$, $n = 9$) (Fig. 3A). Levels of IL-18 in lavage cells from healthy controls were unchanged by BE (median, <25.6 pg/ml) (medium, <25.6 pg/ml, $n = 4$) (data not shown). Stimulation of lavage cell IL-18 by LPS was comparable (4.4-fold increase) in CBD and BE-sensitized groups (data not shown). In freshly isolated bronchoalveolar lavage fluids from which lavage cells had been removed, IL-18 values were below detectable levels (<25.6 pg/ml) in 4/5 CBD, 4/5 BE-sensitized, and 5/5 healthy controls.

The effects of NO on IL-18 were examined in lavage cells from three CBD subjects (Fig. 3B). DNO significantly decreased BE-stimulated lavage cell IL-18 levels in all cases. The mean percentage of reduction was 64.0 ± 3.9% ($P < 0.001$ compared to that in untreated cells) (Table 1).

Caspase 1 inhibitor attenuates IFNγ and IL-18 responses. Caspase 1 represents a potential target of NO and may be required for posttranslational processing of active IL-18 protein (21, 24). We therefore investigated whether BE-stimulated IL-18 or IFNγ responses required caspase 1. Treatment of lavage cells

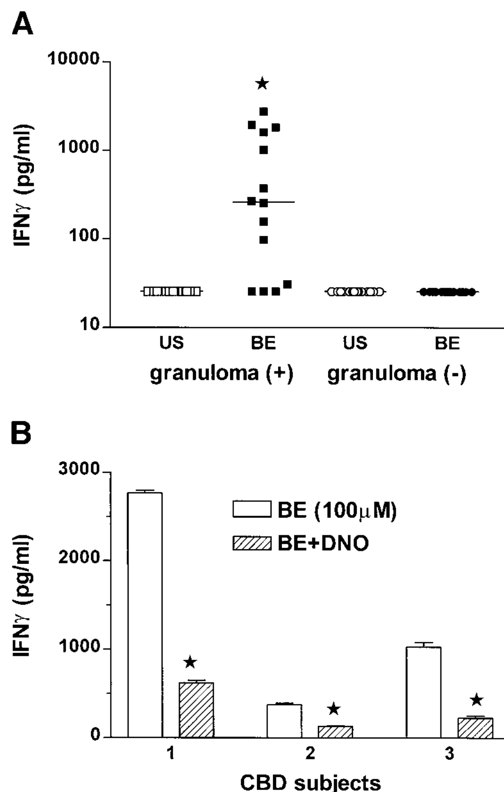

FIG. 2. IFNγ production in CBD lavage cells is enhanced by BE and reduced by DNO. (A) Lavage cells from 14 CBD (granuloma positive) and 15 BE-sensitized, non-CBD (granuloma negative) subjects were cultured for 24 h in medium alone (unstimulated, US) or in the presence of 100 μM $BeSO_4$ (BE). IFNγ levels (pg/ml) were determined in conditioned media by ELISA assay. Medians are shown; $*P = 0.0004$ compared to all other groups. (B) Lavage cells from 3 CBD subjects were cultured for 24 h with BE or BE + 1.0 mM DETA NONOate (DNO). IFNγ (pg/ml) was measured in conditioned media in duplicate for each subject. Means ± SD are shown; $*P < 0.01$ compared to BE alone.

TABLE 1

Percentage Reduction of BE-Stimulated Cytokines by DNO and Caspase 1 Inhibitor (Ac-YVAD-cho)

Treatment of BE-exposed CBD lavage cells	Percentage of reduction of	
	IL-18	IFNγ
Vehicle (DMSO)	8.5 ± 7.0	0
Ac-YVAD-cho (10 mM)	70.8 ± 7.5	43.1 ± 7.9
DNO (1.0 mM)	64.0 ± 3.9	73.6 ± 4.2

Note. Bronchoalveolar lavage cells from CBD patients were cultured for 24 h with 100 μM $BeSO_4$ (BE) in the presence or the absence of the reagents shown and conditioned media were evaluated for IL-18 and IFNγ ($n = 3$). Data represent percentages of reduction (± SEM) of cytokine compared to that of BE alone.

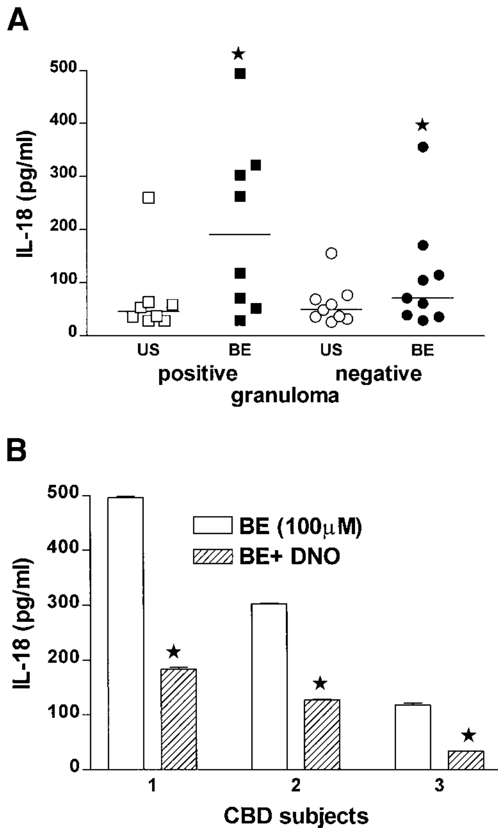

FIG. 3. Lavage cell IL-18 production is enhanced by BE and reduced by DNO. (A) Lavage cells from 8 CBD (granuloma positive), and 9 BE-sensitized, non-CBD (granuloma negative) subjects were cultured for 24 h in medium alone (unstimulated, US) or with 100 μM $BeSO_4$ (BE). IL-18 levels (pg/ml) were determined in conditioned media by ELISA assay. Medians are shown: $*P = 0.03$ compared to US in CBD; $*P = 0.008$ compared to US in BE-sensitized, non-CBD. (B). IL-18 levels (pg/ml) were determined in conditioned media from CBD lavage cells cultured for 24 h with BE or BE + 1.0 mM DETA NONOate (DNO). Means ± SDs are shown; $*P < 0.001$ compared to BE alone.

with the caspase 1 inhibitor Ac-YVAD-cho (10 mM) significantly ($P < 0.01$) reduced BE-induced IFNγ levels in 2/3 CBD lavage cell preparations compared to those in BE + vehicle (DMSO) (Fig. 4A). The mean reduction was 43.1 ± 7.9% compared to 0% for vehicle ($P = 0.03$) (Table 1). Ac-YVAD-cho also significantly ($P < 0.05$) reduced IL-18 levels in 3/3 CBD lavage cell preparations (Fig. 4B). The mean reduction of IL-18 was 70.8 ± 7.5% compared to that of vehicle (8.5 ± 7.0%, $P < 0.01$) (Table 1). Since caspase 1 is also involved in IL-1β processing, the possible involvement of IL-1β in BE-stimulated lavage responses was examined (24). BE challenge of CBD lavage cells showed no consistent evidence of IL-1β stimulation ($P > 0.05$, $n = 7$; data not shown).

Both DNO and Ac-YVAD-cho have been reported to be effective inhibitors of LPS-induced IL-1β (22, 25). To verify the efficacy of DNO and Ac-YVAD-cho in our experiments, reagents were tested for capacity to block IL-1β production by LPS-stimulated lavage cells. Portions of CBD lavage cells tested for IL-1β responses to BE were cultured separately for 24 h with LPS alone and LPS plus DMSO vehicle, DNO, or Ac-YVAD-cho. IL-1β levels in conditioned media were strongly stimulated by LPS (61.4-fold) and significantly reduced by either DNO (80.4 ± 9.1%) or Ac-YVAD-cho (76.1 ± 2.1%) compared to those in vehicle (0%, $P < 0.02$) (data not shown). Findings confirmed the efficacy of DNO and Ac-YVAD-cho in CBD lavage cell cultures and in addition indicated that the failure of BE to stimulate IL-1β was not due to impairment of CBD IL-1β responses.

The extent of DNO and Ac-YVAD-cho inhibition of BE-stimulated IL-18 was comparable, suggesting that caspase 1 constituted a major target of DNO activity against IL-18 (Table 1). Interestingly, the overall percentage of reduction of IFNγ by Ac-YVAD-cho (43.1 ± 7.9%) was significantly ($P = 0.03$) less than the percentage of reduction by DNO (73.6 ± 4.2%, Table 1). Increasing the dosage of Ac-YVAD-cho to 20 mM did

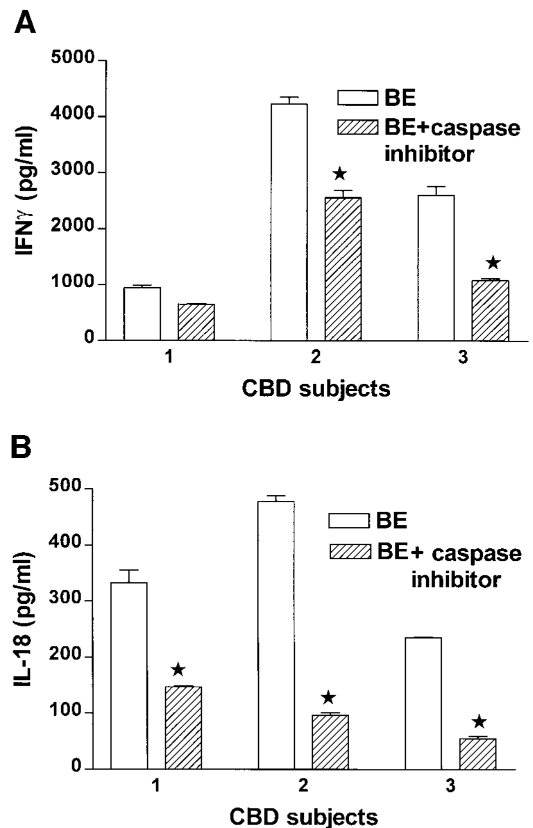

FIG. 4. Effects of caspase inhibitor on BE-stimulated cytokines. Lavage cells from 3 CBD subjects were cultured for 24 h with BE or BE + 10 mM Ac-YVAD-cho, a caspase 1 inhibitor. IFNγ and IL-18 were determined in conditioned media in duplicate for each subject. Means ± SDs are shown. (A) IFNγ. $*P < 0.001$ compared to BE alone. (B) IL-18. $*P < 0.001$ compared to BE alone.

not increase inhibition (data not shown). Data suggested that caspase 1 was not a major target of DNO activity against IFNγ.

BE induces IFNγ but not IL-18 in CBD PBMC. Previous studies have shown that PBMC of CBD patients also produce IFNγ in response to BE challenge (26). The question arose, therefore, whether PBMC IFNγ production would be accompanied by IL-18. CBD PBMC were cultured for 24–48 h in the presence and the absence of BE, and both IFNγ and IL-18 levels of conditioned media were measured (Fig. 5A). Despite dose-dependent IFNγ production in response to BE, no IL-18 was detectable (<25.6 pg/ml) up to 48 h (Fig. 5A). As observed previously in lavage cells, IL-12 was also not detectable in PBMC unless cells were stimulated with mitogen (data not shown).

To determine whether PBMC IFNγ production was sensitive to IL-18, recombinant IL-18 was added to 24-h PBMC cultures in the presence or the absence of BE. In the absence of BE, IL-18 at 10 and 50 ng/ml had no effects on IFNγ (Fig. 5B). In the presence of BE, however, IL-18 augmented IFNγ levels in a dose-dependent manner, suggesting an accessory function for IL-18 in BE stimulation of IFNγ (Fig. 5B).

Depletion of IL-18 only partially affects lavage cell IFNγ production. Although IL-18 was below detectable levels in PBMC-conditioned media, IL-18 was readily detectable in CBD lavage cells. To determine if IL-18 was required for lavage cell IFNγ responses, neutralizing monoclonal anti-IL-18 antibody or control, irrelevant murine IgG1 was added to CBD lavage cultures challenged with BE (Table 2). Anti-IL-18 treatment blocked 94.7 ± 0.7% (mean ± SEM, $P < 0.01$, $n = 2$ experiments) of IL-18 production but only 18.9 ± 12.7% of IFNγ output (Table 2). These results suggested that BE-induced IFNγ production was only partially dependent on IL-18.

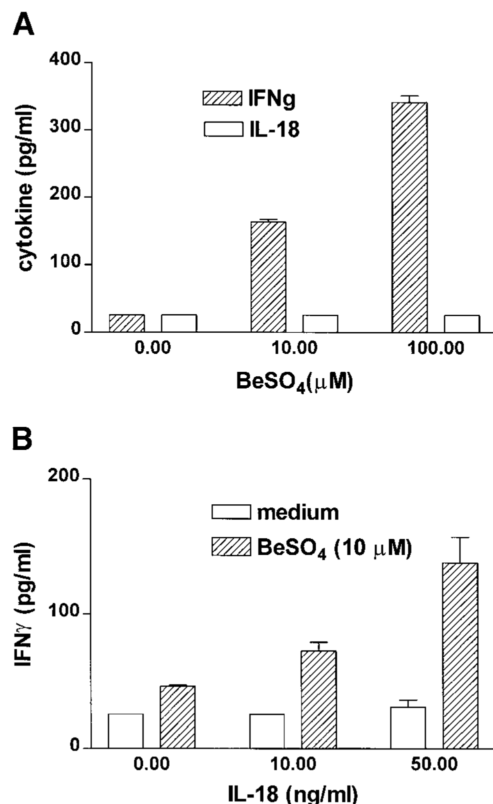

FIG. 5. Production of IFNγ by CBD peripheral blood mononuclear cells (PBMC). (A) IL-18 and IFNγ (pg/ml) were determined in conditioned media from PBMC cultured for 48 h with 0, 10, and 100 μM $BESO_4$ (B) IFNγ levels were determined in conditioned media from PBMC cultured for 24 h with or without BE and the doses of rIL-18 shown. Results from one of three CBD subjects are shown in each figure. Cytokine determinations were carried out in duplicate (means ± SD).

DISCUSSION

Data reported here are the first to describe a potential role for NO in regulating pulmonary IFNγ responses to a pathogenic antigen associated with pulmonary disease. A second novel finding is the demonstration that BE stimulates IL-18, a cytokine whose regulation is incompletely understood. Third, we show that the pulmonary cytokine repertoire elicited by BE in CBD differs from that of BE-exposed, non-CBD subjects even though both groups display systemic BE hypersensitivity. Finally, data indicate that BE-induced IFNγ, although highly sensitive to NO, is only partially reduced by IL-18 depletion, suggesting that IL-18 is not a major target of NO action in CBD and that NO mechanisms are likely to involve other sites.

IL-18, a member of the IL-1 family, participates in both innate and acquired immune activities (9, 27). IL-18 is elevated after bacterial and viral infections and strikingly increased in some forms of autoimmune disorders (28). While IL-18 actions have been extensively studied, less is known about the agents and signaling pathways responsible for upregulating IL-18. Our results indicate that BE-stimulated IL-18 secretion is dependent upon caspase 1 activity and that caspase 1 may be a major target of NO down-regulation of IL-18. IL-18 may also be stimulated by IFNγ itself through activation of the transcription factors AP-1 and IFN consensus sequence-binding protein (29). Thus, the fourfold increase in IL-18 observed in BE-challenged CBD lavage cells may be due in part to positive feedback by IFNγ. Finally, the modest but significant increase in IL-18 observed in the BE-sensitized, non-CBD study population requires further study. Whether BE stimulation of IL-18 occurs by similar or different pathways in CBD and non-CBD subjects is unknown. MHC involvement may represent a

TABLE 2

Effect of Anti-IL-18 Antibody against BE-Induced Cytokines

		Treatment of CBD lavage cells			
Experiment No.	Cytokine	Medium	$BeSO_4$ + control Ig	$BeSO_4$ + anti-IL-18	Percentage of reduction by anti-IL-18
1	IL-18	64 ± 1.5	482 ± 16.3	22 ± 3.2	95.4*
	IFNγ	<25.6	2542 ± 48	1745 ± 48.9	31.4
2	IL-18	53 ± 6.7	200 ± 1.0	13 ± 0.1	94.0*
	IFNγ	<25.6	191 ± 0	179 ± 3.2	6.3

Note. Data represent pg/ml (± SD) of IL-18 or IFNγ in 24-h conditioned media from CBD lavage cells exposed to 100 μM $BeSO_4$ in the presence of 1.0 μg/ml of control murine IgG1 or monoclonal anti-human IL-18 antibody. Lavage cells from two different CBD patients were studied.

* $P < 0.01$, $n = 2$.

key element in this issue since CBD but not BE hypersensitivity without disease has been associated with expression of the HLA-DPGlu69 phenotype (30).

The current findings concerning IL-12 in CBD may have diagnostic relevance with respect to other granulomatous diseases. The lack of detectable IL-12 production in CBD distinguishes it from sarcoidosis, a Th1-associated granulomatous disease of unknown cause. Elevated production of IL-12 has been reported in lavage cells from sarcoidosis patients (31, 32). The stimulatory effect of BE on lavage cell IL-18 in CBD may also prove to be of diagnostic importance in future studies. Taken together, our findings and those of others suggest that the cytokine requirements for maintenance of Th1-associated granulomatous diseases are not uniform but may vary with the pathogenic stimulus.

Elevated airway NO is present in a number of inflammatory lung diseases (33, 34). NO can reduce IFNγ production in human peripheral blood lymphocytes activated nonspecifically by anti-CD3 (20, 35). Despite such findings, the effects of NO on airway cell IFNγ responses to specific antigens associated with disease have not been investigated previously. Our prior studies demonstrated that the inflammatory cytokine production of LPS-stimulated human alveolar macrophages was sensitive to NO down-regulation (22). The current data indicate that NO may also have a potent dampening effect on the capacity of BE to induce IFNγ responses in CBD lavage cells and to stimulate lavage cell IL-18. Hypothetical relationships between NO and cytokines in CBD are depicted in Fig. 6. Results suggest that the increased NO found in CBD and other inflammatory lung diseases may represent an autoregulatory feedback mechanism down-regulating inappropriate cytokine production. The current observations further suggest that exploration of NO pathways regulating BE-induced IFNγ responses may provide insight into the nature of the CBD-associated immune response to beryllium and offer an opportunity to uncover novel targets for future therapeutic intervention.

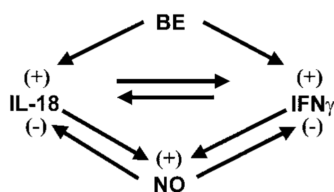

FIG. 6. Cytokine–NO signaling in CBD. Hypothetical relationships between NO and cytokines in CBD are depicted.

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