GM-CSF Regulates Bleomycin-Induced Pulmonary Fibrosis Via a Prostaglandin-Dependent Mechanism

Bethany B. Moore, Michael J. Coffey, Paul Christensen, Stephanie Sitterding, Rose Ngan, Carol A. Wilke, Rod McDonald, Susan M. Phare, Marc Peters-Golden, Robert Paine III and Galen B. Toews

*J Immunol* 2000; 165:4032-4039; doi: 10.4049/jimmunol.165.7.4032
http://www.jimmunol.org/content/165/7/4032

**References**

This article cites 34 articles, 13 of which you can access for free at: http://www.jimmunol.org/content/165/7/4032.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
GM-CSF Regulates Bleomycin-Induced Pulmonary Fibrosis Via a Prostaglandin-Dependent Mechanism

Bethany B. Moore, Michael J. Coffey, Paul Christensen, Stephanie Sitterding, Rose Ngan, Carol A. Wilke, Rod McDonald, Susan M. Phare, Marc Peters-Golden, Robert Paine III, and Galen B. Toews

To characterize the role of GM-CSF in pulmonary fibrosis, we have studied bleomycin-induced fibrosis in wild-type mice vs mice with a targeted deletion of the GM-CSF gene (GM-CSF–/– mice). Without GM-CSF, pulmonary fibrosis was worse both histologically and quantitatively. These changes were not related to enhanced recruitment of inflammatory cells because wild-type and GM-CSF–/– mice recruited equivalent numbers of cells to the lung following bleomycin. Interestingly, recruitment of eosinophils was absent in GM-CSF–/– mice. We investigated whether the enhanced fibrotic response in GM-CSF–/– animals was due to a deficiency in an endogenous down-regulator of fibrogenesis. Analysis of whole lung homogenates from saline- or bleomycin-treated mice revealed that GM-CSF–/– animals had reduced levels of PGE\(_2\). Additionally, alveolar macrophages were harvested from wild-type and GM-CSF–/– mice that had been exposed to bleomycin. Although bleomycin treatment impaired the ability of alveolar macrophages from wild-type mice to synthesize PGE\(_2\), alveolar macrophages from GM-CSF–/– mice exhibited a significantly greater defect in PGE\(_2\) synthesis than did wild-type cells. Exogenous addition of GM-CSF to alveolar macrophages reversed the PGE\(_2\) synthesis defect in vitro. Administration of the PG synthesis inhibitor, indomethacin, to wild-type mice during the fibrogenic phase postbleomycin worsened the severity of fibrosis, implying a causal role for PGE\(_2\) deficiency in the evolution of the fibrotic lesion. These data demonstrate that GM-CSF deficiency results in enhanced fibrogenesis in bleomycin-induced pulmonary fibrosis and indicate that one mechanism for this effect is impaired production of the potent antifibrotic eicosanoid, PGE\(_2\).

The Journal of Immunology, 2000, 165: 4032–4039.

Pulmonary fibrosis is the consequence of diverse insults that result in damage to the alveolar surface of the lung. Lungs obtained from patients with fibrotic disease show alveolar epithelial cell injury and hyperplasia, inflammatory cell accumulation, fibroblast hyperplasia, and deposition of fibrous stroma with scar formation (1, 2). Pulmonary macrophages are important regulators of the repair processes that are initiated following lung injury. They have the capacity either to increase tissue damage and fibrosis or to promote repair. Their pathogenetic potential is a result of their production of molecules that increase cell injury (reactive oxygen and nitrogen intermediates) (1–3), enhance inflammation through the recruitment and activation of other inflammatory cells (chemokines and/or cytokines) (4), or increase the number and activity of fibroblasts (growth factors) (5, 6). However, macrophages can also express molecules that inhibit fibrosis and promote repair in the proper milieu. These include substances that promote the clearance of fibrin matrix (such as uPA) (7–9) and down-regulate inflammation, fibroblast proliferation, and collagen deposition (such as PGE\(_2\)) (10–14).

The functional activity of a macrophage is likely determined by its maturational path and the local cytokine milieu. GM-CSF is a 23-kDa glycoprotein member of the hemopoietic cytokine family, which regulates the proliferation and differentiation of cells in the granulocyte-macrophage lineage (15). GM-CSF also has potent effects on the function of mature hemopoietic cells. The lung is a rich source of GM-CSF, but the functional aspects of GM-CSF in lung tissue injury and repair/fibrosis remain uncertain.

GM-CSF likely plays a complex role in the processes of fibrosis and tissue repair at epithelial surfaces. Intradermal administration of GM-CSF to leprosy patients with skin wounds leads to enhanced wound healing with increased numbers and layers of keratinocytes (16). GM-CSF stimulates keratinocyte proliferation at nanogram per milliliter concentrations (17, 18). Transgenic overexpression of GM-CSF in the lung under the control of the surfactant protein C promoter results in enhanced lung growth and alveolar type II cell hyperplasia (19). In bleomycin-induced pulmonary fibrosis in mice, administration of GM-CSF-neutralizing antisera increases the numbers of macrophages recoverable by bronchoalveolar lavage (BAL)\(^3\) and increases the deposition of hydroxyproline (20). Conversely, when overexpressed in the lung using a recombinant replication-defective adenoviral vector, GM-CSF is fibrogenic, leading to accumulation of macrophages and eosinophils in the lung, lung tissue injury, and varying degrees of fibrosis (21).

Our understanding of the role that GM-CSF plays in the complex interplay of the processes of repair and fibrosis has been limited by the inability to eliminate GM-CSF from the system completely and irreversibly, especially over the duration required to

\(^{3}\) Abbreviations used in this paper: BAL, bronchoalveolar lavage; LTC\(_4\), leukotriene \(C_4\); i.t., intratracheal(ly); WT, Wright-Giemsa; EIA, enzyme immunoassay; COX, cyclooxygenase; IPF, idiopathic pulmonary fibrosis.
assess the fibrotic response. The development of transgenic mice with a targeted deletion of the GM-CSF gene allows the development of an animal model to test the importance of GM-CSF in lung injuries that progress to fibrosis. Intratracheal instillation of the chemotherapeutic agent bleomycin in C57BL/6 mice is a widely accepted animal model for studying the lung fibrotic response. In response to this insult, numerous cytokines, chemokines, and growth factors are produced that mediate the fibrotic/repair processes. In this study we investigate the importance of GM-CSF for the development of bleomycin-induced fibrosis. Comparing GM-CSF/-/- mice with GM-CSF+/- (wild-type) controls, we evaluate the importance of GM-CSF for collagen deposition, inflammatory cell recruitment, and leukotriene and PG production.

Materials and Methods

Mice

A breeding pair of GM-CSF+/- mice, generated by G. Dranoff and bred extensively onto the C57BL/6 background, were obtained from J. A. Whitsett (Cincinnati, OH) and have been previously described (22). The mice were bred in the University Laboratory Animal Medicine facilities under specific pathogen-free conditions at the University of Michigan. Control C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). As GM-CSF+/- mice may be larger than age-matched controls, animals were matched for weight (20–25 g) in all experiments. The University of Michigan Committee on the Use and Care of Animals approved these experiments.

Bleomycin injection

Control and GM-CSF+/- mice were anesthetized with sodium pentobarbital. A single incision was made at the neck, and the salivary glands were parted by blunt dissection. The muscle covering the trachea was snipped to expose the tracheal rings. A single 30-μl injection containing 0.025 U of bleomycin (Sigma, St. Louis, MO) diluted in normal saline was injected using a Tridak stepper (Brookfield, CT) and a 30-gauge needle.

Hydroxyproline assays

Hydroxyproline is a modified amino acid found at a uniquely high percentage in collagen. Therefore, we determined the hydroxyproline content of the lungs as a quantitative measure of collagen deposition. Mice were euthanized by CO2 asphyxiation and perfused via the heart with 5 ml of 10% buffered saline. The lungs were brought up to 10 ml with complete media. An equal volume of 40% Percoll (Baxter Scientific, Miami, FL) and 10% FCS, antibiotics, 1 mg/ml collagenase (Boehringer Mannheim, Chicago, IL) and 30 μg/ml DNase (Sigma). The cell suspension and undigested fragments were further dispersed by repeated passage through the bore of a 10-ml syringe without a needle. The total cell suspension was pelleted, then baked at 110°C for 12 h. Aliquots (5–10 μl) were then assayed by adding a hydroxyproline standard (Sigma). Droxyproline is a modified amino acid found at a uniquely high percentage in collagen. Therefore, we determined the hydroxyproline content of the lungs as a quantitative measure of collagen deposition. Mice were euthanized by CO2 asphyxiation and perfused via the heart with 5 ml of 10% buffered saline. The lungs were brought up to 10 ml with complete media. An equal volume of 40% Percoll (Baxter Scientific, Miami, FL) and 10% FCS, antibiotics, 1 mg/ml collagenase (Boehringer Mannheim, Chicago, IL) and 30 μg/ml DNase (Sigma). The cell suspension and undigested fragments were further dispersed by repeated passage through the bore of a 10-ml syringe without a needle. The total cell suspension was pelleted, then baked at 110°C for 12 h. Aliquots (5–10 μl) were then assayed by adding a hydroxyproline standard (Sigma).

Histology

Animals were euthanized and perfused via the left ventricle with 3 ml normal saline. Lungs were inflated with 1 ml 1% neutral buffered formalin, removed, and fixed overnight in formalin before being dehydrated in 70% ethanol. Lungs were then processed using standard procedures and embedded in paraffin. Sections (3–5 μm) were cut, mounted on slides, and stained with Masson’s Trichrome Blue for collagen deposition.

Collagenase digestions of whole lung

To analyze lung leukocytes, lungs were excised, minced, and enzymatically digested for 30 min using 15 ml of lung digestion buffer (RPMI, 5% FCS, antibiotics, 1 mg/ml collagenase) (Boehringer Mannheim, Chicago, IL) and 30 μg/ml DNase (Sigma). The cell suspension and undigested fragments were further dispersed by repeated passage through the bore of a 10-ml syringe without a needle. The total cell suspension was pelleted, and any contaminating erythrocytes were eliminated by lysis in ice-cold NH4Cl buffer (0.829% NH4Cl, 0.1% KHCO3, and 0.0372% Na2EDTA, pH 7.4). The pellet was resuspended in 5 ml of complete medium (RPMI, 10% FCS, 1% penicillin/streptomycin) and dispersed by 20 passages through a 5-ml syringe. Then the dispersed cells were filtered through a Nytex filter (Tetko, Kansas City, MO) to remove clumps. The total volume was brought up to 10 ml with complete media. An equal volume of 40% Percoll was added and the cells were centrifuged at 3000 rpm for 30 min. The cell pellets were resuspended in complete media and counted on a hemocytometer in the presence of trypan blue. Cells were >90% viable by trypan blue exclusion. Cytospins of recovered cells were prepared for differential staining as described below.

BAL

Mice were euthanized via CO2 asphyxiation, the trachea was cannulated with polyethylene tubing (PE50, Intramedic; Clay Adams, Parsippany, NJ) attached to a 25-gauge needle on a tuberculin syringe, and the lungs were lavaged twice with 0.75 ml PBS/5% EDTA (Sigma) for a total lavage volume of 1.5 ml. In >95% of the mice, the recovery volume was 1.3–1.4 ml. The BAL fluid was spun at 1500 rpm, and the supernatant was removed. The pellet cells were collected, and erythrocytes were lysed as described above in ice-cold NH4Cl buffer for 3 min before being washed and pelleted in complete media. Total BAL cells were enumerated by counting on a hemocytometer in the presence of trypan blue. Cytospins were prepared from resuspended BAL cells.

Differential staining

Cytospins of either collagenase digestions or BAL were made by centrifuging 50,000–100,000 cells onto microscope slides using a Shandon Cytoprimer (Shandon, Astmoore, U. K.). The slides were dried, and were then stained using a modified Wright-Giemsa (WG) stain. For WG staining, the slides were fixed/prestained for 2 min with a one-step methanol-based WG stain (Harleco; EM Diagnostics, Gibbstown, NJ) followed by steps 2 and 3 of the Diff-Quick whole blood stain (Diff-Quick; Baxter Scientific, Miami, FL). This modification of the Diff-Quick stain procedure improves the resolution of eosinophils from neutrophils in the mouse. A total of 300 cells were counted from randomly chosen high power microscope fields for each sample. The differential percentage was multiplied by the total leukocyte number to derive the number of monocyte/macrophages, neutrophils, and eosinophils per sample.

FACS analysis

Lung cells (1 × 106) pooled from the collagenase digestions of three separate animals were incubated for 15 min on ice in Fc block (PharMingen, San Diego, CA) before washing and centrifugation. Then cells were stained in a 100-μl total volume with 1-μg combinations of the following Abs: CD45 (YW62.3; Caltag Laboratories, Burlingame, CA), CD4 (RM4-4; PharMingen), CD8 (53-6.7; PharMingen), CD19 (1D3; PharMingen), TCR β (H57-597; PharMingen), TCR γδ (GL3; PharMingen), or DX5 (PharMingen). Stained samples were stored in the dark at 4°C until analysis on a flow cytometer (FACScan; Becton Dickinson, Mountain View, CA). All samples were stained to identify a leukocyte/epithelial marker. The absolute number of a type of leukocyte in the lungs was determined as the percentage of that cell type times the total number of cells in the lungs.

Determination of PGE2 synthesis by isolated alveolar macrophages

Following anesthesia and euthanasia by exsanguination, the tracheobronchial tree was exposed and alveolar macrophages were harvested by BAL using two 0.75-ml lavages in PBS containing 5 mM EDTA. Isolated alveolar cells were resuspended in serum-free DMEM at 0.5 × 106/ml and plated at 0.2 ml/well in 96-well plates. Nonadherent cells were removed by washing twice with DMEM, and adherent cells were cultured in DMEM containing 10% FCS overnight, with or without exogenous murine recombinant GM-CSF (17 ng/ml; R&D Systems, Minneapolis, MN). Following overnight culture, the alveolar macrophages were washed three times in DMEM and subsequently stimulated with Ca2+ ionophore A23187 (1 μM) for 30 min to trigger arachidonate metabolism. Cell-free supernatants were analyzed by enzyme immunoassay (EIA) for the predominant cyclooxygenase (COX) product PGE2 (Cayman Chemicals, Ann Arbor, MI).

PGE2 and leukotriene C4 (LTC4) analysis in lung homogenates

Mice were euthanized and the lungs were harvested as described above. Lung tissue was homogenized (Tissue Tearor, model 958-370; Biospec Products, Bartlesville, OK) at 5 ml in a 1-ml volume of ice-cold DMEM. Following homogenization, tissue fragments and intact cells were further fragmented by sonication (Sonifier model 250; Branson Ultrasonecs, Danbury, CT) on ice for 30 s at power level 1. Lipids were then methanol extracted from the homogenate using Sep-Pak cartridges (Waters). The samples were dried down under a steady stream of nitrogen to evaporate the solvent, then resuspended in 250 μl of DMEM. PGE2 and LTC4 levels were measured by EIA (Cayman Chemicals, Ann Arbor, MI).
In vivo indomethacin studies

C57BL/6 mice were anesthetized and injected with 0.025 U bleomycin intratracheally (i.t.) as described. Mice were divided into two groups with one group receiving daily i.p. injections of the PG synthesis inhibitor, indomethacin, at a dose of 1.2 mg/kg as previously described (24). Indomethacin injections were started at day 10 following bleomycin injection to specifically inhibit PG synthesis during the postinflammatory, fibroproliferative phase of the bleomycin response.

Statistics

Statistical significance was analyzed using the InStat 2.01 program (GraphPad Software, San Diego, CA) on a Power Macintosh G3. Student t tests were run to determine p values. Values of p < 0.05 were considered significant.

Results

Bleomycin-induced pulmonary fibrosis is more severe in GM-CSF−/− mice

Weight-matched GM-CSF−/− mice and C57BL/6 wild-type mice were injected with bleomycin or saline as a control. Animals were euthanized at day 21 postinoculation, and lungs were analyzed histologically for the extent of fibrosis (Fig. 1). Lung tissues were stained with Masson’s Trichrome Stain. No fibrotic changes were noted in saline-treated wild-type (A) or GM-CSF−/− mice (B). Following bleomycin injection, GM-CSF−/− animals (D) demonstrated more severe and more extensive fibrosis than their wild-type counterparts (C). Thus, histological analysis of wild-type and GM-CSF−/− mice documented the presence of more severe fibrosis in GM-CSF−/− mice.

To quantitatively measure the extent of pulmonary fibrosis in these mice, wild-type and GM-CSF−/− mice were injected with saline or bleomycin, and assayed for hydroxyproline content at day 21. Results are expressed as µg/ml hydroxyproline/g weight of the mouse (Fig. 2). GM-CSF−/− mice treated with bleomycin contained significantly more lung hydroxyproline than bleomycin-treated wild-type mice (*, p = 0.02). There were no significant differences in hydroxyproline content in wild-type or GM-CSF−/− mice injected with saline (Fig. 2). Thus, fibrosis in response to bleomycin was significantly more severe in the GM-CSF−/− mice than in wild-type mice.

Day 7 is the peak inflammatory response following bleomycin injection

To characterize the inflammatory response to bleomycin, we performed a kinetic analysis in wild-type mice to determine when the peak inflammatory response occurred. Wild-type mice were injected with either saline or bleomycin on day 0. Animals were then euthanized at days 1, 3, 7, 14, and 21. BAL fluid was collected at each time point, and the number of total leukocytes was determined. The peak inflammatory response to bleomycin in wild-type mice occurred at day 7 postinjection (Fig. 3A). Results were similar in the GM-CSF−/− mice, where bleomycin injection resulted in a peak inflammatory response at day 7 as well (Fig. 3B). The total number of BAL cells in GM-CSF−/− mice was greater at day 7 than that in wild-type mice (5.9 ± 1.5 ± 10^6 vs 1.05 ± 0.01 ± 10^6). Baseline numbers of BAL cells were also higher in the GM-CSF−/− mice (1.02 ± .36 ± 10^5 vs 0.078 ± 0.02 ± 10^5 for wild type). Because day 7 was the peak response for both groups of mice, this time point was chosen for further study of the inflammatory response to bleomycin.

Recruitment of inflammatory cells was similar in wild-type and GM-CSF−/− mice

Histological analysis revealed that the inflammatory response involved both the alveolar and interstitial spaces of the lung. Therefore, we performed collagenase digestions on excised whole lung...
Day 7 is the peak inflammatory response in both GM-CSF−/− and wild-type animals, bleomycin was injected into GM-CSF−/− animals. The recovered cells were analyzed both by differential counting and flow cytometry. Table I gives values for inflammatory cell numbers in the lung at baseline compared with bleomycin treatment at day 7. Table II compares total lymphocyte populations in the same groups of mice. Cells of monocytic origin accounted for the largest numbers of cells in the lung digests. By differential analysis, we consistently observed more monocytic cells at baseline in GM-CSF−/− animals than in wild-type controls (#, p = 0.04). Following bleomycin treatment, the absolute numbers of monocytes and macrophages further increases. Similarly, neutrophils are increased in GM-CSF−/− animals compared with wild-type animals at baseline and following bleomycin. Eosinophils are absent in GM-CSF−/− animals. Interestingly, although absolute numbers of lymphocytes are increased in wild-type mice following bleomycin, they decrease or are unchanged in GM-CSF−/− animals following bleomycin. Given that there are few significant differences in the magnitude of the inflammatory response in the wild-type and GM-CSF−/− mice, we hypothesized that the differences in the fibrotic responses between wild-type and GM-CSF−/− mice might be due to altered production of pro- or anti-inflammatory mediators.

GM-CSF−/− mice are deficient in the production of the lipid mediator, LTC₄, in response to bleomycin

One possibility to explain the increased fibrotic response in GM-CSF−/− mice was elevated leukotriene levels. Leukotrienes have a variety of possible proinflammatory/profibrotic actions. To investigate this possibility, lung homogenates from wild-type or GM-CSF−/− mice were analyzed for the predominant murine lung leukotriene product, LTC₄, at day 7 following bleomycin treatment. GM-CSF−/− mice had significantly reduced levels of LTC₄ (Fig. 5, p = 0.01) as compared with wild-type animals, demonstrating differences in the fibrotic responses between wild-type and GM-CSF−/− mice might be due to altered production of pro- or anti-inflammatory mediators.

The number of monocytes/macrophages is increased in GM-CSF−/− mice

The recovered cells were analyzed both by differential counting and flow cytometry. Table I gives values for inflammatory cell numbers in the lung at baseline compared with bleomycin treatment at day 7. Table II compares total lymphocyte populations in the same groups of mice. Cells of monocytic origin accounted for the largest numbers of cells in the lung digests. By differential analysis, we consistently observed more monocytic cells at baseline in GM-CSF−/− animals than in wild-type controls (#, p = 0.04). Following bleomycin treatment, the absolute numbers of monocytes and macrophages further increases. Similarly, neutrophils are increased in GM-CSF−/− animals compared with wild-type animals at baseline and following bleomycin. Eosinophils are absent in GM-CSF−/− animals. Interestingly, although absolute numbers of lymphocytes are increased in wild-type mice following bleomycin, they decrease or are unchanged in GM-CSF−/− animals following bleomycin. Given that there are few significant differences in the magnitude of the inflammatory response in the wild-type and GM-CSF−/− mice, we hypothesized that the differences in the fibrotic responses between wild-type and GM-CSF−/− mice might be due to altered production of pro- or anti-inflammatory mediators.

GM-CSF−/− mice are deficient in the production of the lipid mediator, LTC₄, in response to bleomycin

One possibility to explain the increased fibrotic response in GM-CSF−/− mice was elevated leukotriene levels. Leukotrienes have a variety of possible proinflammatory/profibrotic actions. To investigate this possibility, lung homogenates from wild-type or GM-CSF−/− mice were analyzed for the predominant murine lung leukotriene product, LTC₄, at day 7 following bleomycin treatment. GM-CSF−/− mice had significantly reduced levels of LTC₄ (Fig. 5, p = 0.01) as compared with wild-type animals, demonstrating differences in the fibrotic responses between wild-type and GM-CSF−/− mice might be due to altered production of pro- or anti-inflammatory mediators.

The number of monocytes/macrophages is increased in GM-CSF−/− mice

The recovered cells were analyzed both by differential counting and flow cytometry. Table I gives values for inflammatory cell numbers in the lung at baseline compared with bleomycin treatment at day 7. Table II compares total lymphocyte populations in the same groups of mice. Cells of monocytic origin accounted for the largest numbers of cells in the lung digests. By differential analysis, we consistently observed more monocytic cells at baseline in GM-CSF−/− animals than in wild-type controls (#, p = 0.04). Following bleomycin treatment, the absolute numbers of monocytes and macrophages further increases. Similarly, neutrophils are increased in GM-CSF−/− animals compared with wild-type animals at baseline and following bleomycin. Eosinophils are absent in GM-CSF−/− animals. Interestingly, although absolute numbers of lymphocytes are increased in wild-type mice following bleomycin, they decrease or are unchanged in GM-CSF−/− animals following bleomycin. Given that there are few significant differences in the magnitude of the inflammatory response in the wild-type and GM-CSF−/− mice, we hypothesized that the differences in the fibrotic responses between wild-type and GM-CSF−/− mice might be due to altered production of pro- or anti-inflammatory mediators.

GM-CSF−/− mice are deficient in the production of the lipid mediator, LTC₄, in response to bleomycin

One possibility to explain the increased fibrotic response in GM-CSF−/− mice was elevated leukotriene levels. Leukotrienes have a variety of possible proinflammatory/profibrotic actions. To investigate this possibility, lung homogenates from wild-type or GM-CSF−/− mice were analyzed for the predominant murine lung leukotriene product, LTC₄, at day 7 following bleomycin treatment. GM-CSF−/− mice had significantly reduced levels of LTC₄ (Fig. 5, p = 0.01) as compared with wild-type animals, demonstrating differences in the fibrotic responses between wild-type and GM-CSF−/− mice might be due to altered production of pro- or anti-inflammatory mediators.

The number of monocytes/macrophages is increased in GM-CSF−/− mice

The recovered cells were analyzed both by differential counting and flow cytometry. Table I gives values for inflammatory cell numbers in the lung at baseline compared with bleomycin treatment at day 7. Table II compares total lymphocyte populations in the same groups of mice. Cells of monocytic origin accounted for the largest numbers of cells in the lung digests. By differential analysis, we consistently observed more monocytic cells at baseline in GM-CSF−/− animals than in wild-type controls (#, p = 0.04). Following bleomycin treatment, the absolute numbers of monocytes and macrophages further increases. Similarly, neutrophils are increased in GM-CSF−/− animals compared with wild-type animals at baseline and following bleomycin. Eosinophils are absent in GM-CSF−/− animals. Interestingly, although absolute numbers of lymphocytes are increased in wild-type mice following bleomycin, they decrease or are unchanged in GM-CSF−/− animals following bleomycin. Given that there are few significant differences in the magnitude of the inflammatory response in the wild-type and GM-CSF−/− mice, we hypothesized that the differences in the fibrotic responses between wild-type and GM-CSF−/− mice might be due to altered production of pro- or anti-inflammatory mediators.

GM-CSF−/− mice are deficient in the production of the lipid mediator, LTC₄, in response to bleomycin

One possibility to explain the increased fibrotic response in GM-CSF−/− mice was elevated leukotriene levels. Leukotrienes have a variety of possible proinflammatory/profibrotic actions. To investigate this possibility, lung homogenates from wild-type or GM-CSF−/− mice were analyzed for the predominant murine lung leukotriene product, LTC₄, at day 7 following bleomycin treatment. GM-CSF−/− mice had significantly reduced levels of LTC₄ (Fig. 5, p = 0.01) as compared with wild-type animals, demonstrating differences in the fibrotic responses between wild-type and GM-CSF−/− mice might be due to altered production of pro- or anti-inflammatory mediators.

The number of monocytes/macrophages is increased in GM-CSF−/− mice

The recovered cells were analyzed both by differential counting and flow cytometry. Table I gives values for inflammatory cell numbers in the lung at baseline compared with bleomycin treatment at day 7. Table II compares total lymphocyte populations in the same groups of mice. Cells of monocytic origin accounted for the largest numbers of cells in the lung digests. By differential analysis, we consistently observed more monocytic cells at baseline in GM-CSF−/− animals than in wild-type controls (#, p = 0.04). Following bleomycin treatment, the absolute numbers of monocytes and macrophages further increases. Similarly, neutrophils are increased in GM-CSF−/− animals compared with wild-type animals at baseline and following bleomycin. Eosinophils are absent in GM-CSF−/− animals. Interestingly, although absolute numbers of lymphocytes are increased in wild-type mice following bleomycin, they decrease or are unchanged in GM-CSF−/− animals following bleomycin. Given that there are few significant differences in the magnitude of the inflammatory response in the wild-type and GM-CSF−/− mice, we hypothesized that the differences in the fibrotic responses between wild-type and GM-CSF−/− mice might be due to altered production of pro- or anti-inflammatory mediators.

GM-CSF−/− mice are deficient in the production of the lipid mediator, LTC₄, in response to bleomycin

One possibility to explain the increased fibrotic response in GM-CSF−/− mice was elevated leukotriene levels. Leukotrienes have a variety of possible proinflammatory/profibrotic actions. To investigate this possibility, lung homogenates from wild-type or GM-CSF−/− mice were analyzed for the predominant murine lung leukotriene product, LTC₄, at day 7 following bleomycin treatment. GM-CSF−/− mice had significantly reduced levels of LTC₄ (Fig. 5, p = 0.01) as compared with wild-type animals, demonstrating differences in the fibrotic responses between wild-type and GM-CSF−/− mice might be due to altered production of pro- or anti-inflammatory mediators.
that in these mice enhanced leukotriene synthesis is not responsible for the exaggerated fibrosis; indeed, it occurs despite a reduction in LTC₄.

**GM-CSF⁻/⁻ mice are deficient in the production of the lipid mediator PGE₂ in response to bleomycin**

Another possible mechanism for the more aggressive fibrotic response in GM-CSF⁻/⁻ mice than in the wild-type mice was defective production of an antifibrotic mediator in the GM-CSF⁻/⁻ mice. PGE₂ potently down-regulates fibroblast proliferation and collagen synthesis (11–14). To assess whether a defect in PGE₂ production existed in the GM-CSF⁻/⁻ mice, wild-type and GM-CSF⁻/⁻ mice were injected with saline or bleomycin, and whole lung homogenates were assayed for PGE₂ levels at days 7 and 21. The lungs of GM-CSF⁻/⁻ animals contained lower levels of PGE₂ than wild-type animals at both time points for both the saline- and bleomycin-treated mice (Fig. 6). Thus, increased fibrosis in GM-CSF⁻/⁻ mice treated with bleomycin was associated with relatively impaired PGE₂ production at both days 7 and 21.

**Alveolar macrophages from GM-CSF⁻/⁻ mice are deficient in PGE₂ synthesis**

Because monocyte/macrophages accounted for the largest number of inflammatory cells in the lung, and pulmonary macrophages have a high capacity for PGE₂ synthesis, we assessed whether a defect in PGE₂ production existed in pulmonary macrophages in GM-CSF⁻/⁻ mice. To assess maximal capacity for PGE₂ synthesis, alveolar macrophages isolated from BAL of wild-type and GM-CSF⁻/⁻ mice were stimulated with calcium ionophore A23187 (1 μM) for 30 min, and PGE₂ was measured in the supernatant by EIA. PGE₂ production by unstimulated cells was below the level of assay detection in both wild-type and GM-CSF⁻/⁻ mice (data not shown). Stimulated alveolar macrophages from bleomycin-untreated GM-CSF⁻/⁻ mice produced significantly less PGE₂ than did cells from wild-type animals (Fig. 7, left). Bleomycin treatment was associated with a significant reduction in alveolar macrophage capacity for PGE₂ synthesis in wild-type animals (Fig. 7, right). Cells from GM-CSF⁻/⁻ animals treated with bleomycin were even more profoundly impaired in PGE₂ production.

To determine whether this defective PGE₂ production could be reversed by the addition of GM-CSF in vitro, the same experiment was performed with or without the addition of exogenous GM-CSF (17 ng/ml) in the overnight culture. The addition of GM-CSF to the alveolar macrophages from GM-CSF⁻/⁻ mice restored the ability of these cells to produce PGE₂ in response to stimulus (Fig. 8, right). GM-CSF administration restored the levels of PGE₂ seen in alveolar macrophages from GM-CSF⁻/⁻ animals back to the level seen in cells from wild-type animals. Furthermore, GM-CSF treatment superinduced the alveolar macrophages from wild-type animals to produce more PGE₂ upon stimulation (Fig. 8, left).

**Pharmacological blockade of PG synthesis increases bleomycin-induced pulmonary fibrosis in wild-type mice**

To determine whether the increased fibrosis seen in the GM-CSF⁻/⁻ mice could be causally linked to the diminished PGE₂ levels observed, experiments were performed in which PG synthesis was pharmacologically blocked by the in vivo administration of the COX inhibitor, indomethacin. Wild-type mice were treated with bleomycin on day 0, and then given daily i.p. administrations of indomethacin starting at day 10 postbleomycin. Fig. 9 demonstrates that indomethacin treatment started at day 10 significantly increases bleomycin-induced pulmonary fibrosis (*, p = 0.02) at day 21. The level of fibrosis seen in the day 10 indomethacin-treated mice is comparable to that measured in the GM-CSF⁻/⁻ mice treated with bleomycin (see Fig. 2). Thus, pharmacological inhibition of PGE₂ synthesis worsens bleomycin-induced pulmonary fibrosis.

---

**Table I. Inflammatory cell numbers in the lung (×10⁶) at baseline and at day 7 postbleomycin**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Wild-Type Baseline</th>
<th>GM-CSF Knockout Baseline</th>
<th>Wild-Type + Bleomycin</th>
<th>GM-CSF Knockout + Bleomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monocyte/macrophages</td>
<td>6.74 ± 1.01</td>
<td>11.01 ± 1.11</td>
<td>13.8 ± 1.21</td>
<td>17.17 ± 1.18</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0.795 ± 0.27</td>
<td>2.14 ± 0.28</td>
<td>3.71 ± 1.64</td>
<td>4.86 ± 0.44</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.21 ± 0.11</td>
<td>0</td>
<td>0.285 ± 0.14</td>
<td>0</td>
</tr>
</tbody>
</table>

*Values for monocytic cells, neutrophils and eosinophils were calculated as described in Materials and Methods. Values presented for lymphocyte subpopulations represent pooled FACS analysis of three mice per group.

**Table II. Lymphocyte subpopulations in the lung (×10⁶) at baseline and at day 7 postbleomycin**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Wild-Type Baseline</th>
<th>GM-CSF Knockout Baseline</th>
<th>Wild-Type + Bleomycin</th>
<th>GM-CSF Knockout + Bleomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4⁺</td>
<td>0.69</td>
<td>2.2</td>
<td>1.15</td>
<td>2.2</td>
</tr>
<tr>
<td>CD8⁺</td>
<td>0.77</td>
<td>1.17</td>
<td>1.15</td>
<td>0.88</td>
</tr>
<tr>
<td>NK</td>
<td>0.4</td>
<td>0.58</td>
<td>0.43</td>
<td>0.44</td>
</tr>
<tr>
<td>B</td>
<td>1.8</td>
<td>3.3</td>
<td>2.08</td>
<td>2.2</td>
</tr>
</tbody>
</table>

*Values for monocytic cells, neutrophils and eosinophils were calculated as described in Materials and Methods. Values presented for lymphocyte subpopulations represent pooled FACS analysis of three mice per group.
deficient (−/−) and wild-type (WT) animals (n = 4, day 7; *p = 0.009, **p = 0.03, n = 4, day 21).

Discussion

Our studies demonstrate that endogenous GM-CSF plays a beneficial protective role in the setting of pulmonary fibrosis. In particular, our studies yield several major points: 1) in the absence of GM-CSF, bleomycin-induced pulmonary fibrosis is worse, both histologically and quantitatively; 2) despite the absence of GM-CSF, recruitment of inflammatory cells to the lung in response to bleomycin is equivalent in magnitude to wild-type animals; 3) in the absence of GM-CSF, bleomycin-exposed lungs are devoid of eosinophils, cells that have been strongly implicated in the pathogenesis of bleomycin-induced fibrosis (25, 26); 4) GM-CSF−/− mice are impaired in the production of the potentially profibrotic lipid mediator, LTC4, following bleomycin injection; 5) lung levels of the anti-fibrotic lipid mediator, PGE2, are diminished in GM-CSF−/− mice; 6) in the absence of GM-CSF, pulmonary macrophages are impaired in their ability to synthesize PGE2 (Bleomycin treatment further blunts the ability of monocytes to synthesize PGE2); 7) in vitro addition of GM-CSF to GM-CSF−/− monocytes restores their capacity to synthesize PGE2; and 8) pharmacologic blockade of PGE2 results in worse bleomycin-induced pulmonary fibrosis.

Several aspects of the animal model used in this study are worthy of mention. First, the GM-CSF−/− mice lack GM-CSF at the genomic, mRNA, and protein levels. Thus, the lack of GM-CSF is specific, absolute, and irreversible. The use of animals that are genetically deficient in GM-CSF offers a definitive system for studying the role of GM-CSF in the evolution of pulmonary fibrosis. This approach avoids the use of neutralizing Abs, thereby eliminating the concerns about neutralization efficiency and complement-mediated tissue injury. Second, young animals were used in all studies. As GM-CSF−/− mice age, they develop progressive accumulation of surfactant lipids and proteins in the alveolar space (27). However, minimal or no evidence of pulmonary alveolar proteinosis was noted in the young animals used in our studies (Fig. 1, A and B). Most importantly, no fibrotic abnormalities are found at baseline in these mice. No histological findings suggestive of fibrosis are noted, and normalized hydroxyproline content in untreated wild-type and GM-CSF−/− mice do not differ (Fig. 2). Third, bleomycin-induced pulmonary fibrosis is a clinically relevant model of human disease (28).

Our findings documenting a beneficial role for GM-CSF in repair following lung injury are at variance with previously reported studies that used rats, in which GM-CSF was overexpressed in their lungs. High, transient overproduction of murine GM-CSF in rat lungs led to the accumulation of eosinophils and macrophages, which was associated with tissue injury, and was followed by histological evidence of fibrosis (21). Adenoviral delivery of murine GM-CSF to rat lungs resulted in an increase in granuloma formation, fibroblast accumulation, and expression of the fibrogenic cytokine TGF-β1 (29). Several features must be considered when evaluating these previously published studies. Concentrations of GM-CSF induced in this model were supraphysiological, and high levels of GM-CSF were expressed transiently during the early post injury

FIGURE 7. Reduced PGE2 production in alveolar macrophages from wild-type and GM-CSF−/− animals. Alveolar macrophages were isolated from untreated or bleomycin-treated mice at day 7. Cells were plated at 0.5 × 10⁶/ml overnight in DMEM containing 10% FCS. Maximal PGE2 production was determined following stimulation with A23187 (1 μM) for 30 min at 37°C. Medium was analyzed for PGE2 by EIA and expressed as pg PGE2/ml (n = 3). Alveolar macrophages from GM-CSF−/− animals produce less PGE2 than wild-type alveolar macrophages (*, p < 0.05 compared with untreated wild type). If alveolar macrophages are purified from animals treated for 7 days with bleomycin, the synthesis of PGE2 is further blunted in both GM-CSF-deficient (−/−) and wild-type (WT) animals (**, p < 0.05).

FIGURE 6. Reduced PGE2 levels in the lung homogenates from GM-CSF−/− mice treated with saline or bleomycin. Levels of PGE2 were measured in lung homogenates from wild-type or GM-CSF−/− animals analyzed at day 7 or 21 post saline or bleomycin injection. The animals were euthanized, and the lungs were harvested and processed for EIA as described in Materials and Methods (*, p = 0.002, **, p = 0.001, n = 8, day 7; sp = 0.009, **, p = 0.03, n = 4, day 21).

FIGURE 8. Exogenous GM-CSF augments PGE2 synthesis in alveolar macrophages from GM-CSF−/− mice. Alveolar macrophages were isolated from wild-type or GM-CSF−/− animals as described in Materials and Methods, adhered at 0.5 × 10⁶/ml in DMEM, and incubated overnight in DMEM containing 10% FCS with or without exogenous recombinant murine GM-CSF (17 ng/ml). Maximal PGE2 release was determined following stimulation with A23187 (1 μM) for 30 min at 37°C. Medium was analyzed for PGE2 by EIA and expressed as picograms per milliliter, n = 6, for all groups. As shown previously, alveolar macrophages from GM-CSF−/− mice produce less PGE2 than alveolar macrophages from wild-type mice (*, p = 0.001). Exogenous GM-CSF restores PGE2 synthesis in GM-CSF−/− alveolar macrophages to wild-type levels (#, p = 0.03 compared with GM-CSF−/− alone). Exogenous GM-CSF augments PGE2 production from wild-type alveolar macrophages (**, p = 0.02).
phase. Furthermore, the injury associated with an adenoviral vector alone complicated interpretation. Finally, the murine GM-CSF gene was inserted in rat lungs. Thus, GM-CSF likely plays a complex role in the processes of fibrosis and repair following injury. Whether the process results in scarring or healing is likely determined by the location, timing, and amount of GM-CSF produced.

The profibrotic phenotype observed in GM-CSF−/− mice can be explained by the absence of an anti-inflammatory/antifibrotic agent and/or the presence of a proinflammatory/profibrotic agent. We have excluded the possibility that GM-CSF−/− mice produce increased levels of the proinflammatory/profibrotic eicosanoid, LTC4. However, we cannot exclude the possibility that alternative proinflammatory or profibrotic mediators might be overexpressed in the GM-CSF−/− animals. This will be of interest for future studies. One might have predicted, given the decreased leukotriene levels in the GM-CSF−/− mice, that arachidonic acid metabolism might be shunted to favor COX products. Instead, we found that GM-CSF−/− animals also have impaired synthesis of PGE2 in their lungs compared with wild-type animals at both early and late time points in the course of this disease model. Earlier work from our laboratories has demonstrated that GM-CSF augments the release of arachidonic acid in alveolar macrophages via increased phospholipase A2 (PLA2) activity (30). Therefore, it seems likely that diminished availability of arachidonic acid may be a plausible mechanism to explain the in vivo diminution of both LTC4 and PGE2 in GM-CSF−/− mice.

Although PGE2 is produced by immune and inflammatory cells, fibroblasts, epithelial cells, and endothelial cells, we have directed our studies toward monocytes/macrophages because of their prominence in the pulmonary response to bleomycin and because GM-CSF is known to have profound effects on mononuclear cell function. Alveolar macrophages purified from GM-CSF−/− animals exhibit an impaired ability to synthesize the antifibrogenic lipid, PGE2. The defect was correctable with exogenous (recombinant) GM-CSF, demonstrating that GM-CSF deficiency was indeed responsible for the PGE2 synthesis defect. Furthermore, we show that a fibrosing insult itself (bleomycin) blunts the ability of alveolar macrophages purified from either GM-CSF−/− or control mice to synthesize PGE2. These data suggest that diminished alveolar macrophage PGE2 production may be a generalized hallmark of fibrotic disease, and that this defect is exaggerated in GM-CSF−/− animals. Patients with the progressive fibrotic disorder idiopathic pulmonary fibrosis (IPF) have sustained reduction of PGE2 levels (10, 31). Whether the PGE2 synthesis defect identified in macrophages from GM-CSF−/− mice extends to other lung cells, including epithelial cells and fibroblasts themselves, is the subject of future studies.

The defect in PGE2 production contributes to the exuberant proliferation of fibroblasts associated with fibrotic lung disease. PGE2 is a known inhibitor of both fibroblast proliferation (11, 12) and fibroblast collagen synthesis (13, 14). In addition, PGE2 has been documented to promote the degradation of collagen, the major extracellular matrix component of fibrionic tissue (32). Therefore, decreased production of PGE2 could lead to unchecked fibroblast proliferation and deposition of extracellular matrix. Interestingly, fibroblasts purified from the lungs of patients with IPF have decreased PGE2 production and decreased expression of COX-2, the inducible isoform of COX, as compared with fibroblasts isolated from normal human lung (10). Taken together, these data from GM-CSF−/− mice and humans with IPF raise the possibility that diminished PGE2 production is a central mechanism of pulmonary fibrotic diseases in general. Our indomethacin experiments demonstrate that pharmacologic blockade of PGs can increase bleomycin-induced pulmonary fibrosis. Further support for the notion that PGE2 exerts important down-regulatory actions on the evolution of pulmonary fibrosis comes from recent preliminary work showing that COX-2−/− mice have an enhanced fibrotic response to bleomycin (33).

GM-CSF has profound effects on the proliferation, differentiation, and survival of hemopoietic cells in the granulocyte-macrophage lineage, especially macrophages, neutrophils, and eosinophils (15). Eosinophils are common participants in pulmonary fibrosis and portend a worse outcome in human IPF (34). They have been strongly implicated in the pathogenesis of fibrosis in the bleomycin model (25, 26). However, eosinophils are totally absent in the inflammatory response to bleomycin in GM-CSF−/− mice at day 7. Thus, our data demonstrate that eosinophils are not required for this fibrotic host response to lung injury; in the absence of GM-CSF (and the absence of eosinophils) the fibrosis is worse than in wild-type mice.

The role of alveolar macrophages in the pathogenesis of pulmonary fibrosis is quite complex. In some settings, these inflammatory cells may contribute to the initial lung injury that eventually leads to fibrosis. Alternatively, by the removal of provisional matrix from the alveolar space, or by secreting factors that promote repair (such as hepatocyte growth factor) or limit collagen production (such as PGE2), alveolar macrophages may promote normal repair rather than fibrosis. Interestingly, despite the absence of a factor that is mitogenic (15) and chemotactic (35) for alveolar macrophages, GM-CSF−/− mice have more cells in their lungs at baseline than wild-type animals. Furthermore, baseline levels of neutrophils, B, NK, and T cells are slightly elevated in GM-CSF−/− mice. One possibility to explain this phenomenon is that
the GM-CSF−/− mice may have cells of a more immature phenotype as compared with wild-type mice, and that this results in the loss of a feedback inhibition loop that would limit cellular recruitment to the lung. Despite this, wild-type and GM-CSF−/− mice recruit similar numbers of cells into the lung during the first week following bleomycin injury. As a result, GM-CSF−/− mice have 23% more cells in their lungs at day 7 following bleomycin than wild-type mice. We have demonstrated that alveolar macrophages from GM-CSF−/− mice produce significantly less PGE2 than macrophages from wild-type mice. Whether other abnormalities in either proinflammatory or antifibrotic activities of the pulmonary macrophages from GM-CSF−/− mice contribute to the severity of pulmonary fibrosis in these mice awaits further study.

Bleomycin-induced fibrosis is significantly worse in GM-CSF−/− mice than in their wild-type counterparts. These studies indicate that GM-CSF is an important regulator of the number and phenotypic state of local mononuclear cells. Alveolar macrophage synthesis of the antifibrotic prostanooid PGE2 is crucially dependent on the presence of GM-CSF. This loss-of-function experiment documents an important role for GM-CSF in processes that minimize the development of fibrosis or alternatively preserve normal alveolar architecture following lung injury.

References