TNF and IL-6 mediate MIP-1α expression in bleomycin-induced lung injury

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Abstract: Previously, macrophage inflammatory protein-1α (MIP-1α), a member of the C-C chemokine family, has been implicated in bleomycin-induced pulmonary fibrosis, a model of the human disease idiopathic pulmonary fibrosis. Neutralization of MIP-1α protein with anti-MIP-1α antibodies significantly attenuated both mononuclear phagocyte recruitment and pulmonary fibrosis in bleomycin-challenged CBA/J mice. However, the specific stimuli for MIP-1α expression in the bleomycin-induced lesion have not been characterized. In this report, two mediators of the inflammatory response to bleomycin, tumor necrosis factor (TNF) and interleukin-6 (IL-6), were evaluated as putative stimuli for MIP-1α expression after bleomycin challenge in CBA/J mice. Elevated levels of bioactive TNF and IL-6 were detected in bronchoalveolar lavage (BAL) fluid and lung homogenates from bleomycin-treated CBA/J mice at time points post-bleomycin challenge, which precede MIP-1α protein expression. Treatment of bleomycin-challenged mice with soluble TNF receptor (sTNFr) or anti-IL-6 antibodies significantly decreased MIP-1α protein expression in the lungs. Furthermore, normal alveolar macrophages secreted elevated levels of MIP-1α protein in response to treatment with TNF plus IL-6 or bleomycin plus IL-6, but not TNF, bleomycin, or IL-6 alone. Finally, leukocytes recovered from the BAL fluid of bleomycin-challenged mice secreted higher levels of MIP-1α protein, compared to controls, when treated with TNF alone. Based on the data presented here, we propose that TNF and IL-6 are part of a cytokine network that modulates MIP-1α protein expression in the profibrotic inflammatory lesion during the response to intratracheal bleomycin challenge. *J. Leukoc. Biol.* 64: 528–536; 1998.

Key Words: *pulmonary fibrosis · C-C chemokines · CBA/J mice*

INTRODUCTION

Intratracheal instillation of bleomycin in CBA/J mice results in a characteristic inflammatory lesion that culminates in pulmonary fibrosis [1–6]. Factors that mediate this response to bleomycin challenge include many types of inflammatory cells, stromal cells, cytokines, and growth factors [7]. One member of the C-C chemokine family, macrophage inflammatory protein-1α (MIP-1α), mediates mononuclear phagocyte recruitment during the response to bleomycin [8]. In addition, neutralization of MIP-1α protein with anti-MIP-1α antibodies significantly attenuates the pulmonary fibrotic response in CBA/J mice [8]. Although these data establish a central role for MIP-1α in the bleomycin model, the specific stimuli for MIP-1α protein expression have not been identified.

One likely stimulus for MIP-1α protein expression is tumor necrosis factor (TNF). Neutralization of TNF after bleomycin challenge also results in a dramatic decrease in inflammatory cell accumulation and the subsequent pulmonary fibrosis [9, 10]. It is interesting to note that transient expression of TNF protein and mRNA has been detected in whole lung and bronchoalveolar lavage (BAL) preparations 3–8 days post-bleomycin challenge, whereas MIP-1α protein expression peaks at 2 and 12 days post-challenge [8, 9, 11]. Although the expression of TNF protein before the early 2-day peak of MIP-1α protein expression has not been characterized, the temporal association of TNF and MIP-1α expression in the bleomycin model leads us to hypothesize that TNF may be a direct stimulus for the early (2 day) and late (12 day) peaks of MIP-1α protein expression after bleomycin challenge.

Another candidate stimulus for MIP-1α expression is interleukin-6 (IL-6). IL-6 is secreted by bleomycin-challenged rat aortic endothelial cell or murine peritoneal macrophage cultures but, in contrast to TNF and MIP-1α, the specific contribution of IL-6 to the profibrotic lesion has not been demonstrated [12, 13]. However, IL-6 serves as an important cofactor for neutrophil activation and B cell stimulation, suggesting that the contribution of IL-6 may be overlooked when singly evaluated [14, 15]. Therefore, we evaluated IL-6 as a factor that may synergize with other inflammatory mediators, such as TNF, to stimulate MIP-1α expression after bleomycin challenge.

In this study, we investigated TNF and IL-6 as putative stimuli of MIP-1α protein expression in bleomycin-induced

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Abbreviations: MIP-1α, macrophage inflammatory protein 1α; TNF, tumor necrosis factor; IL-6, interleukin-6; sTNFr, soluble tumor necrosis factor receptor; BAL, bronchoalveolar lavage; ELISA, enzyme-linked immunosorbent assay; BALF, BAL fluid; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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lung injury. Elevated levels of bioactive TNF and IL-6 were detected in BAL fluid and lung homogenates from bleomycin-treated CBA/J mice at time points post-bleomycin challenge, which preceded MIP-1α protein expression. Treatment of bleomycin-challenged mice with soluble TNF receptor (sTNFr) or anti-IL-6 antibodies significantly decreased MIP-1α protein expression in the lungs. By *in situ* hybridization, we identified alveolar macrophages as a source of MIP-1α mRNA and as a target for *in vitro* studies. Furthermore, normal alveolar macrophages secreted elevated levels of MIP-1α protein in response to treatment with TNF plus IL-6 or bleomycin plus IL-6, but not TNF, bleomycin, or IL-6 alone. Finally, leukocytes recovered from the BAL fluid of bleomycin-challenged mice secreted higher levels of MIP-1α protein than normal alveolar macrophages, both constitutively and when treated with TNF alone. These findings suggest that TNF, IL-6, and bleomycin are stimuli for MIP-1α expression and establish that these mediators are participants in a cytokine network present in the profibrotic inflammatory lesion during the pulmonary response to bleomycin challenge.

MATERIALS AND METHODS

Animals and reagents

Recombinant murine MIP-1α, murine TNF, and murine IL-6 were purchased from R & D Systems, Minneapolis, MN. Serial dilutions of pyrogen-free cytokines were prepared in sterile RPMI 1640 (Whitaker Biomedical Products, Whitaker, CA), 1 mM glutamine, 25 mM HEPES, 100 units/mL penicillin, 100 ng/mL streptomycin (Hazelton Research Products, Denver, PA) (complete media). CBA/J mice were purchased from Jackson Laboratories, Bar Harbor, ME. Female New Zealand White rabbits were purchased from Hazelton Research Products, Kalamazoo, MI. Blenoxane (bleomycin sulfate) was purchased from Bristol-Myers Squibb Co., Evansville, IN. Ketalar (ketamine HCl) was purchased from Parke-Davis, Morris Plains, NJ.

Bleomycin administration

Bleomycin was administered to CBA/J mice as previously described [8]. Briefly, CBA/J mice were anesthetized with 250 μL of 12.5 μg/mL of ketamine injected intraperitoneally, followed by intratracheal instillation of 0.025 units of bleomycin (in 25 μL of sterile saline) or 25 μL of sterile isotonic saline. At 2 and 10 days post-instillation, animals were killed, cold sterile saline was perfused into the right ventricle of the heart to remove blood from the lung vascular beds, and both lungs removed for homogenization with a hand held tissue tearor (Whatman, Hillsboro, OR). Whole lung homogenates were spun at 30,000 *g* for 35 min and supernatants removed for enzyme-linked immunosorbent assay (ELISA) analysis. In separate experiments, at various times post-bleomycin instillation, mice were killed and the lungs lavaged with 1.5–2.0 mL sterile saline. BAL fluid (BALF) was centrifuged at 900 *g* for 10 min and the supernatant frozen for ELISA or bioassay analysis. Pellets were resuspended in 0.5 mL normal saline, counted on a hemacytometer, and cytospun for differential analysis. Alternatively, lungs were lavaged with 10 mL sterile saline, the lavage fluid centrifuged at 900 *g* for 10 min, and the lavage cells cultured in 96-well plates at 200,000 cells/well. BAL cell culture experiments are a compilations of samples gathered on four separate occasions with different litter groups of age matched CBA/J mice.

MIP-1α-specific ELISA

Antigenic murine MIP-1α was quantitated using a modification of the double ligand method [16]. Briefly, flat-bottomed 96-well microtiter plates (Nunc Immuno-Plate I 96-F) were coated with 50 μL/well of rabbit anti-murine MIP-1α antibody (1 ng/μL in 0.6 M NaCl, 0.26 M H_3BO_4, and 0.08 N NaOH, pH 9.6) for 16 h at 4°C and then washed with phosphate-buffered saline (PBS), pH 7.5, 0.05% Tween-20 (wash buffer). Microtiter plate nonspecific binding sites were blocked with 2% bovine serum albumin (BSA) in PBS and incubated for 90 min at 37°C. Plates were rinsed four times with wash buffer and 50 μL/well of whole-lung homogenate or BALF was added and incubated for 1 h at 37°C. Plates were washed four times with wash buffer, then 50 μL/well of biotinylated rabbit anti-MIP-1α (3.5 ng/μL in PBS, pH 7.5, 0.05% Tween-20, and 2% fetal calf serum) was added, and plates incubated for 30 min at 37°C. Plates were washed four times, streptavidin-peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA) was added, and the plates incubated for 30 min at 37°C. Plates were washed four times and chromogen substrate (Bio-Rad Laboratories, Richmond, CA) was added. The plates were incubated at room temperature to the desired extinction, and the reaction terminated with 50 μL/well of 3 M H_2SO_4. Plates were read at 490 nm in an ELISA reader. Standards were 1/2 log dilutions of recombinant murine MIP-1α, from 1 pg/mL to 100 ng/mL (50 μL/well). This ELISA method consistently detected MIP-1α concentrations in a linear fashion greater than 50 pg/mL.

TNF and IL-6 bioassay

IL-6 levels were assayed from whole-lung homogenates with the use of a sensitive and specific proliferation assay involving the IL-6-dependent murine hybridoma cell line B13.29, Clone 9 (B9). Serial dilutions of samples were incubated with 100 μL of B9 cells in 96-well plates at a concentration of 5×10^4 cells/mL for 72 h in a humidified incubator at 37°C with 5% CO_2. Proliferation was measured in a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) purchased from Sigma. During the final 6 h of the incubation, 20 μL of MTT (5 mg/mL in PBS) were added to each sample. The supernatant was aspirated from each well and 100 μL of acidified isopropanol was added. Absorbance was measured at 550 nm. IL-6 concentrations in experimental samples were calculated using a standard curve generated by serial dilutions of human rIL-6. This bioassay consistently detected IL-6 concentrations >0.10 pg/mL.

TNF levels were assayed from whole lung homogenates with the use of a cell death assay utilizing the TNF-sensitive WEHI 164 subclone 13 cell line. Serial dilutions of test samples were incubated with 100 μL of WEHI cells at a concentration of 5×10^5 cells/mL and 0.5 μg/mL actinomycin D for 24 h in a humidified incubator at 37°C with 5% CO_2. After an additional 6-h incubation with 20 μL of MTT/well (5 mg/mL in PBS) the supernatant was aspirated from each well, and 100 μL of acidified isopropanol was added, and the plates isolated from light for 24 h. Absorbance was measured at 550 nm. TNF concentrations in experimental samples were calculated using a standard curve generated by serial dilutions of human rTNF. This bioassay consistently detected TNF concentrations >0.10 pg/mL.

In situ hybridization

Three-micrometer paraffin-embedded serial lung sections from bleomycin-challenged mice were deparaffinized by sequential treatment with xylene (15 min), 100% ETOH, 95% ETOH, 2× SSC, 0.2 N HCl (20 min), 2× SSC, 0.25% acetic anhydride in triethanolamine (10 min), 2× SSC (70°C, 30 min), and 2× SSC. All treatments were 5 min unless otherwise noted. Slides were incubated with pre-hybridization buffer [pre-hyb: 10% dextran sulfate (final), 3× SSC (final), 2× Denhardts (final), 0.1 mg/mL salmon sperm DNA, 0.125 mg/mL yeast tRNA, 0.01 mg/mL poly-A-poly-C, 1 mg/mL sodium pyrophosphate, and 50% deionized formamide (final), in DEPC H_2O (to 100 mL)] for 2 h at 44°C. About 15 μL pre-hyb solution was added to each tissue section. Some sections were pretreated with 20 μL of 50 μg/mL solution of DNase-free RNase (Boehringer Mannheim, Indianapolis IN). Anti-sense (5'-GAA-GCA-GCA-GGC-AGT-CGG-GGT-GTC-AGC-TCC-3') and sense probes for MIP-1α were end-labeled with ^{35}S-labeled ATP (DuPont-NEN, Wilmington, DE), purified by gel electrophoresis, and assayed for specific activity. Pre-hyb was removed and 20 μL of hybridization buffer [hyb: pre-hyb plus ^{35}S-labeled sense or anti-sense probe (750,000 cpm/section) and 0.2 mg/mL dithiothreitol] were placed on each tissue section. Slides were covered with an RNase-free coverslip and incubated for 12–16 h at 44°C in a humidified chamber. Next, slides were washed in 2× SSC for 2 h (changed six times), 1× SSC for 1 h, 0.5× SSC for 0.5 h, 0.5× SSC for 0.5 h at 44°C, 0.5× SSC for 0.5 h, 70% ETOH with 0.3 M

ammonium acetate for 2 min, and 100% ETOH with 0.3 M ammonium acetate for 2 min. Slides were then air dried, dipped in NBT-2 emulsion (Kodak, Rochester, NY), air dried again, and incubated for 18–21 days at 4°C in a desiccated chamber. Slides were developed with D-19 developer, fixed with Kodak rapid fix, counterstained with hematoxylin, and fixed with Permount (Fisher Scientific, Fair Lawn, NJ).

Soluble TNF receptor (sTNFr) and anti-IL-6 antibody therapy

sTNFr was a generous gift from Immunex, Seattle, WA. One hour before bleomycin challenge, CBA/J mice were given an intraperitoneal injection of 100 μg of sTNFr and/or 0.5 mL of anti-IL-6 immune serum and killed at 2 days post challenge. In separate experiments, lungs from animals injected intraperitoneally with 100 μg sTNFr or 0.5 mL of anti-TNF immune serum 6 and 8 days post-bleomycin administration were harvested 10 days post-challenge. CBA/J mice treated as described above were analyzed for total lung MIP-1α protein levels, BAL differential cell counts, and total cell counts. The *in vivo* half-life of the sTNFr construct as previously reported is approximately 24 h [17]. To determine dosage parameters and the *in vivo* half-life of anti-IL-6 immune serum, mice injected intraperitoneally with anti-IL-6 serum or pre-immune serum were bled at 6, 12, 24, and 48 h post-injection. Serum samples were diluted 10^2–10^7 in sterile PBS and analyzed as described below. Ninety-six-well plates (Nunc Immuno-Plate I 96-F) were coated with 50 μL of recombinant murine IL-6 (1 μg/mL) and incubated at 4°C overnight. The plates were blocked with 2% normal goat serum in PBS for 1 h at 37°C. Next, plates were washed three times with wash buffer (PBS, pH 7.5, and 0.05% Tween-20). Fifty microliters of diluted sample was added to each well and the plate incubated for 0.5 h at 37°C. The plates were again washed three times in wash buffer and 50 μL of peroxidase-labeled goat-anti-rabbit IgG diluted 1:400 in dilution buffer (0.6 M NaCl, 0.26 M H_3BO_4, and 0.08 N NaOH, pH 9.6) was added to each well and incubated for 0.5 h at 37°C. Plates were washed three times with wash buffer, and 100 μL fresh chromogen substrate/well (Bio-Rad Laboratories) was added. The plates were incubated at room temperature for 5 min. The reaction was stopped with 50 μL/well of 3 M H_2SO_4. Plates were read at 490 nm in an ELISA reader. A best fit curve was calculated and a plasma half-life of 60 h for rabbit anti-IL-6 was calculated assuming linear clearance kinetics.

Statistical analysis

Data were evaluated by one-way analysis of variance and where appropriate, further evaluated with either the parametric Student-Newman-Keuls test for multiple comparisons or with the non-parametric Mann-Whitney rank sum test.

RESULTS

TNF protein is expressed immediately after bleomycin challenge

Previous studies have characterized TNF mRNA and protein expression in bleomycin-challenged CBA/J mice 4 and 7 days post-challenge, which is before the late (12 day) peak of MIP-1α protein expression reported in similarly treated mice [8, 9, 11]. In addition, passive immunization with TNF antibodies or soluble TNF receptor has been shown to abrogate the fibrotic response to bleomycin [9, 18]. Consequently, we hypothesized that TNF stimulates MIP-1α production in the bleomycin model. To test this hypothesis we first completely characterized the expression of TNF and another pro-inflammatory cytokine, IL-6, in CBA/J mice at early time points after bleomycin challenge. Elevated levels of bioactive TNF and IL-6 protein were detected in BAL fluid from CBA/J mice at 6, 12, and 24 h post-bleomycin challenge, compared to controls (**Fig. 1**, **A** and **B**). In contrast, MIP-1α was not increased in BAL fluid at 6 or 12 h, but was elevated at 24 h post-bleomycin challenge (Fig. 1C). In separate experiments, we characterized TNF and IL-6 expression at later time points. Bioactive TNF was elevated in the BAL fluid of CBA/J mice 2, 8, and 16 days post-bleomycin challenge (**Fig. 2B**), whereas bioactive IL-6 was elevated in whole lung homogenates of bleomycin-challenged mice at all time points evaluated (Fig. 2A). The temporal sequences of TNF and IL-6 expression, which precede both the early and late peaks of MIP-1α protein expression, are compatible with the hypothesis that TNF and IL-6 are stimuli for MIP-1α production in the bleomycin model.

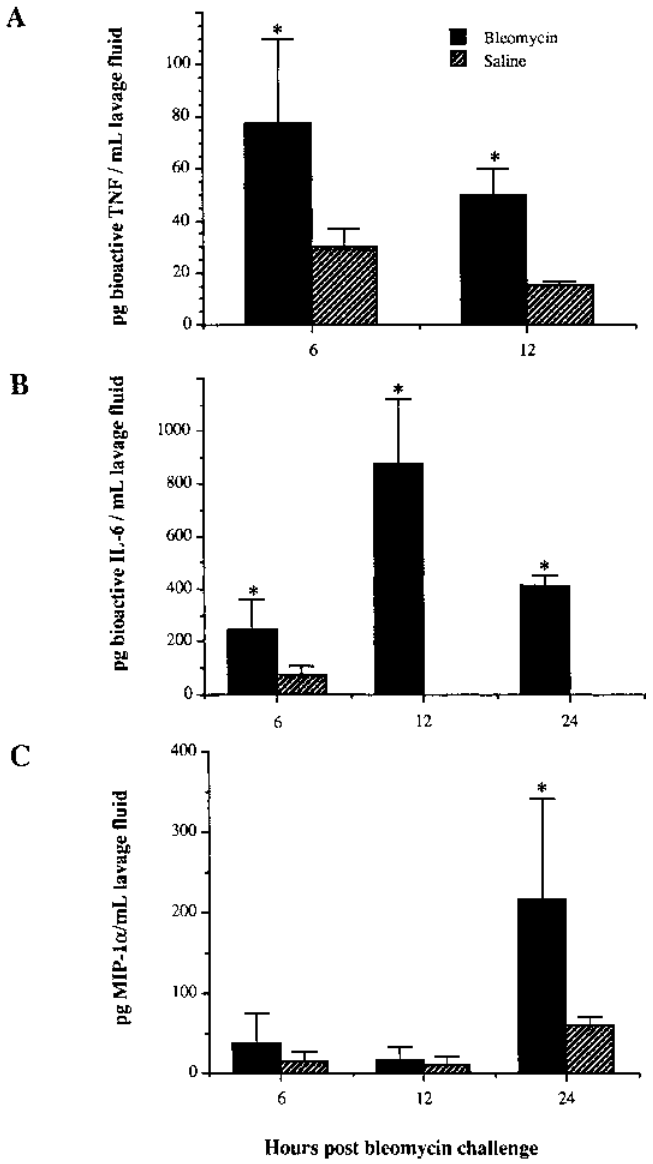

Fig. 1. Time course of TNF (A), IL-6 (B), and MIP-1α (C) ELISA (C) or bioassay(A, B) analysis of BALF from bleomycin- or saline-challenged CBA/J mice. Data expressed as mean ± SEM, $n = 5$, $^*P < 0.05$.

Treatment with sTNFr attenuates inflammatory cell accumulation and MIP-1α expression

To further investigate the hypothesis that TNF modulates MIP-1α expression during the inflammatory response to bleomycin, CBA/J mice were treated with a soluble TNF receptor (sTNFr) construct that possesses potent neutralizing activity *in*

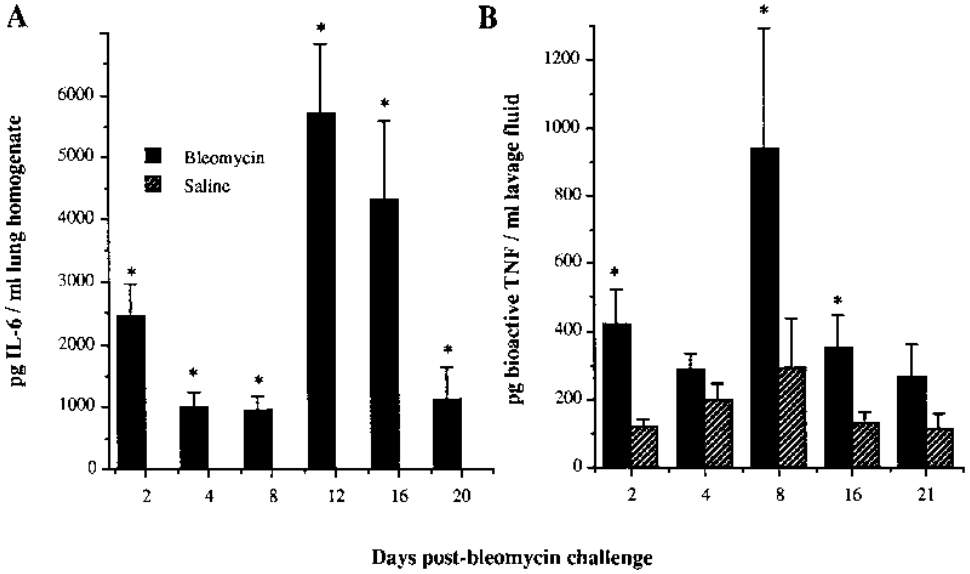

Fig. 2. Time course of IL-6 (A) and TNF (B) bioassay analysis of BALF and whole lung homogenates, respectively, from bleomycin- or saline-challenged CBA/J mice. Data expressed as mean ± SEM, $n = 5$, $^*P < 0.05$.

vivo [17]. Because MIP-1α protein and mRNA expression peak at approximately 2 and 12 days post-challenge, we hypothesized that sTNFr treatment would be most effective at the time of bleomycin challenge (day 0) or before the second MIP-1α peak (days 6 and 8) [8]. CBA/J mice treated with sTNFr, IgG, or saline were challenged with 25 μL of 1 U/mL solution of bleomycin or 25 μL of sterile saline. BAL and whole lung homogenization were performed at 2 and 10 days post challenge for quantitation of inflammatory cells or MIP-1α protein levels. Lavage cells were quantitated and differential analysis was performed to confirm the anti-inflammatory effects of the sTNFr reagent. No significant differences in total cell counts (**Fig. 3A**) or differential cell counts (data not shown) were detected 2 days post-bleomycin challenge, consistent with previous findings that total lavage cell populations are not elevated until 4 days post-challenge [8]. However, at 10 days post-bleomycin challenge, sTNFr-treated mice had significantly reduced total lavage cell populations compared to bleomycin and IgG-treated controls (Fig. 3A). Differential analysis of lavage cells 10 days post-challenge from sTNFr-treated mice detected reductions in lymphocyte and mononuclear phagocyte but not granulocyte populations compared to controls (**Table 1**). These results are consistent with previous reports which demonstrate that neutralization of TNF attenuates cellular accumulation in inflammatory lesions [10, 19, 20]. After BAL, lungs from the above animals were homogenized and analyzed for MIP-1α protein levels. MIP-1α protein expression in CBA/J mice passively immunized with sTNFr was significantly reduced at 2 days post-bleomycin challenge, compared to IgG- and bleomycin-treated controls, suggesting that TNF stimulates MIP-1α protein expression (Fig. 3B). In CBA/J mice treated with sTNFr 6 and 8 days post-bleomycin challenge, MIP-1α protein expression was not significantly decreased at day 10 (Fig. 3B). However, in similar experiments, in CBA/J mice passively immunized with anti-TNF polysera 6 and 8 days post-bleomycin challenge, MIP-1α protein expression was significantly decreased at day 10 (141 ± 36 pg MIP-1α/animal) compared to non-immune serum controls (549 ± 78 pg MIP-1α/animal, $P < 0.05$). These data strongly support the hypothesis that TNF modulates both the early and late peaks of MIP-1α protein expression *in vivo*.

Treatment with anti-IL-6 antibodies attenuates MIP-1α expression

In experiments similar to the sTNFr studies above, CBA/J mice were treated with IP injections of IgG, anti-IL-6 immune serum, and/or sTNFr either before bleomycin administration or at 6 and 8 days post challenge. Lungs were homogenized and the concentration of MIP-1α protein determined by ELISA analysis at 2 and 10 days post challenge. MIP-1α protein expression in CBA/J mice passively immunized with anti-IL-6 antibodies alone was significantly reduced at 2 days post-bleomycin challenge, compared to IgG- and bleomycin-treated controls (**Fig. 4**). Combination of anti-IL-6 immune serum and sTNFr therapy resulted in a larger decrease in MIP-1α expression at 2 days post challenge than that mediated by anti-IL-6 alone (Fig. 4). In CBA/J mice treated with anti-IL-6 immune serum 6 and 8 days post-bleomycin challenge, MIP-1α protein expression was not significantly decreased at day 10 (data not shown). These data suggest that IL-6 contributes to stimulation of the first peak of MIP-1α protein expression at 2 days post challenge, but not the second peak at 10 days.

In situ hybridization: localization of MIP-1α mRNA 6 h post-bleomycin challenge

To determine whether TNF and IL-6 are directly or indirectly stimulating MIP-1α expression, we moved to an *in vitro* model where exogenous stimuli could be easily controlled. To confirm that alveolar macrophages produce MIP-1α during the response to bleomycin *in vivo*, we challenged CBA/J mice with 50 mU bleomycin sulfate, killed the animals 6 h post-challenge, and removed the lungs for processing. In situ hybridization using an anti-sense probe for MIP-1α mRNA specifically labeled alveolar macrophages, identifying this cell type as a potential source of MIP-1α protein (**Fig. 5**, **A** and **B**). Serial sections probed with a sense MIP-1α probe (Fig. 5, C and D) or pretreated with RNase (Fig. 5, inset E and inset F) were not

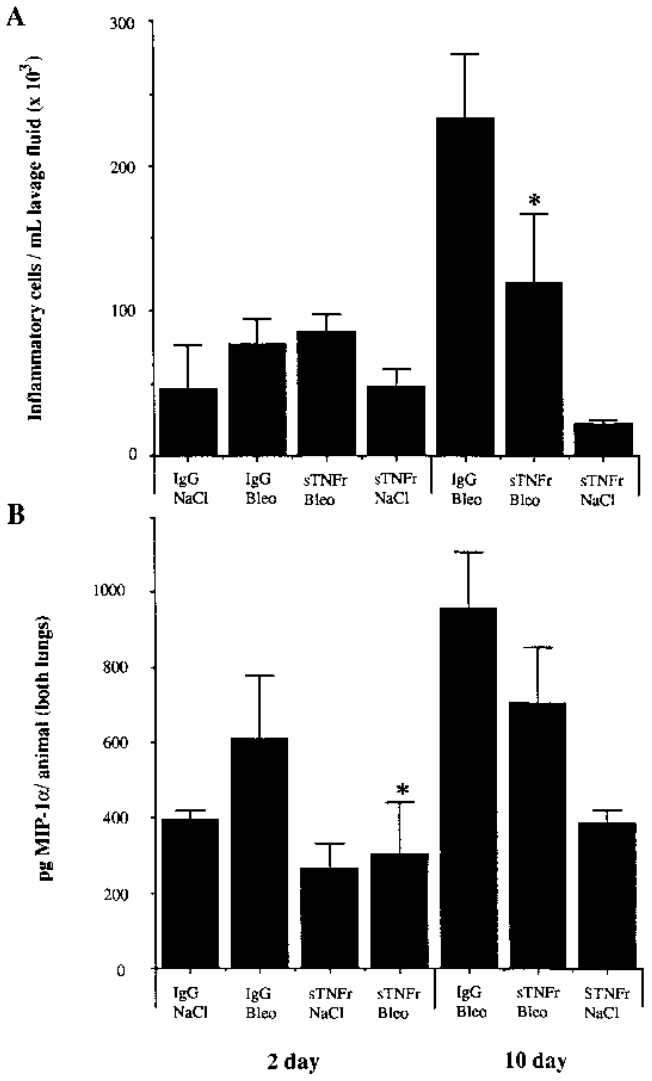

Fig. 3. Total BAL cell counts (A) and whole lung homogenate MIP-1α ELISA analysis (B) at 2 and 10 days post-bleomycin or saline challenge in CBA/J mice passively immunized with sTNFr or IgG. Data are expressed as mean ± SEM, $n = 5$, $^*P < 0.05$.

specifically labeled. Animals challenged with saline had a similar cellular labeling pattern, but with much less frequency and intensity (data not shown). These results are consistent with previous experiments demonstrating immunohistochemical staining of MIP-1α protein in lung sections of CBA/J mice 1–2 days post-bleomycin challenge, and identify the alveolar macrophage as a potential source of MIP-1α protein expression in the bleomycin-induced lesion [8].

Stimulus-specific MIP-1α secretion from alveolar macrophages *in vitro*

To directly evaluate putative stimuli for MIP-1α expression, normal alveolar macrophages isolated from CBA/J mice were incubated with various factors known to be present in the bleomycin-induced pulmonary lesion. Cells obtained via BAL were ≥95% macrophages as determined by differential analysis. We detected significantly elevated MIP-1α protein in the supernatants of lavage cells incubated with 5 ng/mL IL-6 plus either 5 mU/mL bleomycin sulfate or 5 ng/mL TNF, compared to controls (**Fig. 6A**). MIP-1α levels in supernatants from cells stimulated with similar concentrations of IL-6, bleomycin, and/or TNF alone were not significantly different from controls (Fig. 6A). These data suggest that TNF alone is not sufficient for stimulation of MIP-1α secretion in resting alveolar macrophages and that IL-6 may be an important co-stimulatory molecule in this system.

Although analysis of normal lavage cells is a reasonable approximation of events early in the response to bleomycin, evaluation of TNF and IL-6 as putative stimuli for the late MIP-1α peak required a different approach. Here we challenged CBA/J mice with bleomycin and isolated BAL cells 8 days post-challenge. Previously, we demonstrated that the 8-day BAL cell population contained 64% macrophages, 21% polymorphonuclear neutrophils, and 15% lymphocytes by differential analysis [8]. Cells were stimulated with combinations of IL-6, bleomycin, and/or TNF for 24 h and the supernatants analyzed for MIP-1α protein. BAL cells from bleomycin-challenged animals constitutively secreted an order of magnitude more MIP-1α protein per 2×10^5 cells than BAL cells from unchallenged animals (Figs. 6, A and B). In addition, in contrast to normal alveolar macrophages, BAL cells from bleomycin-challenged animals secreted significantly elevated amounts of MIP-1α protein in response to stimulation with TNF, but not IL-6 or bleomycin (Fig. 6B). These data suggest that TNF can directly stimulate the late peak of MIP-1α expression present in the bleomycin model.

DISCUSSION

In this study, we detected elevated TNF and IL-6 protein expression in the BAL fluid of CBA/J mice 6 and 12 h post-bleomycin challenge, whereas elevated MIP-1α protein expression was not detected until 24 h (Fig. 1, A–C). In addition, IL-6 and TNF protein were elevated 8, 12, and 16 days post-challenge, preceding the late peak of MIP-1α expression 12–16 days post-challenge (Fig. 2, A and B) [8]. These data suggest that TNF and IL-6 may stimulate MIP-1α expression *in vivo*. Directly supporting this contention, expression of MIP-1α mRNA was attenuated by neutralization of TNF protein in an LPS-induced murine model of septic shock [21]. However, while primary cultures of both peritoneal and alveolar macrophages expressed elevated levels of MIP-1α mRNA after LPS challenge, peritoneal macrophages did not secrete elevated levels of MIP-1α protein [22]. This finding suggests that

TABLE 1. Differential Cell Counts of Lavage Fluid From IgG-, sTNFr-, Saline-, and Bleomycin-Treated CBA/J Mice 10 Days Post-Challenge[a]

Intraperitoneal treatment Intratracheal treatment	sTNFr Saline	sTNFr Bleomycin	IgG Bleomycin
Lymphocytes	1.2 ± 0.3	12.7 ± 4.0[b]	34.0 ± 9.0
Mononuclear phagocytes	21.6 ± 1.0	80.1 ± 34.3[b]	140 ± 21.5
Granulocytes	2.1 ± 0.7	27.1 ± 12.3	59.1 ± 25.4

[a] CBA/J mice were challenged intratracheally with saline or bleomycin, followed by intraperitoneal injection of sTNFr or IgG 6 and 8 days post-challenge; $n = 5$ ([b] $P < 0.05$).

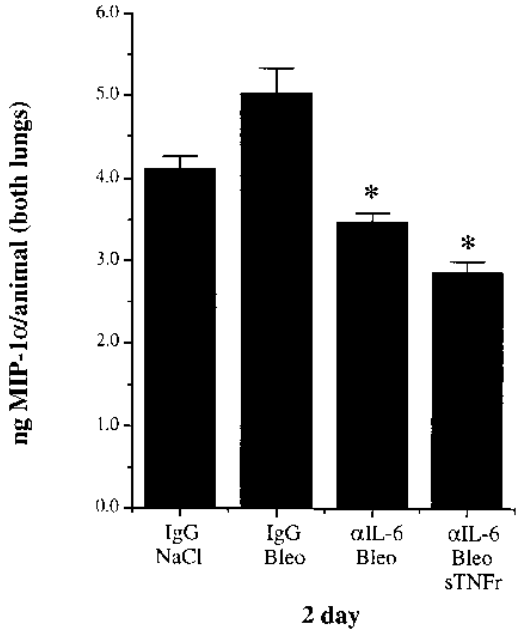

Fig. 4. Whole lung homogenate MIP-1α ELISA analysis at 2 days post-bleomycin or saline challenge in CBA/J mice passively immunized with IgG, anti-IL-6 antibodies, or anti-IL-6 antibodies plus sTNFr. Data are expressed as mean ± SEM, $n = 5$, $^*P < 0.05$.

investigation of MIP-1α mRNA expression is alone insufficient to evaluate a putative stimuli for MIP-1α protein expression. Preliminary results in a model of airway hyperreactivity have demonstrated that neutralization of TNF may abrogate MIP-1α protein expression *in vivo* [23]. Consequently, we treated bleomycin-challenged CBA/J mice with sTNFr or anti-TNF antibodies and measured MIP-1α protein expression by ELISA. Consistent with the results in the septic shock and airway hyperreactivity models, we observed decreases in the early and late peaks of MIP-1α protein expression of 80 and 35%, respectively. These findings unequivocally demonstrate that TNF is a requisite for stimulation of MIP-1α protein *in vivo*.

In similar experiments, neutralization of IL-6 resulted in a significant decrease in MIP-1α expression at 2 days, but not 10 days, post-bleomycin challenge. This result suggests that IL-6 may mediate MIP-1α expression during the acute inflammatory response in the first 2 days after bleomycin challenge, but have a lesser role during the maturation of the inflammatory lesion. Neutralization of both IL-6 and TNF decreased MIP-1α protein levels more than anti-IL-6 therapy alone, consistent with our expectation that stimulation of MIP-1α protein expression is multifactorial.

Previously, we demonstrated that MIP-1α mediates macrophage, but not lymphocyte, accumulation in the lungs of bleomycin-challenged CBA/J mice [8]. It is interesting to note that neutralization of TNF attenuated both macrophages and lymphocyte accumulation, suggesting that TNF is more proximal than MIP-1α in a cytokine network where an additional unidentified mediator(s) is responsible for lymphocyte recruitment (Table 1). Candidates for lymphocyte recruitment include other members of the C-C chemokine family, such as MCP-1, MCP-2, MCP-3, RANTES, eotaxin, C10, and MIP-1β [24–26]. Many of these chemokines are chemotactic for subsets of lymphocytes *in vitro*. Because our TNF and MIP-1α neutralization experiments have only analyzed total lymphocyte populations, we may be overlooking significant changes in subsets of lymphocytes. Nevertheless, it is reasonable to postulate that

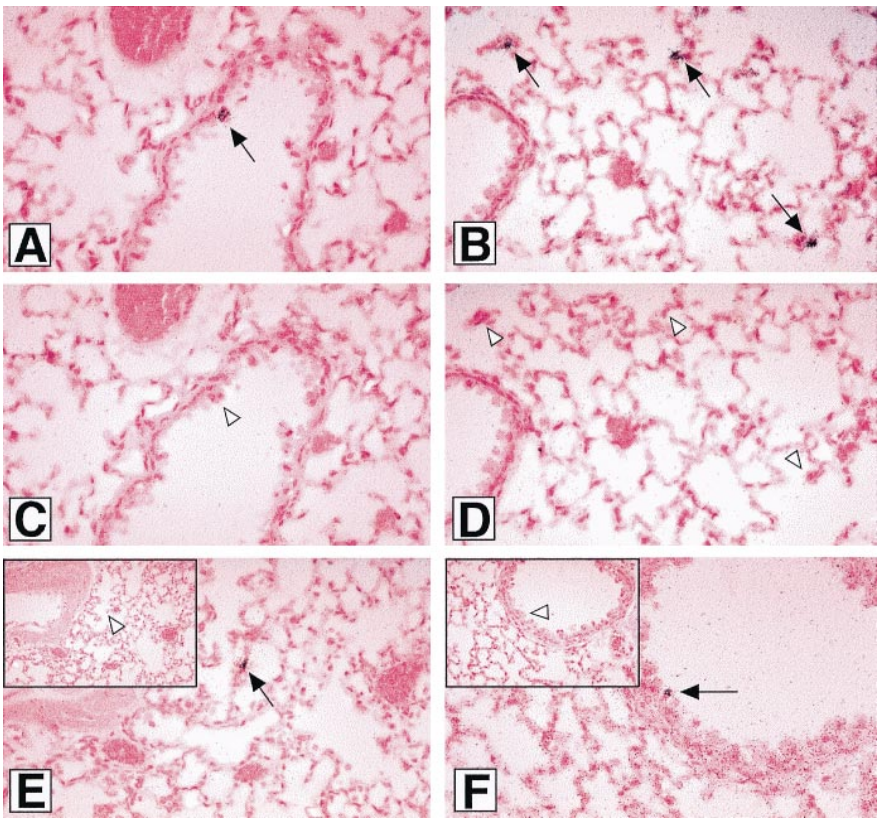

Fig. 5. Serial lung sections from bleomycin- (A–F) or saline- (not shown) challenged CBA/J mice analyzed by *in situ* hybridization for MIP-1α mRNA. Sections were probed with anti-sense (A, B, E, F) or sense probe (C, D) and/or pretreated with RNAase (inset, E, F). Sections were counterstained with hematoxylin and photographed at ×400 magnification. Arrows indicate positive cells and the corresponding area on the serial section.

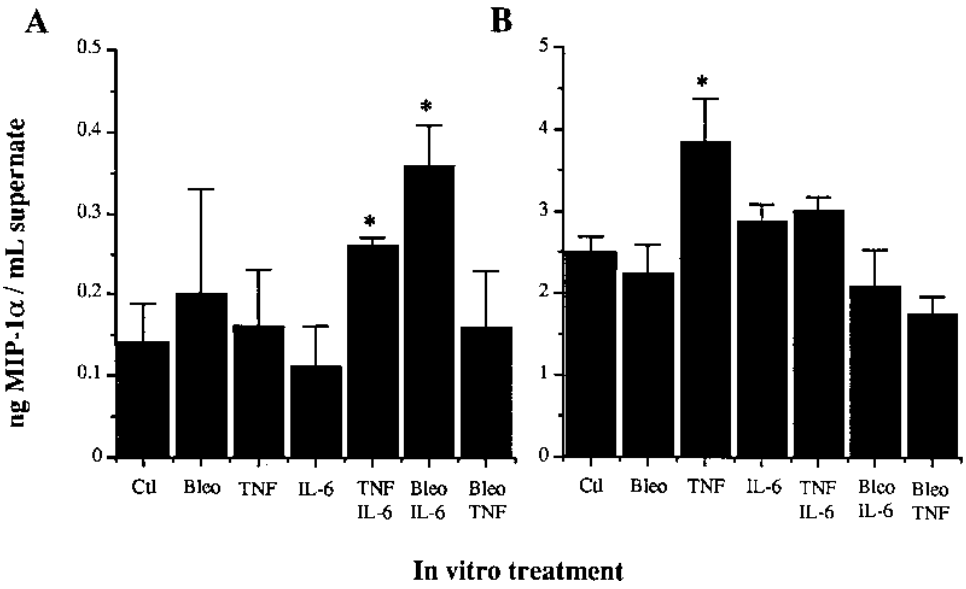

Fig. 6. MIP-1α ELISA analysis of normal alveolar macrophage (A) or 8-day post-bleomycin challenge lavage cell (B) culture supernatants. Cells were treated for 24 h with 5 ng/mL TNF, 5 ng/mL IL-6, and/or 5 mU bleomycin. Data expressed as mean ± SEM, $n = 3–5$, $*P < 0.05$.

TNF is stimulating expression of chemokines other than MIP-1α, which are mediating lymphocyte recruitment to the lungs of bleomycin-challenged mice.

Although the above data provide significant insights into the pulmonary cytokine responses to bleomycin challenge, they do not provide definitive evidence for which mediators directly stimulate MIP-1α expression. TNF has previously been shown to be a weak, if not equivocal, stimulus for MIP-1α protein expression from normal alveolar macrophages *in vitro* [27–29]. Consistent with this data, we found that normal alveolar macrophages treated with TNF alone did not secrete significantly elevated levels of MIP-1α protein (Fig. 6A). However, normal alveolar macrophages did secrete significantly elevated levels of MIP-1α protein when treated with TNF plus IL-6, consistent with our observation that neutralization of IL-6 and/or TNF *in vivo* attenuates MIP-1α expression (Fig. 6A). It is interesting that IL-6 has been described as a co-stimulus in other systems. For example, IL-6 plus IL-10 modulated immunoglobulin secretion from B cells, whereas IL-6 alone did not have a similar effect [15]. Treating alveolar macrophage cultures with TNF, which stimulates IL-6 expression from these cells, could theoretically stimulate MIP-1α expression. One reason this theoretical effect was not observed may be that IL-6 and TNF need to be encountered simultaneously by the alveolar macrophage. Once the macrophage encounters TNF in the absence of IL-6, it may no longer be responsive to costimulation by IL-6. Similar to TNF plus IL-6, bleomycin plus IL-6 stimulated significantly increased MIP-1α protein expression from normal alveolar macrophages, compared to controls, whereas bleomycin or IL-6 alone had no effect (Fig. 6A). The rapid, bleomycin-dependent stimulation of TNF from alveolar macrophages along with exogenously added IL-6 may be synergizing to stimulate MIP-1α protein expression *in vitro*. Alternatively, bleomycin may be directly synergizing with IL-6 to stimulate MIP-1α expression. Regardless of the mechanism, these results provide insight into the components of the cytokine network present in the alveolar compartment after administration of bleomycin *in vivo*. During the first 12 h after bleomycin instillation, epithelial cells, alveolar macrophages, and endothelial cells are the likely sources of the profoundly elevated IL-6 levels, whereas alveolar macrophages are the predominant source of TNF (Figs. 1B and 2A) [12, 13, 30–32]. Next, at 12–24 h post-challenge, TNF and IL-6 probably synergize to stimulate MIP-1α protein secretion from alveolar macrophages. This putative cytokine network is consistent with the kinetics of TNF, IL-6, and MIP-1α protein expression, and strongly supported by the findings of decreased MIP-1α protein levels after neutralization of TNF and IL-6 *in vivo* [8].

In contrast to the effects attributable to IL-6 in normal alveolar macrophages, treatment with IL-6 plus TNF or bleomycin did not significantly affect MIP-1α expression in lavage cells isolated from animals challenged 8 days previously with bleomycin. Addition of bleomycin to this already maximally stimulated cell population probably had a toxic effect, whereas addition of exogenous IL-6 was probably insignificant given the levels of IL-6 detected *in vivo* at this time point. TNF alone was the only treatment that stimulated a modest but significant increase in MIP-1α production from the 8-day lavage cell cultures. Supporting the finding of the modest effect of TNF on these cultures, fibroblasts grown out of arthritic lesions and alveolar macrophages isolated from mice previously challenged with mineral dust also secreted only very modest amounts of MIP-1α in response to stimulation with TNF [27, 28]. The cells isolated from these inflammatory lesions were already significantly activated, possibly accounting for the modest increases in MIP-1α protein expression observed in response to additional stimuli. Supporting this contention, levels of MIP-1α secreted from the untreated control group of lavage cells isolated from bleomycin-challenged animals were 10 times higher than MIP-1α levels secreted from the control group of normal alveolar macrophages isolated from animals not challenged with bleomycin. Although these results suggest the lavage cells isolated 8 days after bleomycin challenge are a poor target for the evaluation of potential stimuli of MIP-1α expression *in vitro*, the modest effects attributable to TNF support the hypothesis that it is one of the stimuli for MIP-1α protein expression late (8–16 days) during the response to bleomycin challenge.

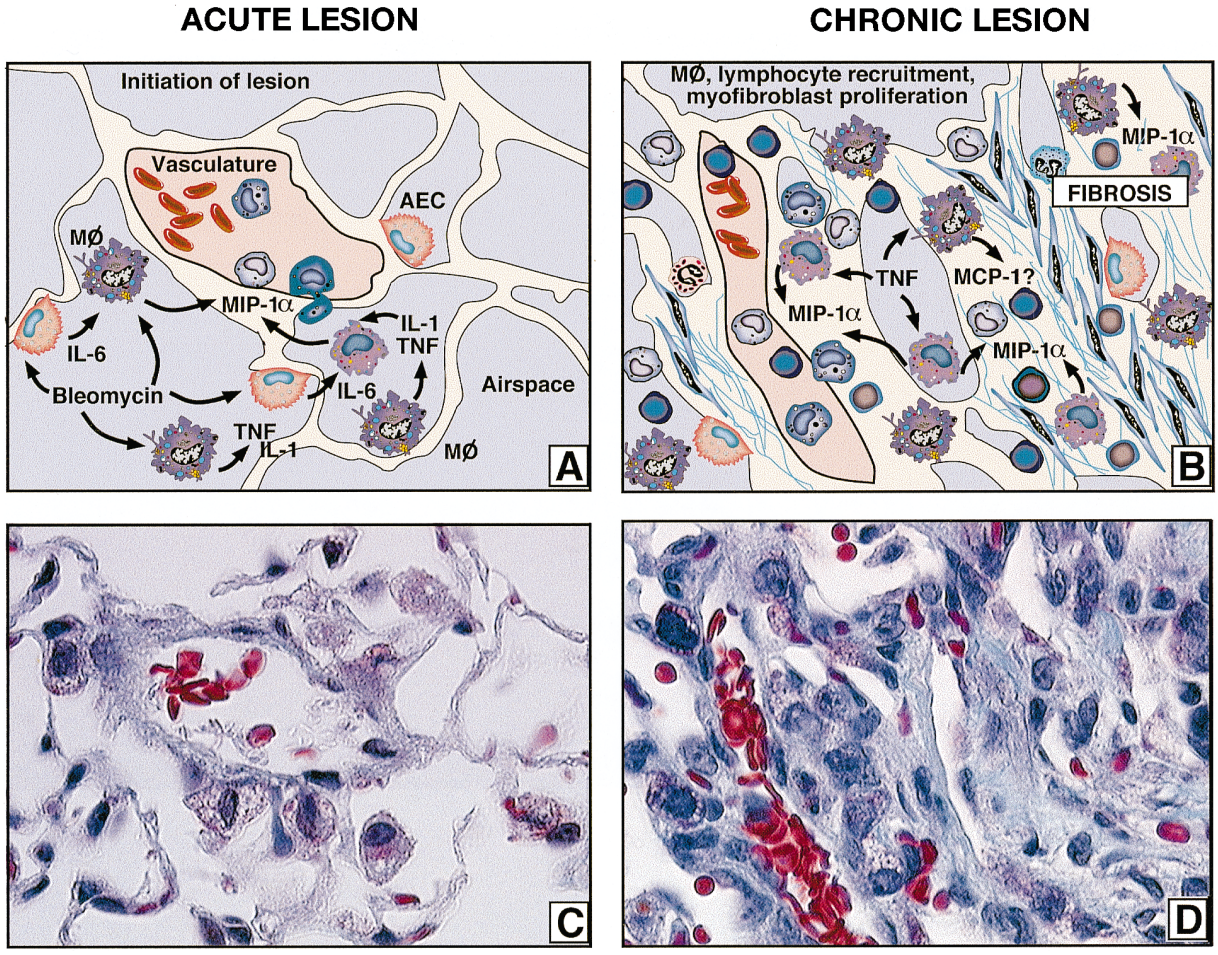

Fig. 7. Proposed cytokine networks activated in the lungs of CBA/J mice 24 h (A) and 8 days (B) post-bleomycin challenge. Corresponding light micrographs of lung sections 24 h (C) and 8 days (D) post-bleomycin challenge stained with hematoxylin and eosin and photographed at ×400 magnification.

Another likely stimulus for MIP-1α expression is IL-1. Previous work has established that IL-1 is a potent *in vitro* stimulus for MIP-1α. Cultures of peripheral blood monocytes, alveolar macrophages, and inflammatory fibroblasts all secreted significantly elevated levels of MIP-1α protein after stimulation with IL-1 [22, 33]. Although administration of IL-1 receptor antagonist protein to bleomycin-challenged mice putatively attenuated fibrosis, no link between IL-1 and expression of specific chemoattractants, like MIP-1α, has been demonstrated in the bleomycin model [34].

The data presented in this study support the hypothesis that cytokine networks are activated in the lungs after bleomycin challenge. We propose the following cytokine networks. (1) The acute lesion: bleomycin instillation stimulates TNF, IL-6, and possibly IL-1 secretion from airway epithelial cells and resident alveolar macrophages. Next, TNF and IL-6 synergize to stimulate MIP-1α expression from alveolar macrophages, contributing to the expansion of the lesion via recruitment of mononuclear phagocytes (**Fig. 7**, **A** and **C**), (2) The chronic lesion: stimulated alveolar macrophages are likely secreting TNF and IL-1, stimulating expression of MIP-1α and other chemokines such as MCP-1, which in turn recruit additional macrophages and lymphocytes to the expanding inflammatory lesion (Fig. 7, B and D). The inflammatory cells recruited and modulated by these cytokine networks are a component of the profibrotic inflammatory nidus responsible for dysregulated collagen synthesis and deposition. Similar to IPF, these processes result in significant pulmonary pathophysiology. Although the precise mechanism of stimulation of the profibrotic lesion is unclear, we have identified important components of this process, providing insights for future investigations and interventions.

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REFERENCES

1. Chandler, D. B. (1990) Possible mechanisms of bleomycin-induced fibrosis. Clin. Chest Med. 11, 21–30.
2. Schrier, D. J., Kunkel, R. G., Phan, S. H. (1983) The role of strain variation in murine bleomycin-induced pulmonary fibrosis. Am. Rev. Respir. Dis. 127, 63–66.
3. Adamson, I. Y., Bowden, D. H. (1974) The pathogenesis of belomycin-induced pulmonary fibrosis in mice. Am. J. Pathol. 77, 185–197.
4. Aso, Y., Yoneda, Y. (1976) Morphological and biochemical study of pulmonary changes induced by bleomycin in mice. Lab. Invest. 35, 558–568.

5. Bedrossian, C. M. W., Grennberg, S. D., Yawn, D. H., O'Neal, R. M. (1977) Experimentally induced bleomycin sulfate pulmonary toxicity. Arch. Pathol. Lab. Med. 101, 248–254.
6. Bowden, D. H. (1984) Unraveling pulmonary fibrosis: the bleomycin model. Lab. Invest. 50, 487–488.
7. Smith, R. E., Strieter, R. M., Phan, S. H., Kunkel, S. L. (1996) C-C chemokines: novel mediators of the profibrotic inflammatory response to bleomycin challenge. Am. J. Respir. Cell Mol. Biol. 15, 693–702.
8. Smith, R. E., Strieter, R. M., Phan, S. H., Lukacs, N. W., Huffnagle, G. B., Wilke, C. A., Burdick, M. D., Lincoln, P., Evanoff, H., Kunkel, S. L. (1994) Production and function of murine macrophage inflammatory protein-1α in bleomycin-induced lung injury. J. Immunol. 153, 4704–4712.
9. Piguet, P. F., Collart, M. A., Grau, G. E., Kapanci, Y., Vassalli, P. (1989) Tumor necrosis factor/cachectin plays a key role in bleomycin-induced pneumopathy and fibrosis. J. Exp. Med. 170, 655–663.
10. Zhang, K., Gharaee, M., McGarry, B., Remick, D. Phan , S. H. (1997) TNF-α mediated lung cytokine networking and eosinophil recruitment in pulmonary fibrosis. J. Immunol. 158, 954–959.
11. Phan, S. H., Kunkel, S. L. (1992) Lung cytokine production in bleomycin-induced pulmonary fibrosis. Exp. Lung Res. 18, 18–43.
12. Micallef, M., Hosokawa, M., Togashi, Y., Kobayashi, H. (1992) Rat macrophage activation after treatment with the bleomycin group of antitumour antibiotics *in vivo*. Cancer Immunol. Immunother. 35, 106–112.
13. Karmiol, S., Remick, D. G., Kunkel, S. L., Phan, S. L. (1993) Regulation of rat pulmonary endothelial cell interleukin-6 production by bleomycin: effects of cellular fatty acid composition. Am. J. Respir. Cell Mol. Biol. 9, 628–636.
14. Biffl, W. L., Moore, E. E., Moore, F. A., Barnett, C. C., Jr. (1996) Interleukin-6 delays neutrophil apoptosis via a mechanism involving platelet activating factor. J. Trauma 40, 575–579.
15. Burdin, N., Van Kooten, C., Galibert, L., Abrams, J. S., Wijdenes, J., Banchereau, J., Rousset, F. (1995) Endogenous IL-6 and IL-10 contribute to the differentiation of CD-40-activated human B lymphocytes. J. Immunol. 154, 2533–2544.
16. Burdick, M. D., Kunkel, S. L., Lincoln, P. M., Wilkie, C. A., Strieter, R. M. (1993) Specific ELISAs for the detection of human macrophage inflammatory protein-1 alpha and beta. Immunol. Invest. 22, 441–449.
17. Mohler, K. M., Torrence, D. S., Smith, C. A., Goodwin, R. G., Stremler, K. B., Fung, V. P., Madani, H., Widmer, M. B. (1993) Soluble tumor necrosis factor (TNF) receptors are effective therapeutic agents in lethal endotoxemia and function simultaneously as both TNF carriers and TNF antagonists. J. Immunol. 151, 1548.
18. Piguet, P. F., Vesin, C. (1994) Treatment by human recombinant soluble TNF receptor of pulmonary fibrosis induced by bleomycin or silica in mice. Eur. Respir. J. 7, 515–518.
19. Lukacs, N. W., Chensue, S. W., Strieter, R. M., Warmington, K., Kunkel, S. L. (1994) Inflammatory granuloma formation is mediated by TNF-α-inducible intercellular adhesion molecule-1. J. Immunol. 152, 5883–5889.
20. Lukacs, N. W., Strieter, R. M., Chensue, S. W., Widmer, M., Kunkel, S. L. (1995) TNF-α mediates recruitment of neutrophils and eosinophils during airway inflammation. J. Immunol. 154, 5411–5417.
21. Standiford, T. J., Kunkel, S. L., Lukacs, N. W., Greenberger, M. J., Danforth, J. M., Kunkel, R. G., Strieter, R. M. (1995) Macrophage inflammatory protein-1α mediates lung leukocyte recruitment, lung capillary leak, and early mortality in murine endotoxemia. J. Immunol. 155, 1515–1524.
22. VanOtteren, G. M., Standiford, T. J., Kunkel, S. L., Danforth, J. M., Burdick, M. D., Abruzzo, L. V., Strieter, R. M. (1994) Expression and regulation of macrophage inflammatory protein-1α by murine alveolar and peritoneal macrophages. Am. J. Respir. Cell Mol. Biol. 10, 8–15.
23. Lukacs, N. W., Strieter, R. M., Chensue, S. W., Kunkel, S. L. (1996) Activation and regulation of chemokines in allergic airway inflammation. J. Leukoc. Biol. 59, 13–17.
24. Taub, D. D., Conlon, K., Lloyd, A. R., Oppenheim, J. J., Kelvin, D. J. (1993) Preferential migration of activated $CD4^+$ and $CD8^+$ T cells in response to MIP-1 alpha and MIP-1 beta. Science 260, 355–358.
25. Schall, T. J., Bacon, K., Camp, R. D., Kaspari, J. W., Goeddel, D. V. (1993) Human macrophage inflammatory protein alpha (MIP-1 alpha) and MIP-1 beta attract distinct populations of lymphocytes. J. Exp. Med. 177, 1821–1826.
26. Rand, M. L., Warren, J. S., Mansour, M. K., Neuman, W., Ringler, D. J. (1996) Inhibition of T cell recruitment and cutaneous delayed-type hypersensitivity-induced inflammation with antibodies to monocyte chemoattractant protein-1. Am. J. Pathol. 148, 855–864.
27. Koch, A. E., Kunkel, S. L., Harlow, L. A., Mazarakis, D. D., Haines, G. K., Burdick, M. D., Pope, R. M., Strieter, R. M. (1994) Macrophage Inflammatory protein-1α: A novel chemotactic cytokine for macrophages in rheumatiod arthritis. J. Clin. Invest. 93, 921–928.
28. Driscoll, K. E., Hassanbein, D. G., Carter, J., Poynter, J., Asquith, T. N., Grant, R. A., Whitten, J., Purdon, M. P., Takigiku, R. (1993) Macrophage inflammatory proteins 1 and 2: Expression by rat alveolar macrophages, fibroblasts, and epithelial cells and in rat lung after mineral dust exposure. Am. J. Respir. Cell Mol. Biol. 8, 311–318.
29. Lukacs, N. W., Kunkel, S. L., Allen, R., Evanoff, H. L., Shaklee, C. L., Sherman, J. S., Burdick, M. D., Strieter, R. M. (1995) Stimulus and cell-specific expression of C-X-C and C-C chemokines by pulmonary stromal cell populations. Am. J. Physiol. 268, L856–L861.
30. Scheule, R. K., Perkins, R. C., Hamilton, R., Holian, A. (1992) Bleomycin stimulation of cytokine secretion by the human alveolar macrophage. Am. J. Physiol. 262, 386–391.
31. Crestani, B., Cornillet, P., Dehoux, M., Rolland, C., Guenounou, M., Aubier, M. (1994) Alveolar type II epithelial cells produce interleukin-6 *in vitro* and *in vivo*. Regulation by alveolar macrophage secretory products. J. Clin. Invest. 94, 731–740.
32. Cromwell, O., Hamid, Q., Corrigan, C. J., Barkans, J., Meng, Q., Collins, P. D., Kay A. B. (1992) expression and generation of interleukin-8, IL-6, and granulocyte-macrophage colony stimulating factor by bronchial epithelial cells and enhancement by IL-1 beta and tumor necrosis factor alpha. Immunol. 77, 330–337.
33. Lukacs, N. W., Chensue, S. W., Smith, R. E., Strieter, R. M., Warmington, K., Wilke, C., Kunkel, S. L. (1994) Production of monocyte chemoattractant protein-1 and macrophage inflammatory protein-1α by inflammatory granuloma fibroblasts. Am. J. Pathol. 144, 711–718.
34. Piguet, P. F., Vesin, C., Grau, G. E., Thompson, R. C. (1993) Interleukin 1 receptor antagonist (IL-1ra) prevents or cures pulmonary fibrosis elicited in mice by bleomycin or silica. Cytokine 5, 57–61.