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Collagen Deposition in a Non-Fibrotic Lung Granuloma Model after Nitric Oxide Inhibition

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Recent studies support the concept that pulmonary granulomatous inflammation directed by interferon (IFN)-γ, interleukin (IL)-12, and nitric oxide usually resolves in the absence of fibrosis. To determine whether nitric oxide participates in modulating the fibrotic response during the development of pulmonary granulomas in response to purified protein derivative (PPD), mice presensitized to PPD received daily intraperitoneal injections of N^G-nitro-D-arginine-methyl ester (D-NAME), N^G-nitro-L-arginine-methyl ester (L-NAME), or aminoguanidine after delivery of PPD-coated beads to the lungs. Eight days later, morphometric analysis of lung granulomas revealed that L-NAME-treated mice when challenged with PPD *in vitro* for 36 hours had the largest pulmonary granulomas and the greatest collagen deposition among the treated groups. In addition, equivalent numbers of dispersed lung cells from L-NAME- and aminoguanidine-treated mice produced significantly higher levels of IL-4, monocyte chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-1α and significantly lower levels of eotaxin compared with D-NAME-treated mice. Cultures of dispersed lung cells from L-NAME-treated mice also produced significantly more IL-10 and less IL-12 compared with similar numbers of dispersed lung cells from D-NAME-treated mice. Cultures of isolated lung fibroblasts from L-NAME-treated mice expressed higher levels of C-C chemokine receptor 2 (CCR2) and CCR3 mRNA and contained less MCP-1 and eotaxin protein than a similar number of fibroblasts from D-NAME-treated mice. Thus, nitric oxide appears to regulate the deposition of extracellular matrix in lung granulomas through the modulation of the cytokine and chemokine profile of these lesions. Alterations in the cytokine, chemokine, and procollagen profile of this lesion may be a direct effect of nitric oxide on the pulmonary fibroblast and provide an important signal for regulating fibroblast activity during the evolution of chronic lung disease. ***(Am J Pathol 1998, 153:1861–1872)***

It is not uncommon for chronic pulmonary granulomatous inflammation to result in irreversible tissue injury and end-stage fibrosis.[1] For reasons that are presently unclear, the reparative process associated with interstitial pulmonary inflammation can progress uncontrollably, as evidenced by increased lung fibroblast proliferation and the deposition of collagenous material.[2] Clinical and laboratory evidence suggest that the progressive and unregulated reparative process in the lung is potentially related to the persistence of a variety of inflammatory signals.[3] Unfortunately, clinical strategies directed at preventing deleterious fibrotic responses through the nonselective inhibition of the inflammatory process with corticosteroid, cyclophosphamide, and azathioprine treatments have been both largely unsuccessful and often associated with severe side effects.[4] Previous studies of experimental, antigen-driven granulomatous pulmonary inflammatory responses has demonstrated that inflammatory cytokine profiles have a major role in the degree of extracellular matrix deposition around these lesions.[5] For example, the pulmonary cytokine response to purified peptide derivative (PPD) from *Mycobacteria* is dominated initially by interferon (IFN)-γ and interleukin (IL)-12, and little deposition of extracellular matrix results around Sephadex beads coated with PPD that are embolized in the lungs of PPD-sensitized mice. In contrast, the cytokine response to *Schistosoma mansoni* egg antigen (SEA)-coated beads also embolized to the lungs of SEA-sensitized mice are dominated by IL-4 and accompanied by pulmonary fibroblast activation and excessive extracellular matrix deposition within the interstitium.[5] Thus, the evolution of the fibrotic process within the lung appears to be inherently dependent on the counter-regulatory actions of specific cytokine profiles.[6]

Further examination of the mechanisms through which cytokine profiles dictate the pulmonary fibrotic response has revealed that the chemotactic cytokines or chemokines also play unique roles in this process.[7,8] Monocyte chemoattractant protein (MCP)-1 and macrophage in-

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flammatory protein (MIP)-1α are two such C-C chemokines that have garnered attention because of their demonstrable roles in experimental interstitial fibrosis. MCP-1 has been shown to be necessary for the development of fibrosis in the kidney.[9] Interestingly, studies by Gharaee-Kermani et al[10] have revealed that MCP-1 has direct stimulatory effects on *de novo* synthesis of transforming growth factor (TGF)-β, which in turn augments collagen generation by cultured rodent pulmonary fibroblasts. Other experimental studies have shown that MIP-1α clearly contributes to the pulmonary fibrotic response to bleomycin.[11] Clinical observations also support a role for MCP-1 and MIP-1α in pulmonary fibrosis as both are elevated in the bronchoalveolar lavage and open-lung biopsies from patients with interstitial lung fibrosis.[2,12–14] Eotaxin represents another C-C chemokine that may also exert a prominent role in pulmonary inflammation because of its ability to recruit eosinophils.[15] Although a causal link between eosinophils and pulmonary fibrosis has yet to be established, eosinophils are found in abundance in fibrotic tissue,[16] and activated eosinophils are a rich source of many inflammatory cytokines.[17] Thus, the cumulative balance of pro- and antifibrotic cytokines and chemokines during a pulmonary inflammatory response may be critical in regulating the tissue reparative process.

Endogenous nitric oxide synthesis is a key regulator of interstitial fibrotic responses in numerous organs, including the kidney,[18] heart,[19] and vasculature.[20] This free radical is produced by many cells through the utilization of L-arginine by at least three distinct nitric oxide synthase (NOS) isoforms.[21] The three major classifications of NOS are as follows: NOS I is a constitutive isoform found in neurons, NOS II is an inducible isoform found in activated macrophages and epithelial and smooth muscle cells, and NOS III is a constitutive isoform found in endothelial cells.[22] NOS I and III are calcium-dependent enzymes that produce nanomolar pulses of nitric oxide continuously, whereas NOS II is expressed only after gene induction and can be involved in the generation of micromolar quantities of nitric oxide that may be cytotoxic.[22] The major functions of NOS I through III include neurotransmission, microbicidal and tumoricidal effects, and vasoregulation, respectively.[21] At present, the precise role of each NOS isoform in pulmonary fibrotic disease is unknown, but immunohistochemical studies have shown that NOS II expression is increased whereas NOS III is decreased in patients with early- and intermediate-stage interstitial fibrotic disease.[23] Furthermore, the by-product of protein nitration by nitric oxide and superoxide, nitrotyrosine, has also been detected in interstitial fibrotic lung disease.[23] However, other studies suggest that exogenous nitric oxide has a beneficial role in idiopathic pulmonary fibrosis,[24] but only when it is administered in quantities that are similar to those generated by NOS I and III.[25,26] Thus, nitric oxide may have dichotomous effects on the pulmonary interstitial fibrotic process that are related to the enzymatic source of this mediator.

In this study, we have addressed the role of nitric oxide in the regulation of collagen deposition around PPD-bead pulmonary granulomas. In a previous study, we showed that NOS II was prominent in many cells in the lung by day 4 in the PPD-bead granuloma model, and the granulomatous response on day 4 was markedly augmented after the inhibition of nitric oxide synthesis.[27] Furthermore, the cytokine and chemokine profile of dissociated lung cells from N^G-nitro-L-arginine-methyl ester (L-NAME)-treated, PPD-challenged mice reflected a cytokine and chemokine profile more in line with that observed during a granulomatous response to SEA-beads in SEA-sensitized mice.[5] As previous studies have shown that increased IL-4, MCP-1, and MIP-1α are associated with the development of experimental lung fibrosis,[8] the specific aim of the present study was to determine whether the inhibition of nitric oxide would alter the resolution phase in the PPD-bead granuloma model and modify chemokine and chemokine receptor expression by isolated lung fibroblasts from mice with PPD-bead granulomas.

Materials and Methods

PPD-Bead Pulmonary Granuloma Model

Specific-pathogen free (SPF), female CBA/J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were maintained under SPF conditions before and during experiments. All mice received a subcutaneous and intraperitoneal injection of complete Freund's adjuvant (CFA; Sigma Chemical Co., St. Louis, MO) diluted 1:1 with normal saline as previously described in detail.[5] Immediately after the injection of CFA, all mice were fed a chemically defined diet from Zeigler Bros. (Gardiners, PA) that was deficient in L-arginine. Previous investigators have used this solid pellet chow to effectively reduce baseline nitric oxide synthesis in mice.[28] In the present study we observed that this diet did not alter the normal growth patterns of the mice over the entire course of the experiment, but it did markedly reduce detectable levels of urinary nitric oxide metabolites such as nitrite and nitrate (data not shown). Sixteen days after CFA sensitization, all mice received 3000 Sepharose 4B beads covalently coupled to purified protein derivative (PPD) from *Mycobacterium sp*. by intravenous injection. Introduction of PPD-coated beads in this manner ensures that the beads embolize to the lungs and elicit a granulomatous response. After the introduction of PPD-coated beads, the mice were divided into groups of five and received daily intraperitoneal injections of L-NAME, D-NAME, or aminoguanidine during the PPD-bead embolization period. All compounds were administered at a dose of 8 mg/kg as previously described.[27,29] L-NAME is a nondiscriminating inhibitor of all three isoforms of nitric oxide synthase, whereas D-NAME is a structural enantiomer of L-NAME that lacks nitric oxide synthase inhibitory actions.[30] Aminoguanidine has been used by a number of investigators to more selectively inhibit NOS II activity.[29,31–33] Aminoguanidine has also been shown to inhibit the induction of NOS II in the endotoxin-challenged lung.[34] L-NAME and aminoguanidine were used in the present study to determine the relative contribution of all

of the isoforms nitric oxide synthase (ie, NOS I, II, and III) as compared with the inducible nitric oxide synthase (ie, NOS II) on the development of extracellular matrix around PPD-bead granulomas. Based on results obtained by monitoring urine nitrite/nitrate levels, it appeared that both compounds equally reduced systemic nitric oxide production during the 8-day treatment protocol (data not shown). All mice were maintained on the L-arginine-deficient diet throughout the 8 days of PPD-bead challenge, after which whole lungs were removed and fixed for morphometric analysis or cultured for assessment of cytokine and chemokine synthetic capacity.

Morphometric Analysis and Masson Trichrome Staining of Collagen in Granulomatous Lungs

Whole lungs from D-NAME-, L-NAME-, or aminoguanidine-treated mice 8 days after PPD-bead administration were fully inflated by intratracheal administration of 4% paraformaldehyde. Lungs were then dissected out and fixed in fresh 4% paraformaldehyde for an additional 24 hours. Routine histological techniques were used to paraffin-embed this tissue, and 5-μm sections of whole lung were prepared for Masson trichrome staining as previously described.[35] At the conclusion of this staining protocol, lung sections were counterstained with Mayer's hemotoxylin (Mayer & Myles Laboratories, Coopersburg, PA) for the visualization and identification of nucleated cells composing the granulomas. Quantitative digital morphometric analysis of granulomas was performed using a protocol described in detail elsewhere.[27] A minimum of 10 granulomas were analyzed in each whole-lung tissue section, and granulomas were considered in this analysis if a full cross section of the bead nidus was visible. Using the application program IP Lab Spectrum-R4, images of the granulomas were captured, and copies of the image were made. The color wavelengths of the copied image were transformed into digital readings, allowing for quantification of the various color wavelengths using pixels as the unit of measure. Using the original image for comparison, the color spectra of each copied image was then adjusted until the collagen (highlighted in the original image by Masson trichrome staining) was green while the remaining granuloma was black. Percent collagen was then calculated by dividing the total pixel area of the granuloma by the pixel area corresponding to collagen.

Assessment of Cytokine and Chemokine Profiles from Dissociated Granulomatous Lungs

Granulomatous lungs from D-NAME-, L-NAME-, or aminoguanidine-treated mice were mechanically dissociated over steel mesh using a plunger from a 20-ml syringe. Red blood cells in the dissociated cell suspensions were lysed using a hypotonic lysing buffer (150 mmol/L NH_4Cl, 10 mmol/L $NaHCO_3$, 1 mmol/L EDTA) for 2 minutes at 4°C. The remaining cells were suspended in RPMI containing 10% fetal bovine serum and were added at a density of 4.0×10^5 cells/well in six-well tissue culture plates. The dispersed lung cells were then challenged with 3 μg/ml PPD for 36 hours at 37°C in a humidified CO_2 incubator as previously described[27,36] before 1-ml aliquots were removed and stored at −20°C before ELISA analysis.

Granuloma Lung Fibroblast Culture

Pulmonary granuloma fibroblasts from D-NAME- and L-NAME-treated mice were subsequently grown out from mixed lung cell primary cultures (see above), as previously described.[37] Briefly, these cells were transferred to 175-ml tissue culture flasks and were fed Dulbeco's modified Eagle's medium (DMEM) containing 1% (v/v) antibiotic/antimycotic and 15% (v/v) fetal bovine serum (FBS). After a minimum of three passages, homogeneous populations of fibroblasts were transferred to six-well tissue culture plates for experiments. Before use in any experiment, lung fibroblasts were transferred to two-well Labtek chamber culture slides and stained for α-actin, desmin, and α-naphthyl acetate esterase. After the third passage, lung fibroblasts stained for α-actin, suggesting a myofibroblast-type phenotype, but cultures of these cells were found to be completely free of α-naphthyl acetate esterase-positive cells such as macrophages (data not shown). After ensuring the homogeneity of fibroblast cultures, each well in a six-well tissue culture plate was initially seeded with approximately 1.0×10^6 fibroblasts. When cell confluence was reached, the DMEM was removed and IL-4 (R&D Systems, Minneapolis, MN) or IFN-γ (Genzyme, Cambridge, MA) suspended at 10 ng/ml in RPMI containing 10% FBS was added. Twenty-four hours later, cell-free supernatants were removed for ELISA measurements, and the adherent fibroblasts were washed and subjected to RNA isolation or prepared for flow cytometry. Cultured lung fibroblasts were used in these experiments up to the sixth passage.

Cytokine and Chemokine Measurement

Murine IL-4, IL-10, IL-12, MCP-1, MIP-1α, eotaxin, and TGF-β levels were determined in 50-μl supernatant samples from dispersed lung cells or purified lung fibroblasts using a standardized sandwich ELISA as previously described.[27] Briefly, Nunc-immuno ELISA plates (MaxiSorp) were coated with the appropriate cytokine capture antibody at a dilution of 1 μg/ml coating buffer (0.6 mol/L NaCl, 0.26 mol/L H_3BO_4, 0.08 mol/L NaOH, pH 9.6) for 16 hours at 4°C. Excess capture antibody was washed away and each plate was blocked for 90 minutes with 2% bovine serum albumin (BSA)/PBS at 37°C. After the blocking period, each ELISA plate was washed with PBS/Tween 20 (0.05%, v/v), and 50-μl samples (no dilution or 1:10) were added to wells in duplicate for 1 hour at 37°C. Recombinant murine IL-4, IL-10, IL-12, MCP-1, MIP-1α, eotaxin, and TGF-β standard curves were used to calculate cytokine concentrations. The plates were then thoroughly washed, and the appropriate biotinylated poly-

clonal rabbit anti-cytokine antibody (3.5 μg/ml) was added. After washing the plates 30 minutes later, streptavidin-peroxidase (Bio-Rad Laboratories, Richmond, CA) was added to each well for 30 minutes, and each plate was thoroughly washed again. Chromogen substrate (Bio-Rad Laboratories) was added and plates were read on an ELISA plate scanner at 492 nm. The limit of detection for each cytokine was consistently above 50 pg/ml.

Reverse Transcription Polymerase Chain Reaction

mRNA expression in the lung fibroblasts derived from D-NAME- or L-NAME-treated mice with PPD-bead lung granulomas were examined using reverse transcription polymerase chain reaction (RT-PCR). Briefly, total RNA was isolated from cultured fibroblasts and 1 μg of total RNA was reverse transcribed to yield cDNA using techniques previously described in detail.[27] The following sense and antisense primers, respectively, were used in the PCR reaction: β-actin sense, 5′-GTGGGGCGCCCCAGGCACCA-3′, and antisense, 5′-GCTCGCCCGTGGTGGTGAAGC-3′ (450-bp product); CCR2 sense, 5′CACGAAGTATCCAAGAGCTT3′, and antisense, 5′CATGCTCTTCAGCTTTTTAC3′ (422-bp product); and CCR3 sense, 5′TGGGCAACATGATGGTTGTG3′, and antisense, 5′GCTGTCTTGAGACTCATGGA3′ (385-bp product); procollagen type Iα sense, 5′ TCGTGACCGTGACCTTGCG3′, and antisense, 5′GGATGAGTCGGCAGACACGGA3′ (255-bp product); and procollagen type III sense, 5′ GCTCAGAGTAGCACCATCAG3′, and antisense, 5′GGCTGATGTACACATGCTCC3′ (220-bp product).

PCR samples were initially incubated at 94°C for 5 minutes and then cycled 27 times through denaturation at 95°C for 30 seconds, annealing at 58°C for 45 seconds, and extension at 72°C for 75 seconds. PCR products were then separated on 2% agarose gels containing 0.3% ethidium bromide, and the bands corresponding to the intended products were photographed under ultraviolet illumination.

Statistical Analysis

Data are means ± SEM (SE). Each group contained a minimum of five mice, and statistical analysis was performed using analysis of variance. Multiple comparisons were made using the Dunnett's test. An associate *P* value of ≤0.05 was considered significant.

Results

The Size of the PPD-Bead Granuloma Is Significantly Increased after L-NAME Treatment

Our previous studies showed that the cellularity of the PPD-bead granuloma on day 4 after bead embolization was significantly increased by the inhibition of nitric oxide using L-NAME.[27] The increased cellularity of the pulmonary granulomas in L-NAME-treated mice on day 4 was attributable to augmented numbers of granulocytes such as neutrophils and eosinophils in the lesion. Thus, we first determined whether prolonged inhibition of nitric oxide using an L-arginine-deficient diet in combination with either L-NAME or aminoguanidine in the PPD-bead model was associated with altered pulmonary granuloma size at day 8 after PPD-bead administration. Previous characterization of this granuloma model showed that the inflammatory infiltrate around the PPD-bead is normally resolved by day 8.[5] Consistent with these early observations, the PPD-bead pulmonary granulomas in D-NAME-treated mice, also maintained on the L-arginine-deficient diet, showed little inflammatory response around the day 8 PPD-bead granuloma (Figure 1A). In contrast, mononuclear and polymorphonuclear inflammatory cells were present around PPD beads in L-NAME-treated mice (Figure 1B). Fewer inflammatory cells were detected around beads in aminoguanidine-treated mice (Figure 1C). Morphometric analysis of pulmonary granulomas in L-NAME-treated mice showed that the mean size of these lesions was 24-fold larger than the pulmonary granulomas in the D-NAME-treated group (Figure 2). Although the pulmonary lesions in the aminoguanidine-treated mice were larger than those in the D-NAME-treated mice (Figure 2), this difference did not reach statistical significance. These data suggested that only the nonselective inhibition of nitric oxide synthases (presumably NOS I, II, and III) using L-NAME was associated with increased inflammatory cell number around the PPD-bead.

PPD-Bead Pulmonary Granulomas Contain Collagen after L-NAME and Aminoguanidine Treatment

Masson trichrome staining was used to reveal the deposition of collagen in whole-lung sections from each treatment group. Whereas collagen deposition was detectable in granulomas from each of the treatment groups (see Figure 1, A–C), marked differences in the percentage of collagen present in the pulmonary lesions from the various treatment groups were noted. As summarized in Table 1, 5% of the total area of the pulmonary granulomas analyzed in L-NAME-treated mice was occupied by collagen, a statistically significant increase in collagen content above that measured in D-NAME-treated mice as less than 0.2% of the cross-sectional area of the granulomas found in D-NAME-treated mice was composed of collagen (Table 1). In aminoguanidine-treated mice, the collagen content was calculated to be almost 3% of the total granuloma area. Overall, these data suggested that the inhibition of nitric oxide with either the nonselective L-NAME or the NOS-II-selective aminoguanidine was associated with increased collagen deposition around the PPD-bead. In the next series of experiments, we examined whether nitric oxide regulated the production of profibrotic cytokines and chemokines by dispersed lung granuloma cells from each of the treatment groups.

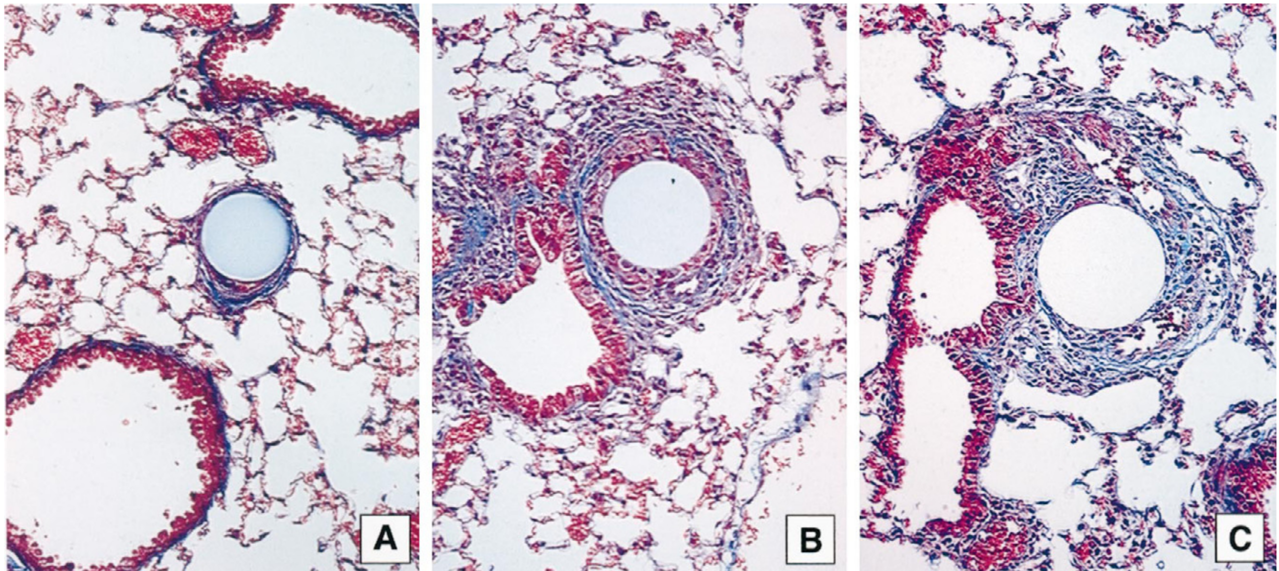

Figure 1. Composite of PPD-bead granulomas from D-NAME-treated (A), L-NAME-treated (B), and aminoguanidine-treated (C) mice. All mice were maintained on an L-arginine-deficient diet for the duration of the study, and mice received D-NAME, L-NAME, or aminoguanidine for the 8-day PPD-bead challenge period. Histological sections of whole lungs from all three treatment groups on day 8 of the PPD-bead challenge period were subjected to Masson trichrome staining, and collagen content in these pulmonary lesions is highlighted by the light blue coloration. Approximately 95% of the cross-sectional area of the granulomas in the D-NAME-treated mice was occupied by the Sepharose bead alone. Furthermore, PPD-bead granulomas in L-NAME- and aminoguanidine-treated mice contained significantly more collagen than similar granulomas in D-NAME-treated mice (see Table 1).

Effect of Nitric Oxide Inhibition on Cytokine Generation by Dispersed Lung Cells

Previous studies have shown that the cytokine profile in the lung is altered after the initiation of antigen-specific granulomatous inflammation.[5] In the PPD-bead model, the inflammatory response at day 4 surrounding PPD-coated beads is dominated by IFN-γ and IL-12.[38] However, the inhibition of nitric oxide synthesis with L-NAME in the PPD-bead model significantly decreased the amounts of both IFN-γ and IL-12 in cultures of dispersed lung cells.[27] In the present study, IFN-γ was not detected in any cultures of dispersed lung cells from day 8 granulomas rechallenged with 3 μg/ml PPD (data not shown), but IL-4, IL-10, and IL-12 were present in cell-free supernatants removed from these cultures 36 hours later (Figure 3, A–C). IL-4 levels were significantly augmented in cultures of dispersed cells from L-NAME- and aminoguanidine-treated mice above those levels detected in cultures of cells removed from D-NAME-treated mice (Figure 3A). When compared with cell supernatants from cultures containing dispersed lung cells from D-NAME-treated mice, significantly more IL-10 was detected in cultures from L-NAME-treated mice whereas significantly less IL-10 was detected in cultures from aminoguanidine-treated mice (Figure 3B). Dispersed lung cells from L-NAME-treated mice also generated significantly less IL-12 than similar numbers of cells from D-NAME- and aminoguanidine-treated mice (Figure 3C). Thus, these data suggest that IL-4 production was greater in the cultures of lung cells taken from animals receiving either pharmacological inhibitor of nitric oxide.

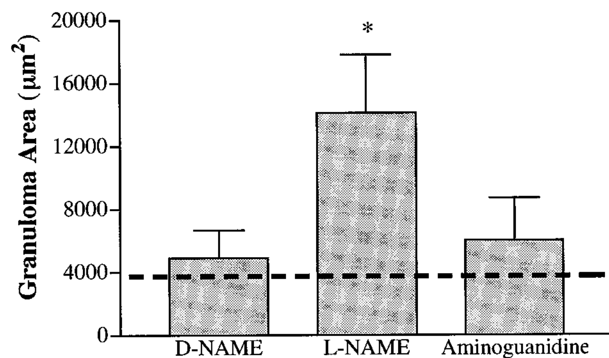

Figure 2. Pulmonary granuloma cross-sectional area (μm^2) in mice presensitized to PPD from *Mycobacteria*, maintained on an L-arginine-deficient diet, and intravenously injected with PPD-coated beads. For 8 days after PPD-bead administration, mice received D-NAME, L-NAME, or aminoguanidine. The dotted line shows the mean size of the Sepharose beads in the pulmonary granulomas. Data shown are means ± SEM from at least three mice. *$P \leq 0.05$ compared with D-NAME-treated group.

Table 1. Percentage of Collagen Content Present in Pulmonary Granulomas from Mice Presensitized to Purified Peptide Derivative (PPD) from *Mycobacteria* and Intravenously Injected with PPD-Coated Beads

Drug treatment group	Collagen content of granuloma (%)
D-NAME	0.2 ± 0.3
L-NAME	4.7 ± 2.0*
Aminoguanidine	2.5 ± 1.2

For 8 days after PPD-bead administration, mice received D-NAME, L-NAME, or aminoguanidine, and all mice were maintained on an L-arginine-deficient diet. Collagen was visualized using a Masson trichrome stain, and the percentages of collagen were calculated by dividing the area of staining by the total area of the granuloma (see Materials and Methods). Data shown are means ± SEM from at least three mice.

*$P \leq 0.05$ compared with D-NAME-treated group.

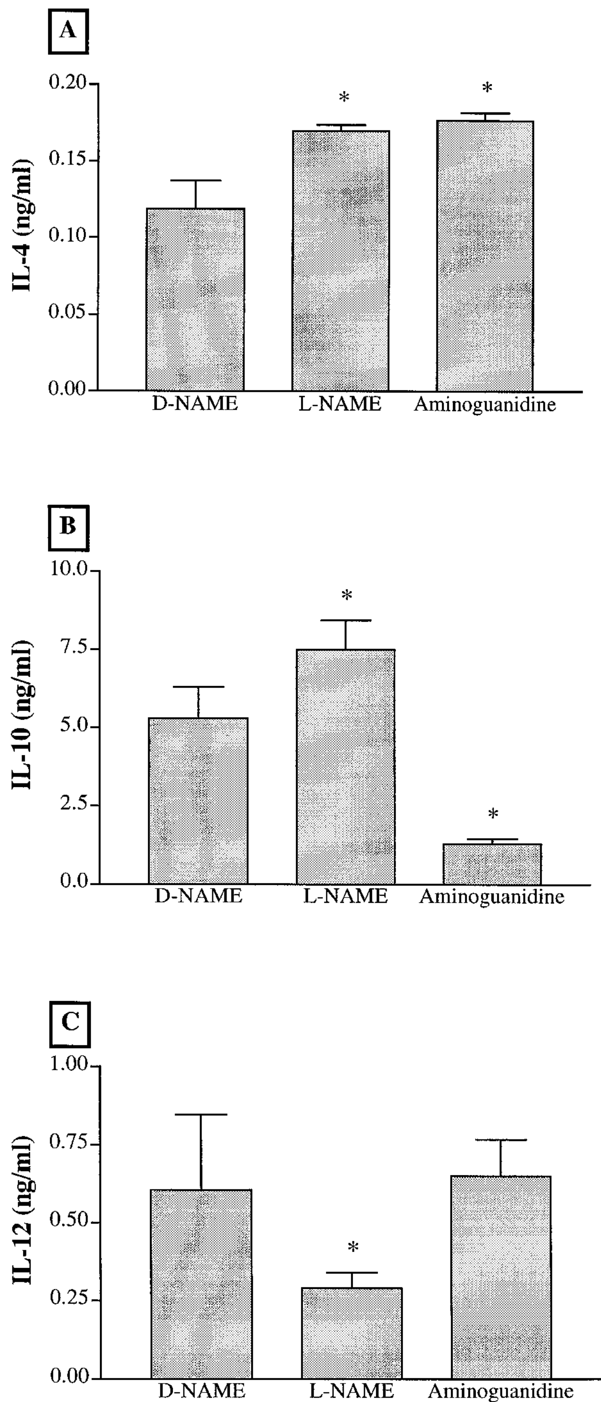

Figure 3. IL-4 (A), IL-10 (B), and IL-12 (C) levels in cell-free supernatants from cultures containing dispersed lung cells from D-NAME-, L-NAME-, and aminoguanidine-treated mice with PPD-bead pulmonary granulomas. All mice were maintained on an L-arginine-deficient diet for the duration of the study, and mice received D-NAME, L-NAME, or aminoguanidine for 8 days after PPD-bead administration. Whole lungs were removed from each group and mechanically dissociated, and 4.0×10^5 dissociated lung cells/well of a six-well tissue culture plate were cultured in the presence of PPD for 36 hours. Data shown are means ± SEM from triplicate wells from each treatment group. $^*P \leq 0.05$ compared with D-NAME-treated group.

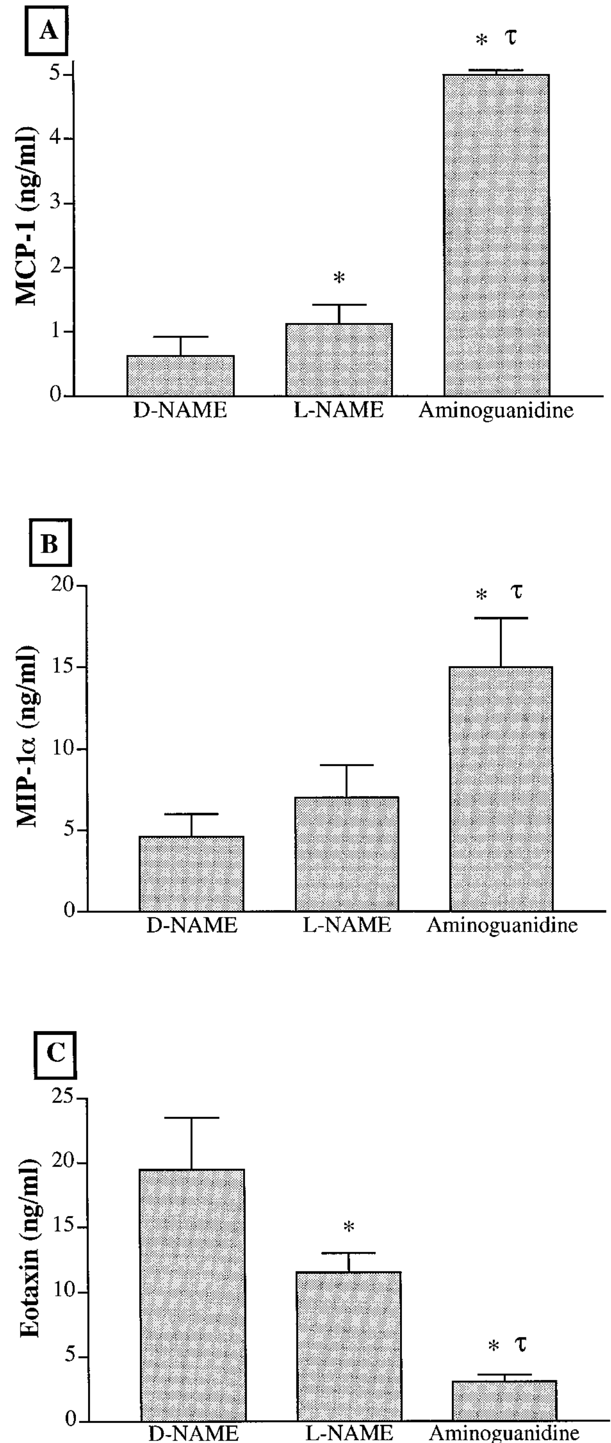

Figure 4. Changes in MCP-1 (A), MIP-1α (B), and eotaxin (C) levels in cell-free supernatants from cultures containing dispersed lung cells from D-NAME-, L-NAME-, and aminoguanidine-treated mice with Th1-type pulmonary granulomas. All mice were maintained on an L-arginine-deficient diet for the duration of the study, and mice received D-NAME, L-NAME or aminoguanidine for 8 days after PPD-bead administration. Whole lungs were removed from each group and mechanically dissociated, and 4.0×10^5 dissociated lung cells/well of a six-well tissue culture plate were cultured in the presence of PPD for 36 hours. Data shown are means ± SEM from triplicate wells from each treatment group. $^*P \leq 0.05$ compared with D-NAME-treated group. $^{\tau}P \leq 0.05$ compared with D-NAME-treated group.

Inhibition of Nitric Oxide Synthesis in the PPD-Bead Granuloma Model Increases Monocyte Chemoattractant Protein-1 and Macrophage Inflammatory Protein-1α

Further examination of cell-free supernatants from dispersed lung cells removed from D-NAME-, L-NAME-, and aminoguanidine-treated mice on day 8 of the PPD-bead granulomatous response revealed differences in the accumulation of C-C chemokines such as MCP-1, MIP-1α, and eotaxin. Dispersed lung cells from L-NAME- and aminoguanidine-treated mice released significantly more MCP-1 than a similar quantity of cells removed from the D-NAME treatment group after PPD rechallenge for 36 hours (Figure 4A). MIP-1α levels in cultures of dispersed lung cells from aminoguanidine-treated mice were significantly increased approximately threefold above the other two treatment groups (Figure 4B). In contrast to the above two C-C chemokines detected in these cultures, eotaxin levels in dispersed lung cell cultures from L-NAME- and aminoguanidine-treated mice were significantly reduced by twofold compared with levels measured in cultures containing similar numbers of cells from the D-NAME treatment group (Figure 4C). In the next series of experiments, cultured fibroblasts from the L-NAME- and D-NAME-treated groups were examined to determine whether these cells were a potential source of MCP-1, MIP-1α, and eotaxin and, in turn, had the ability to recognize these chemokines through the expression of chemokine receptors.

Purified Lung Fibroblasts from L-NAME- and D-NAME-Treated Mice with PPD-Bead Granulomas Release MCP-1 and Eotaxin

Lung fibroblasts from L-NAME- and D-NAME-treated mice were also examined for changes in MCP-1, MIP-1α, and eotaxin production after cytokine stimulation for 24 hours. MIP-1α was not detected in any of the cultures of fibroblasts under any of the conditions tested (data not shown). Cultures of purified lung fibroblasts from the D-NAME and L-NAME treatment groups contained equivalent amounts of MCP-1 when cultured without the addition of cytokines (Figure 5A). However, after IL-4 or IFN-γ treatment of both types of fibroblasts in culture for 24 hours, significantly more MCP-1 was detected in cell-free supernatants removed from fibroblasts cultured from D-NAME-treated mice compared to similar numbers of fibroblasts cultured from L-NAME-treated mice. A constitutive level of approximately 50 pg/ml eotaxin was detected only in cultures of fibroblasts from L-NAME-treated mice. After *in vitro* IL-4 treatment of both fibroblast types, eotaxin levels in the culture supernatants exceeded 1.5 ng/ml, and the greater levels of eotaxin were detected in cultures of fibroblasts from D-NAME-treated mice. Eotaxin levels were identical in both cultures of fibroblasts after IFN-γ treatment for 24 hours (Figure 5B).

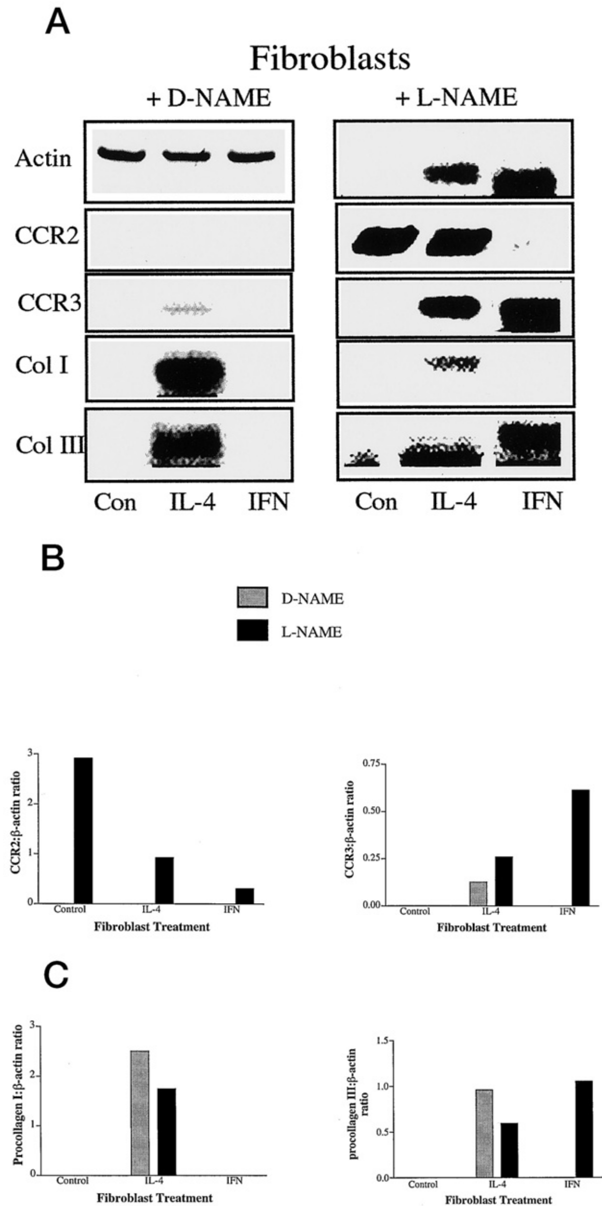

Figure 5. RT-PCR analysis of CCR2, CCR3, and procollagen type I and type III mRNA expression in purified lung fibroblasts from D-NAME- and L-NAME-treated mice (A). All mice were maintained on an L-arginine-deficient diet for the duration of the study, and mice received D-NAME, L-NAME, or aminoguanidine for 8 days after PPD-bead administration. Lung fibroblasts were grown out from cultures of dispersed lung cells from day 8 PPD-bead granulomas. Purified fibroblast cultures were left untreated or treated with IL-4 or IFN-γ for 24 hours before mRNA isolation. Fibroblasts from L-NAME-treated exhibited strong CCR2 and CCR3 mRNA expression (see B for densitometry analysis of RT-PCR) but less procollagen I and III expression than fibroblasts from D-NAME-treated mice. However, procollagen gene expression was noted in cultures of fibroblasts from L-NAME-treated mice after exposure of these cells to IFN-γ for 24 hours.

Increased CCR2, CCR3, and Procollagen Gene Expression in Fibroblasts Isolated from L-NAME-Treated Mice with PPD-Bead Granulomas

As the greatest differences in granuloma size and collagen deposition in the Th1-type pulmonary granuloma model were observed between the D-NAME and L-NAME

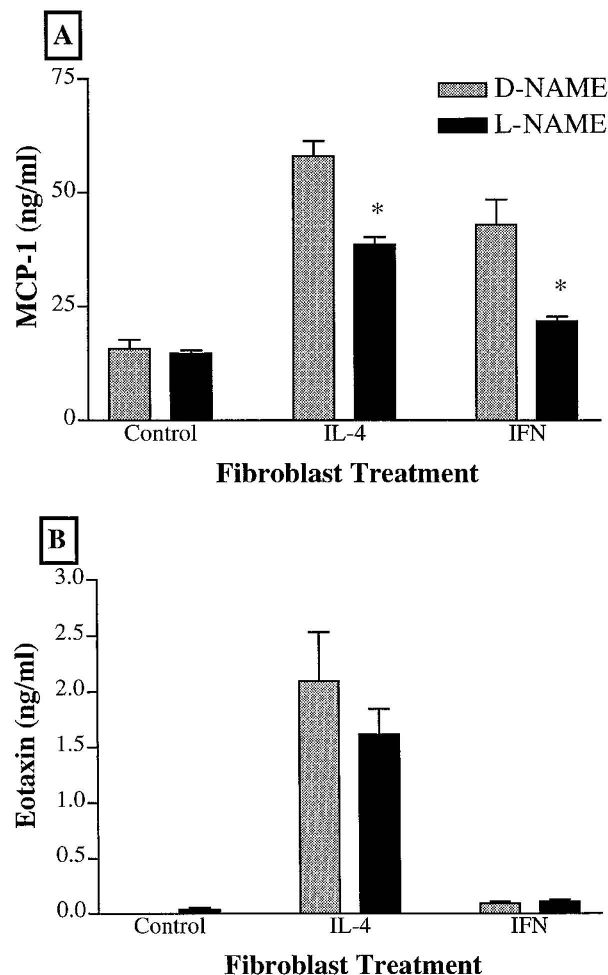

Figure 6. MCP-1 (**A**) and eotaxin (**B**) levels in cell-free supernatants in cultures of purified lung fibroblasts from D-NAME- or L-NAME-treated mice. All mice were maintained on an L-arginine-deficient diet for the duration of the study, and mice received D-NAME, L-NAME, or aminoguanidine for 8 days after PPD-bead administration. Lung fibroblasts were grown out from cultures of dispersed lung cells from day 8 PPD-bead granulomas. Purified fibroblasts cultures were left untreated or treated with IL-4 or IFN-γ for 24 hours before removal of cell-free supernatants for ELISA. Cultures of lung fibroblasts from L-NAME-treated mice with PPD-bead granulomas generated significantly less MCP-1 after cytokine activation than similar numbers of fibroblasts derived from D-NAME-treated mice (**A**). Similarly, less eotaxin was present in cultures of fibroblasts from L-NAME-treated mice than cultures of fibroblasts from D-NAME-treated mice (**B**). Data shown are means ± SEM of three separate experiments.

treatment groups, we examined whether these changes were related to differences in the expression of CCR2 and CCR3 mRNA by purified lung fibroblasts with or without *in vitro* cytokine treatment for 24 hours. CCR2 and CCR3 are the receptors that recognize MCP-1 and eotaxin, respectively. MIP-1α will also bind to CCR3 in the mouse. In pilot studies, these two C-C chemokine receptors were expressed on a higher proportion of normal fibroblasts than other chemokine receptors such as CCR1, CCR4, and CCR5 (C.M. Hogaboam, personal observations). Changes in procollagen I and III gene expression by these fibroblasts were also examined under the same conditions. Figure 6 is a composite of RT-PCR results obtained from cultured fibroblasts grown from day 8 PPD-bead granulomas in D-NAME- and L-NAME-treated mice showing a number of differences in constitutive and cytokine-induced gene expression between these two fibroblast types. First, CCR2 mRNA was not detected in fibroblasts cultured from D-NAME-treated mice, whereas CCR2 mRNA expression was present in cultured fibroblasts from L-NAME-treated mice under all *in vitro* conditions. Based on the ratios of CCR2 to β-actin expression shown in Figure 6B, untreated lung fibroblasts from mice that received L-NAME *in vivo* exhibited the greatest level of CCR2 expression. Next, CCR3 gene expression by both fibroblast types was apparent when IL-4 was present for 24 hours, but the highest expression of CCR3 mRNA was measured in fibroblasts from L-NAME-treated mice that were exposed to IFN-γ for 24 hours in culture (Figure 6B). Finally, procollagen types I and III gene expression were not detected in control cultures of fibroblasts from both *in vivo* treatment groups. Procollagen types I and III mRNA were present in fibroblasts from both mouse treatment groups when these cells received IL-4 in culture. However, greater procollagen gene expression was observed in the fibroblasts from the D-NAME-treated mice when compared with similar numbers of fibroblasts from L-NAME-treated mice (Figure 6B). Interestingly, exposure of fibroblasts from L-NAME-treated mice to IFN-γ for 24 hours appeared to stimulate the gene expression of procollagen III. This effect was not observed in cultures of fibroblasts from D-NAME-treated mice (Figure 6, A and B).

Increased TGF-β Synthesis by Fibroblasts Isolated From L-NAME-Treated Mice with PPD-Bead Granulomas

Fibroblasts from both treatment groups were also examined for the generation of the profibrotic cytokine TGF-β. TGF-β was detected under control conditions in cultures of fibroblasts from D-NAME- and L-NAME-treated mice (Figure 7). After IL-4 treatment, TGF-β levels were increased significantly (sevenfold) in cultures of fibroblasts from L-NAME-treated mice but not in cultures from D-NAME-treated mice. The presence of IFN-γ in cultures of fibroblasts cultured from D-NAME-treated mice abolished TGF-β levels, but a similar treatment did not affect TGF-β synthesis by fibroblasts from L-NAME-treated mice (Figure 7).

Discussion

Nitric oxide synthesis is increased in a variety of experimental and clinical pulmonary inflammatory diseases,[23,39] but it appears that nitric oxide can exert both beneficial and deleterious effects within the lung. The increased presence of NOS II in the inflamed lung has been shown to contribute to complement-induced pulmonary injury,[40] lung transplant rejection,[41] and airway hyperresponsiveness in asthma.[42,43] Conversely, others[44] have shown that increased nitric oxide production by lung epithelial cells protects these cells from polymorphonuclear-cell-mediated injury. Increased nitric oxide

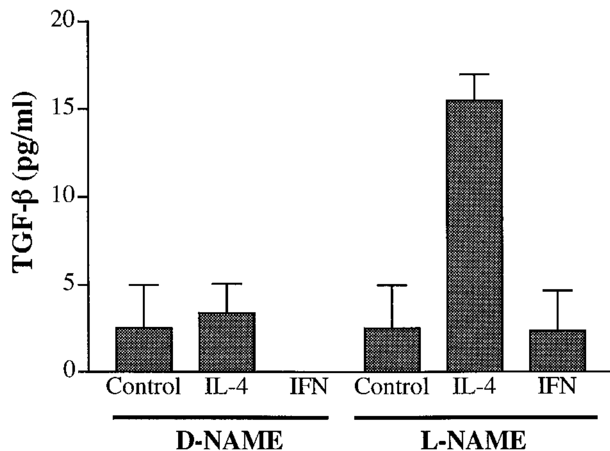

Figure 7. TGF-β levels in cell-free supernatants from cultures of fibroblasts isolated from D-NAME- or L-NAME-treated mice. All mice were maintained on an L-arginine-deficient diet for the duration of the study, and mice received D-NAME, L-NAME, or aminoguanidine for 8 days after PPD-bead administration. Lung fibroblasts were grown out from cultures of dispersed lung cells from day 8 PPD-bead granulomas. Purified fibroblasts cultures were left untreated or treated with IL-4 or IFN-γ for 24 hours before removal of cell-free supernatants for ELISA. Cultures of lung fibroblasts from L-NAME-treated mice with PPD-bead granulomas generated significantly more TGF-β after IL-4 activation than similar numbers of fibroblasts derived from D-NAME-treated mice. Data shown are means ± SEM of three separate experiments.

also prevents the full expression of cytokines by epithelial cells during experimental respiratory distress.[45] Although previous studies have shown that nitric oxide is important in containing or inhibiting the fibrotic response in the kidney during obstructive nephropathy[18] and in the heart,[20,46] the exact role of nitric oxide in pulmonary fibrotic events is not known. Previous immunohistochemical evidence indicates that augmented nitric oxide synthesis promotes the formation of a tissue-damaging oxidant, peroxynitrite, in pulmonary tissue from patients with idiopathic pulmonary fibrosis (IPF).[23] However, the results from the present study indicate that nitric oxide from constitutive and/or inducible nitric oxide synthases regulates the fibrotic process, as evidenced by collagen deposition, within the lung. We observed that the inhibition of nitric oxide synthesis using L-NAME, a nonselective nitric oxide synthase inhibitor, resulted in significantly larger pulmonary granulomas around PPD-coated beads compared with pulmonary lesions from D-NAME-treated mice. This increased cellularity of the day 8 PPD-bead granuloma in L-NAME-treated mice is consistent with our previous study of the day 4 granulomatous response in the context of nonselective nitric oxide depletion.[27] In addition, treatment of mice with a more selective NOS II inhibitor, aminoguanidine,[31] resulted in a PPD-bead lesion that was not more cellular but contained significantly more collagen as compared with PPD-bead lesions in D-NAME-treated mice. It should be noted that the amount of collagen deposition around the PPD-bead granuloma in aminoguanidine-treated mice may have been reduced by the inhibitory effects of aminoguanidine on collagen formation.[47] Thus, the inhibition of nitric oxide production by all three isoforms of nitric oxide synthase or the inducible isoform alone significantly augmented the deposition of collagen around a PPD-bead pulmonary lesion that is not associated with fibrosis.[48]

The manner in which the inhibition of nitric oxide leads to increased collagen deposition around the PPD-bead granuloma is not presently known, but promotion of profibrotic cytokine synthesis in the lung during nitric oxide inhibition may have played a prominent role. The onset of fibrosis during clinical or experimental lung disease appears to follow increases in IL-4, tumor necrosis factor-α and TGF-β levels.[49] Experimental pulmonary granuloma formation is also dependent on the initial cytokine profile elicited.[1,38] The PPD-bead granulomatous response is initially dominated by IFN-γ with little IL-4 synthesis, and this lesion typically resolves without evidence of extracellular matrix deposition around the bead. Interestingly, the resolution of the PPD-bead granuloma can be reversed by the transgene-induced overexpression of IL-4 in the lung.[50] Under conditions of increased IL-4 expression, the PPD-granuloma is markedly increased in size, contains eosinophils, and has increased procollagen gene expression.[50] In the present study, an increase in IL-4 levels was measured in cultures containing dissociated lung cells from L-NAME- and aminoguanidine-treated mice, as compared with cultures of similar numbers of dispersed lung cells from D-NAME-treated controls. In addition, a clear shift in the cytokine profile was apparent in dispersed lung cell cultures from L-NAME-treated mice as IL-4 and IL-10 were significantly increased whereas IL-12 was significantly decreased. It is of note that levels of all cytokines measured in cultures of dispersed lung cells from day 8 PPD-bead lesions were markedly lower than cytokine levels measured in cultures of similar cells from day 4 PPD-bead lesions,[27] confirming earlier studies showing that this lesion is nearly completely resolved by day 8.[5] Thus, the appearance of collagen in the PPD-bead pulmonary granuloma may have resulted from an alteration in the cytokine profile from an antifibrotic (ie, IL-12) to a profibrotic (ie, IL-4) phenotype within the lung.

Recent evidence also points to a unique role for chemotactic cytokines in fibrotic responses.[9] Whereas many investigators have shown that MCP-1 expression is markedly augmented in IPF,[2,12,13,51] its precise role in this pulmonary fibrotic disease is presently unknown. Pulmonary granuloma responses in mice characterized by collagen deposition are MCP-1 dependent,[48] and MCP-1 may promote the fibrotic process through the recruitment of T cells and monocytes,[52] modulation of T cell cytokine synthesis,[53–55] and T cell adhesion to extracellular matrix.[56] In the present study, C-C chemokine generation by PPD-stimulated dispersed lung cells was altered by the *in vivo* pharmacological inhibition of nitric oxide synthesis. MCP-1 was significantly increased in cultures of lung cells from L-NAME-treated mice as compared with cells from D-NAME-treated mice, and the levels of MCP-1 in cultures of cells from aminoguanidine-treated mice were fivefold higher than either of the other treatment groups. The effects of nitric oxide inhibition on MCP-1 generation in fibroblast cultures are consistent with the previous findings of Zeiher et al[57] who observed that nitric oxide modulates MCP-1 expression in vascular endothelial cells through an oxidant-sensitive transcription mecha-

nism. MIP-1α levels in the cultures of dispersed lung cells from aminoguanidine-treated mice were approximately threefold higher than levels measured in dispersed cell cultures from D-NAME- and L-NAME-treated mice. Thus, modulation of nitric oxide production by constitutive and/or inducible nitric oxide synthases during a PPD-bead pulmonary granuloma response promotes the increased synthesis of known profibrotic C-C chemokines such as MCP-1 and MIP-1α from dispersed lung cells.

Previous observations suggest that nitric oxide also directly regulates fibroblast activation.[58–60] In the present study, it was observed that cultured fibroblasts were an excellent source of MCP-1 and eotaxin and that these cells respond to IL-4 and IFN-γ, two potent regulators of the fibrotic process. However, the regulatory effect of nitric oxide on fibroblasts may be related to its effects on the expression of chemokine receptor expression by these cells. CCR2 is the receptor for MCP-1[61,62] that is required for a normal PPD-bead granuloma response.[63] CCR3 was originally described as the receptor for eotaxin, but in the mouse CCR3 will bind MIP-1α.[64] Using RT-PCR, CCR2 and CCR3 gene expression were detected in fibroblasts cultured from L-NAME-treated mice, but mRNA for both chemokine receptors was largely absent from lung fibroblasts derived from D-NAME-treated mice. Therefore, nitric oxide appears to modulate CCR2 and CCR3 expression by fibroblasts, which in turn may prevent fibroblasts from responding to the presence of profibrotic chemokines.

The observation that procollagen types I and III were elevated in the IL-4-pretreated pulmonary fibroblasts from PPD lesions is supportive of other studies showing that IL-4 is a profibrotic cytokine.[65] However, procollagen I and III mRNA expression was greater in fibroblasts cultured from D-NAME-treated mice after an IL-4 stimulus than in similar numbers of fibroblasts from L-NAME-treated mice. The decreased levels of procollagen I and III in cultures of fibroblasts from L-NAME-treated mice may reflect a hyporesponsiveness of these cells to a profibrotic signals (ie, IL-4) because of their previous involvement in the fibrotic PPD-granuloma response. Fibroblasts cultured from fibrotic lesions have been previously described as hyporesponsive to various inflammatory stimuli.[66,67] It is also plausible that fibroblasts from L-NAME-treated mice generate collagen protein much more rapidly than similar cells from D-NAME-treated mice through augmented or altered procollagen mRNA translation.[68] IFN-γ treatment of fibroblasts from L-NAME-treated mice also resulted in procollagen III mRNA expression. Although these findings contrast with previous studies in which IFN-γ was found to inhibit collagen synthesis by normal fibroblasts,[69] these findings may reflect changes in the way that procollagen mRNA is regulated in fibroblasts derived from a nitric-oxide-deprived environment. This observation is also of considerable interest considering that increased procollagen type III occurs early in pulmonary fibrosis and is thought to be predictive of fibrotic diseases in the lung.[49,70] Although constitutive levels of TGF-β did not differ between the two types of fibroblasts, levels of this cytokine were approximately sevenfold higher in cultures of IL-4-stimulated fibroblasts from L-NAME-treated mice compared with their D-NAME-treated counterparts. Furthermore, *in vitro* IFN-γ treatment of fibroblasts from L-NAME-treated mice did not abolish TGF-β synthesis. Taken together, these data suggest that fibroblasts cultured from L-NAME- and D-NAME-treated mice with PPD-bead granulomas differ markedly in their ability to generate extracellular matrix and TGF-β, and these alterations may account for changes in collagen production by these cells *in vivo*.

In summary, these findings suggest that inhibition of nitric oxide synthesis during PPD-bead granuloma formation can markedly change the cellular appearance and the degree of extracellular matrix deposition in this lesion. The results from the present study also suggest that changes in nitric oxide increase the ability of isolated lung cells to generate profibrotic cytokines and chemokines. Furthermore, nitric oxide appears to be important in the regulation of chemokine and chemokine receptor expression by the interstitial pulmonary fibroblast.

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