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### Research Article

Mutation of the granulocyte-macrophage colony-stimulating factor (GM-CSF) gene by homologous recombination caused alveolar proteinosis in mice. To further discern the role of GM-CSF in surfactant homeostasis, the synthesis of GM-CSF was directed to the respiratory epithelium of GM-CSF-hull mutant mice (GM-/-) with a chimeric gene expressing GM-CSF under the control of the promoter from the human surfactant protein-C (SP-C) gene. Transgenic mice bearing the SP-C-GM-CSF construct (SP-C-GM+) were bred to GM-/- mice resulting in complete correction of alveolar proteinosis in bitransgenic GM-/-, SP-C-GM+ mice. No effects of the transgene were found outside the lung. GM-CSF was increased in bronchoalveolar lavage fluid of the bitransgenic mice. Surfactant proteins-A and -B and phospholipid in bronchoalveolar lavage fluid were normalized in the GM-/-, SP-C-GM+ mice. SP-A, -B, and -C mRNAs were unaltered in lungs from GM-CSF-deficient and -replete mice. Expression of GM-CSF in respiratory epithelial cells of transgenic mice restores surfactant homeostasis in GM-/- mice. From these findings, we conclude that GM-CSF regulates the clearance or catabolism rather than synthesis of surfactant proteins and lipids.

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# Pulmonary Epithelial Cell Expression of GM-CSF Corrects the Alveolar Proteinosis in GM-CSF-deficient Mice

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## Abstract

Mutation of the granulocyte-macrophage colony-stimulating factor (GM-CSF) gene by homologous recombination caused alveolar proteinosis in mice. To further discern the role of GM-CSF in surfactant homeostasis, the synthesis of GM-CSF was directed to the respiratory epithelium of GM-CSF-null mutant mice (GM<sup>-/-</sup>) with a chimeric gene expressing GM-CSF under the control of the promoter from the human surfactant protein-C (SP-C) gene. Transgenic mice bearing the SP-C-GM-CSF construct (SP-C-GM<sup>+</sup>) were bred to GM<sup>-/-</sup> mice resulting in complete correction of alveolar proteinosis in bitransgenic GM<sup>-/-</sup>, SP-C-GM<sup>+</sup> mice. No effects of the transgene were found outside the lung. GM-CSF was increased in bronchoalveolar lavage fluid of the bitransgenic mice. Surfactant proteins-A and -B and phospholipid in bronchoalveolar lavage fluid were normalized in the GM<sup>-/-</sup>, SP-C-GM<sup>+</sup> mice. SP-A, -B, and -C mRNAs were unaltered in lungs from GM-CSF-deficient and -replete mice. Expression of GM-CSF in respiratory epithelial cells of transgenic mice restores surfactant homeostasis in GM<sup>-/-</sup> mice. From these findings, we conclude that GM-CSF regulates the clearance or catabolism rather than synthesis of surfactant proteins and lipids. (*J. Clin. Invest.* 1996. 97:649-655.) Key words: alveolar proteinosis • transgenic mice • granulocyte-macrophage colony-stimulating factor (GM-CSF) • surfactant homeostasis • gene correction

## Introduction

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a 23-kD glycoprotein (1) encoded by a single gene containing four exons spanning 2.4 kb of the mouse genome (2). While GM-CSF was initially isolated from conditioned media from pulmonary cells, GM-CSF is expressed in a variety of hematopoietic cell types and nonhematopoietic cells and cell lines (3-8). GM-CSF has potent hematopoietic regulatory properties and was named for its ability to stimulate prolifera-

tion and differentiation of granulocyte and macrophage precursor cells in soft agar culture (4). GM-CSF stimulates proliferation and activation of mature granulocytes and macrophages and induces migration and proliferation of endothelial cells (3, 4, 9-11). Development of GM-CSF-dependent hematopoietic precursor cell lines provided further evidence that established GM-CSF as a regulator of hematopoietic cell proliferation and differentiation (12, 13).

Extensive animal and human studies demonstrated that GM-CSF is a potent hematopoietic growth factor (8). Recombinant murine GM-CSF administered intravenously increased peripheral blood cell populations. Activated peritoneal macrophages, neutrophils, and eosinophils increased after peritoneal injection of GM-CSF without altering blood or bone marrow populations of these cells. Transgenic mice expressing GM-CSF under control of the Moloney murine leukemia virus promoter displayed a phenotype consistent with that of wild-type mice injected intraperitoneally with recombinant GM-CSF. Infusion of recombinant human GM-CSF stimulated hematopoiesis in primates (14). Recombinant human GM-CSF has been used after chemotherapy treatment to counteract bone marrow depression (15).

GM-CSF-null mutant mice (GM<sup>-/-</sup>) were generated by gene targeting (16-18). However, hematopoietic development and function were not disturbed in the GM<sup>-/-</sup> mice. Surprisingly, GM<sup>-/-</sup> animals developed pulmonary disease characteristic of pulmonary alveolar proteinosis (PAP)<sup>1</sup> associated with markedly increased concentrations of surfactant proteins and phospholipids in the alveolar spaces. Pulmonary surfactant is a heterogeneous mixture of phospholipids and associated surfactant proteins-A, -B, -C, and -D (SP-A, SP-B, SP-C, and SP-D) which are synthesized and secreted by pulmonary type II epithelial cells (for review see reference 19). Clearance of surfactant is mediated primarily by type II cells, which take up ~ 80% of surfactant phospholipids and proteins for recycling or degradation; the remainder is cleared by alveolar macrophages (20).

The accumulation of surfactant in the lungs of GM<sup>-/-</sup> mice supports the hypothesis that GM-CSF is required for regulation of pulmonary surfactant homeostasis, perhaps mediating surfactant clearance by alveolar macrophages and alveolar type II epithelial cells. The recent finding that respiratory epithelial cells synthesize GM-CSF is consistent with the hypothesis that GM-CSF plays an important role in surfactant metabolism. In the present work, GM-CSF was selectively expressed in type II epithelial cells of the GM<sup>-/-</sup> mouse, correcting the alveolar proteinosis associated with this animal model.

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1. Abbreviations used in this paper: 3.7SP-C, 3.7-kb SP-C promoter; BAL, bronchoalveolar lavage; PAP, pulmonary alveolar proteinosis; Sat PC, saturated phosphatidylcholine; SP, surfactant protein.

## Methods

**Construction of the SP-C-GM-CSF chimeric gene.** Cloning vector p3.7-tpA, comprised of the human SP-C promoter region, nucleotides -3683 to +18 (3.7SP-C) (21, 22), followed by a multiple cloning site, SV40 small t-intron, and polyadenylation signal, was used to construct the chimeric gene in a pUC18 plasmid, as described previously (23). The coding region of mouse GM-CSF cDNA, bp 174-620, was isolated from pMFGmuGM-CSF (10). Ends were modified with addition of EcoRI linkers and inserted into the EcoRI site of p3.7-tpA to generate p3.7GM-tpA.

**Production of transgenic mice.** The SP-C-GM-CSF chimeric gene was excised from the parental plasmid p3.7GM-tpA by NdeI/NcoI digestion and purified by agarose gel electrophoresis in the absence of ethidium bromide. The DNA was recovered using Qiaex Gel extraction kit (QIAGEN Inc., Chatsworth, CA) and dialyzed in 5 mM Tris-HCl, pH 7.5, and 0.1 mM EDTA for 36 h. Donor eggs from FVB/N mice (GM+/+) were prepared for pronuclear injection. Sperm was obtained from GM-CSF-null mutant mice (GM-/-), described previously (16). Tail DNA was digested with BamHI and Southern blotted, using the EcoRI fragment of GM-CSF cDNA from p3.7GM-tpA to probe for wild-type (9.2 kb), null mutant (4.1 kb), and SP-C-GM-CSF-transgenic (0.5 kb) alleles. SP-C-GM-CSF transgenic founders were back-crossed with GM-/- mice to generate pups that were homozygous GM-/- and heterozygous for the SP-C-GM-CSF transgene (GM-/-, SP-C-GM+). GM-/-, GM+/+ mice, and GM+/- littermates were used as age-matched controls as indicated.

**Processing and staining of tissues for histopathology.** Lungs were inflation fixed with 4% paraformaldehyde/phosphate-buffered saline (PBS), pH 7.4, for 24 h, as described previously (24). Tissue was then washed in PBS, dehydrated in a series of alcohols, and embedded in paraffin. Hematoxylin and eosin staining was used for histological analysis of paraffin sections.

**Immunohistochemistry.** Paraffin sections of 5  $\mu$ m were used to stain for surfactant protein. Anti-SP-B (28031) is a rabbit anti-bovine polyclonal antibody that recognizes mature SP-B (25). Anti-SP-B was detected using a secondary antibody and detection system (Vectastain ABC anti-rabbit immunohistochemical horseradish peroxidase kit; Vector Labs, Inc., Burlingame, CA). Tissues were counterstained with hematoxylin and eosin.

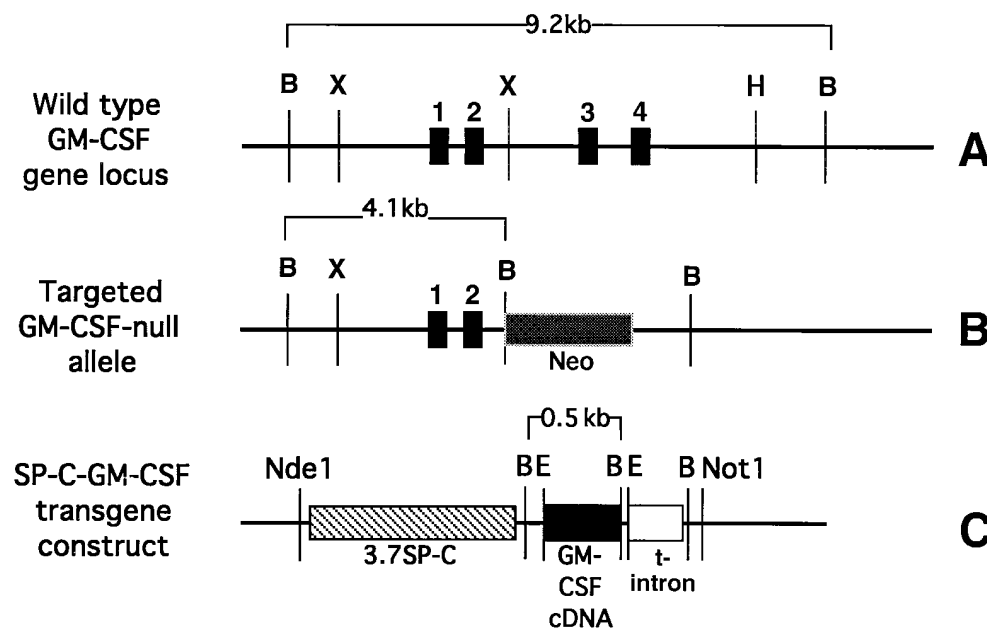
**RNA isolation.** RNA was isolated by a modification of the guanidinium-thiocyanate method described by Chomczynski and Sacchi (26). Briefly, tissues were homogenized in guanidinium-thiocyanate.

A series of phenol-chloroform extractions was followed by precipitation in isopropanol as described in RNA isolation protocol package insert for Phase-Lock Gel IIA Heavy Tubes (5 Prime-3 Prime, Inc., Boulder, CO).

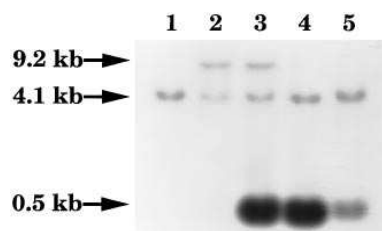
**S1 nuclease protection analysis.** Total lung RNA (2  $\mu$ g) was hybridized in solution at 55°C for 18 h with an excess of  $^{32}$ P-end-labeled linearized probes in 400 mM NaCl, 2 mM EDTA, 40 mM Pipes, pH 6.6, and 80% formamide. Each sample was treated with S1 nuclease at 37°C for 1 h in 100 mM NaCl, 15 mM NaOAc, pH 4.5, 2.5 mM ZnSO<sub>4</sub>, 25 ng denatured salmon sperm DNA, with 110 U S1 nuclease (GIBCO-BRL, Gaithersburg, MD). Protected fragments were resolved on a 6% polyacrylamide/8 M urea gel and visualized by autoradiography. Plasmids containing probe sequences for SP-A, SP-B, SP-C, and L32 were kindly provided by Dr. Cindy Bachurski (Children's Hospital Medical Center, Cincinnati, OH) (16).

**Peritoneal wash, blood collection, and cell counts.** Mice were anesthetized by inhalation of methoxyflurane. 2 ml of PBS (pH 7.4) was injected into the peritoneal cavity using a 25-gauge needle. The abdomen was massaged gently and carefully opened to recover the fluid. Volume, color, and turbidity of each sample was noted to assure that ascites were not present in animals tested. Each sample was stored on ice until manual cell counts were performed. Mice were killed by exsanguination from the posterior vena cava. Blood was placed in microtubes containing EDTA for cell counts or allowed to clot for serum collection. Manual white blood cell and differential cell counts were performed. Cell counts were performed on bronchoalveolar lavage (BAL) fluid by flow cytometry using MAC-1 and Thy-1 antibodies to identify macrophages and T cells, respectively (Pharmingen, San Diego, CA).

**BAL studies.** Mice were anesthetized with pentobarbital injected intraperitoneally, and BAL was performed three times with 1-ml aliquots of PBS which were pooled and the volume measured. Cells were removed by centrifugation at 6,300 g for 10 min, and aliquots were stored at -20°C until analysis. SP-B was measured by ELISA as previously described (27). SP-A was measured using ELISA rabbit anti-mouse SP-A antiserum. GM-CSF was measured using GM-CSF Minikit ELISA (Endogen, Inc., Boston, MA). Horseradish peroxidase-Streptavidin (Zymed Laboratories, Inc., South San Francisco, CA) was diluted to 1:8,000. Substrate was DAKO TMB one-step Substrate System (Dako Corp., Carpinteria, CA). The lower limits of detection with this system are < 5-35 pg/ml, thus, any samples falling within this range or below were reported as not detectable. No samples reported as not detectable were > 12 pg/ml. All samples > 200



**Figure 1.** Gene constructs. (A) Wild-type mouse GM-CSF gene locus. (B) GM-CSF-null allele produced by gene targeting with the neomycin gene replacing exons 3 and 4, intronic sequences, and 3' UTR of the mouse GM-CSF gene. (C) SP-C-GM-CSF chimeric gene containing the human SP-C gene promoter (3.7SP-C), coding sequence of mouse GM-CSF cDNA, SV40 small t-intron, and polyadenylation signal. BamHI digestion of genomic DNA yields diagnostic fragments for wild-type (9.2 kb), GM-/- (4.1 kb), or SP-C-GM-CSF transgene (0.5 kb) as indicated. B, BamHI; X, XbaI; H, HindIII; E, EcoRI; Neo, neomycin.



**Figure 2.** Southern blot analysis of GM<sup>-/-</sup>, SP-C-GM<sup>+</sup> mice. Genotype was determined by Southern blotting of BamHI-digested tail DNA, using the mouse GM-CSF cDNA probe. Fragment sizes are indicated on the left in kilobases. Lanes 1 and 2 contain DNA from line 48, GM<sup>-/-</sup>, and GM<sup>+/-</sup>, respectively. Lanes 3 and 4 represent DNA from line 48, GM<sup>+/-</sup>, SP-C-GM<sup>+</sup>; and GM<sup>-/-</sup>, SP-C-GM<sup>+</sup>, respectively. Lane 5 is representative of line 59 GM<sup>-/-</sup>, SP-C-GM<sup>+</sup> animals.

pg/ml were diluted and repeated. To assay saturated phosphatidylcholine (Sat PC) in BAL fluid, mouse lungs were lavaged and Sat PC was determined as described previously (27a).

## Results

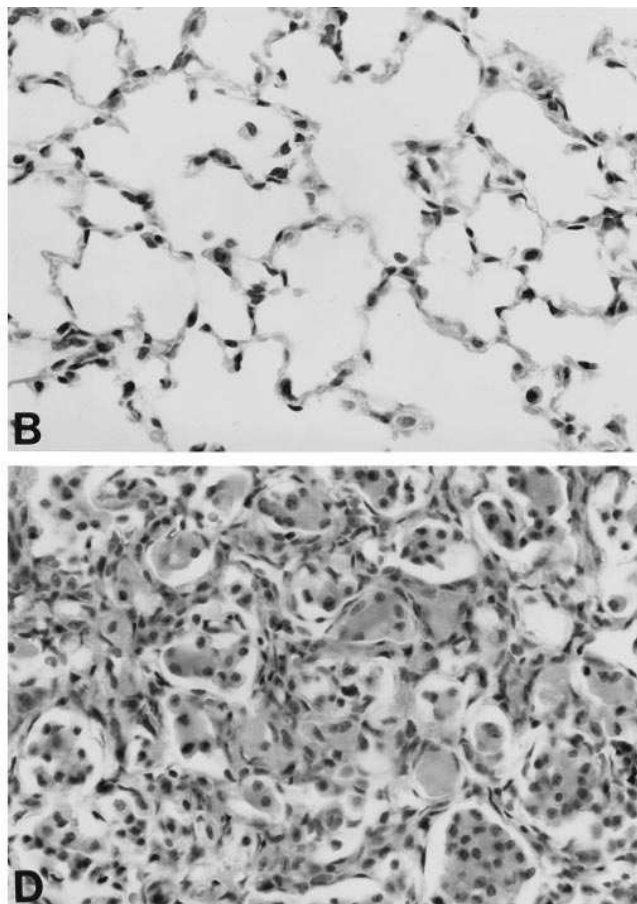
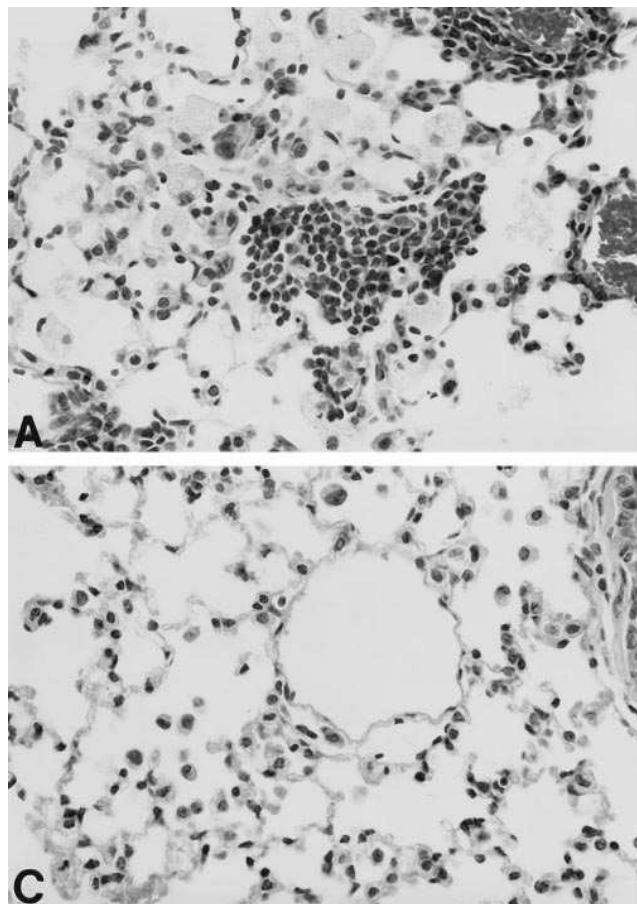
Promoter sequences (3.7SP-C) from the human SP-C gene, nucleotides -3683 to +18, were used to construct a chimeric gene directing expression of mouse GM-CSF cDNA in the re-

**Table I. Differential White Blood Cell Counts**

Genotype	Monocytes	Lymphocytes	Granulocytes
GM <sup>-/-</sup> , SP-C-GM <sup>+</sup>	2.6% ± 1.7	73% ± 3.1	24% ± 3.3
GM <sup>-/-</sup> , SP-C-GM <sup>-</sup>	3.6% ± 1.8	63% ± 10	33% ± 10
GM <sup>+/-</sup> , SP-C-GM <sup>-</sup>	2.4% ± 2.2	76% ± 6.9	22% ± 5.4

White blood cell differentials were performed on peripheral blood smears from GM<sup>-/-</sup>, SP-C-GM<sup>+</sup>; GM<sup>-/-</sup>, SP-C-GM<sup>-</sup>; and GM<sup>+/-</sup>, SP-C-GM<sup>-</sup> animals. Results show percentage of monocytes, lymphocytes, and granulocytes counted; mean ± SD; *n* = 5 individuals of each genotype.

spiratory lung epithelium of wild-type and GM<sup>-/-</sup> mice (Fig. 1, A–C). Ova from GM<sup>+/+</sup> females were fertilized with sperm from GM<sup>-/-</sup> males. At the pronuclear stage of development, the SP-C-GM-CSF chimeric gene was injected into the ova, which were transferred to pseudopregnant foster mothers. Founders were screened by Southern blotting of tail DNA digested with BamHI, using a mouse cDNA probe (Fig. 2). Genotypes GM<sup>+/-</sup>, SP-C-GM<sup>-</sup> or GM<sup>+/-</sup>, SP-C-GM<sup>+</sup> were determined by the presence of 9.2-, 4.1-, or 0.5-kb bands for



**Figure 3.** Lung histology in GM-CSF-deficient and -replete mice. Lungs from adult mice were inflation fixed and stained with hematoxylin and eosin. Alveolar proteinosis, enlarged foamy macrophages, and lymphocytic infiltration were consistent findings in GM<sup>-/-</sup> mice (A). Alveolar proteinosis was corrected in GM<sup>-/-</sup>, SP-C-GM<sup>+</sup> mice from line 48 (B) and line 59 (C) in association with increased numbers of lung macrophages. Marked alveolar infiltration with macrophages was noted in the F16, GM<sup>+/-</sup>, SP-C-GM<sup>+</sup> mouse (D). At least four individual mice were assessed from lines 48 and 59. ×340.

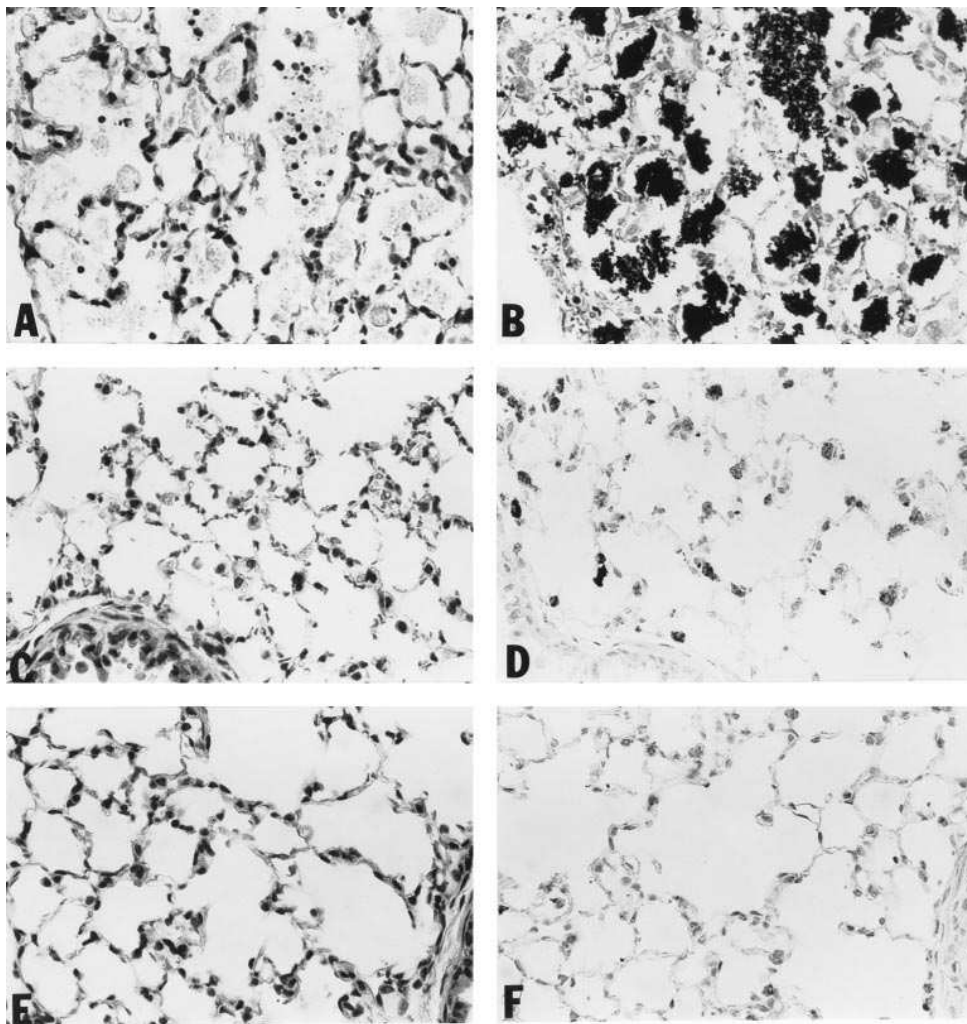
wild-type GM-CSF allele, GM-CSF-null allele, and SP-C-GM-CSF transgene, respectively.

Three distinct GM<sup>+/−</sup>, SP-C-GM<sup>+</sup> transgenic mice founders were backcrossed to GM<sup>−/−</sup> mice to obtain GM<sup>−/−</sup>, SP-C-GM<sup>+</sup> pups. One founder mouse (F16) failed to breed and was moribund at 4 mo; histology of lungs from this animal demonstrated marked pulmonary infiltration with alveolar macrophages and no alveolar proteinosis. Two other GM<sup>+/−</sup>, SP-C-GM<sup>+</sup> mice, designated F48 and F59, were used to establish separate lines. Genotypes of offspring were as expected from Mendelian inheritance, indicating that the transgene had inserted into an autosomal locus in each line. Litters were of expected sizes, and pups were healthy with normal growth and development. Mice from each line have been bred to the F4 generation and are > 9 mo old at present. Founder mice are now > 11 mo old and are apparently healthy.

There were no apparent systemic effects of GM-CSF, and effects of the transgene were restricted to the lung. Peripheral blood counts were similar in GM<sup>−/−</sup>, SP-C-GM<sup>+</sup>; GM<sup>−/−</sup>, SP-C-GM<sup>−</sup>; and GM<sup>+/−</sup>, SP-C-GM<sup>−</sup> mice (Table I). Likewise, cell counts of peritoneal washes from GM<sup>−/−</sup>, SP-C-GM<sup>+</sup>; GM<sup>−/−</sup>, SP-C-GM<sup>−</sup>; and GM<sup>+/−</sup>, SP-C-GM<sup>−</sup> mice were indistinguishable,  $5.0 \pm 1.8$  ( $n = 3$ ),  $4.4 \pm 2.4$  ( $n = 5$ ), and  $3.3 \pm 0.3$  ( $n = 4$ )  $\times 10^6$  cells recovered mean  $\pm$  SD, respectively. Values for peripheral blood and peritoneal wash cell counts

were within ranges established in the literature (28, 29). Histopathologies of spleens from wild-type, GM<sup>−/−</sup>, and bitransgenic mice were identical. The numbers of MAC-1-reactive cells were increased in BAL fluid from the GM<sup>−/−</sup>, SP-C-GM<sup>+</sup> mice compared with wild-type and GM<sup>−/−</sup> mice (lacking the SP-C-GM transgene) as assessed by flow cytometry, a finding consistent with the increased numbers of alveolar macrophages observed histologically (Fig. 3, A–D). Numbers of cells collected by BAL were increased similarly in GM<sup>+/−</sup>, SP-C-GM<sup>+</sup> and GM<sup>−/−</sup>, SP-C-GM<sup>+</sup> lines and were not different in mice from lines 48 or 59. BAL cell counts were  $0.074 \pm 0.02 \times 10^6$  mean  $\pm$  SD ( $n = 4$ ) in GM<sup>+/−</sup> mice compared with  $0.72 \pm 0.3 \times 10^6$  ( $n = 4$ ) in line 48 and  $0.79 \pm 0.32 \times 10^6$  ( $n = 4$ ) in line 59 of the GM<sup>+/−</sup>, SP-C-GM<sup>+</sup> mice. Similarly, cell numbers were  $0.47 \times 10^6$  (line 48) and  $1.1 \times 10^6$  (line 59)  $n = 2$  each in the GM<sup>−/−</sup>, SP-C-GM<sup>+</sup> mice.

Proteinaceous accumulations in the alveolar spaces were not detected in lungs from GM<sup>−/−</sup>, SP-C-GM<sup>+</sup> and GM<sup>+/−</sup>, SP-C-GM<sup>−</sup> (Fig. 4, C and E), or GM<sup>+/+</sup> mice (16), in sharp contrast to lungs from GM<sup>−/−</sup>, SP-C-GM<sup>−</sup> littermates, wherein eosinophilic material filled the alveolar spaces in all lobes of the lungs, that was intensely stained by SP-B antibody (Fig. 4, A and B). The intensity of anti-SP-B staining of type II cells in GM<sup>−/−</sup>, SP-C-GM<sup>+</sup> and GM<sup>+/−</sup>, SP-C-GM<sup>−</sup> mice (Fig. 4, D and F) was similar to that of GM<sup>+/+</sup> wild-type mice



**Figure 4.** SP-B in lungs of GM<sup>−/−</sup> and GM<sup>+/−</sup>, SP-C-GM<sup>+</sup> mice. Lungs were inflation fixed and stained by hematoxylin and eosin (A, C, and E) or stained for SP-B (B, D, and F). Immunohistochemical staining with anti-SP-B antibody selectively stained type II cells in lung sections from GM<sup>−/−</sup>, SP-C-GM<sup>+</sup> mice, but did not stain alveolar material (D and F). Intense staining of alveolar material was noted in the GM<sup>−/−</sup>, SP-C-GM<sup>−</sup> lung (B), corresponding to the proteinaceous infiltrates noted by hematoxylin and eosin staining (A). Lungs from GM<sup>+/−</sup>, SP-C-GM<sup>−</sup> mice stained with hematoxylin and eosin (E) and anti-SP-B (F) were indistinguishable from those of wild-type GM<sup>+/+</sup> animals (not shown). Sections are representative of offspring from two separate founder SP-C-GM-CSF lines, assessing  $n = 3$  mice per line.

(16). Lymphocytic infiltrates characteristic of the GM-/- mice were not observed in the "corrected" bitransgenic mice (Fig. 4, C and D).

SP-A, SP-B, and GM-CSF were measured in BAL fluid from GM-/-, SP-C-GM-; GM-/-, SP-C-GM+; and GM+/-, SP-C-GM- mice (Fig. 5).

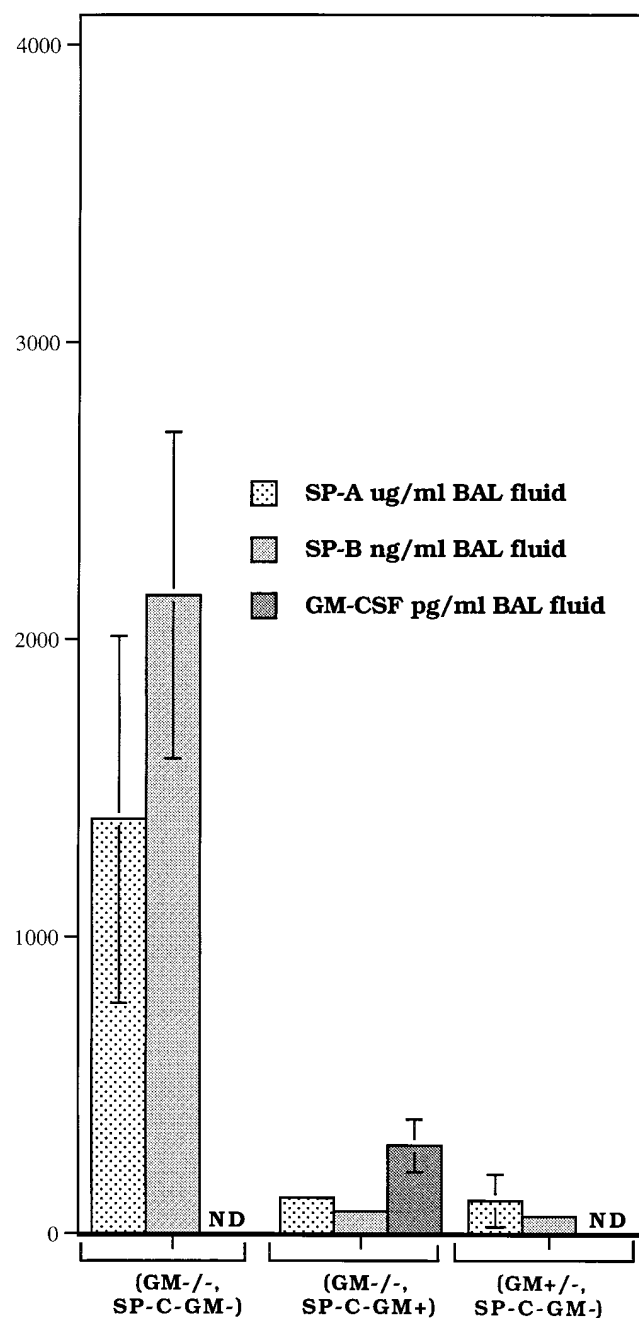


Figure 5. GM-CSF and surfactant proteins in GM-/- and GM-/-, SP-C-GM+ mice. SP-A, SP-B, and GM-CSF were measured in BAL fluid from GM-/-, SP-C-GM-; GM-/-, SP-C-GM+; and GM+/-, SP-C-GM- mice ( $n \geq 4$ ). SP-A and SP-B levels in GM-/-, SP-C-GM- mice were  $\geq 10$ -fold higher than in GM+/-, SP-C-GM- mice; the latter were also within the range of healthy GM+/+ mice (data not shown). GM-CSF was not detectable in BAL from either GM-/-, SP-C-GM- or GM+/-, SP-C-GM- mice. SP-A and SP-B in BAL collected from GM-/-, SP-C-GM+ mice were within the ranges found in GM+/-, SP-C-GM- and GM+/+ mice. Values are mean  $\pm$  SD,  $n \geq 4$  per group. ND, not detectable.

SP-A and SP-B levels in GM-/-, SP-C-GM- mice were  $\geq 10$ -fold higher than in GM+/-, SP-C-GM- mice. The levels of SP-A and SP-B in GM+/-, SP-C-GM- mice did not differ significantly from GM+/+ mice (data not shown). SP-A and SP-B in BAL collected from GM-/-, SP-C-GM+ mice were not different from those in GM+/-, SP-C-GM- and GM+/+ mice. GM-CSF concentrations were below the level of detection in BAL from either GM-/-, SP-C-GM- or GM+/-, SP-C-GM- mice. GM-CSF in BAL fluid from GM-/-, SP-C-GM+ mice ranged from 151 to 438 pg/ml in both mouse lines. GM-CSF was below the level of detection in the serum from all mice, including the SP-C-GM+ transgenic mice (data not shown).

SP-A, SP-B, and SP-C mRNAs in GM-/-, SP-C-GM-; GM+/-, SP-C-GM-; and GM-/-, SP-C-GM+ were not different, suggesting that the correction of alveolar proteinosis in GM-/-, SP-C-GM+ mice was not directly related to changes in expression of surfactant protein mRNAs (Fig. 6).

Sat PC content in BAL from GM-/-, SP-C-GM- mice was  $\geq 10$ -fold higher than that in BAL from GM+/-, SP-C-GM- mice (Fig. 7). Sat PC content in GM-/-, SP-C-GM+ mice did not differ significantly from GM+/-, SP-C-GM- or GM+/+ mice, demonstrating that expression of GM-CSF in the lungs of GM-/- mice normalized alveolar surfactant phospholipids and proteins.

## Discussion

The local expression of murine GM-CSF in respiratory epithelial cells of lungs of transgenic mice corrected the alveolar proteinosis and lymphocytic infiltrates characteristic of the lungs of GM-/- mutant mice. The accumulation of surfactant proteins and phospholipids was resolved in the absence of detectable systemic effects of the transgene, supporting the hypothesis that the local production of GM-CSF by lung cells influenced surfactant homeostasis mediated by respiratory epithelial cells and alveolar macrophages.

The marked accumulations of surfactant in GM-/- mice and the resolution of the alveolar proteinosis in the GM-/-,

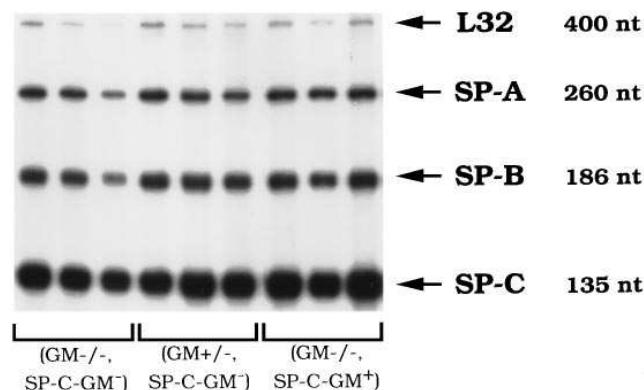


Figure 6. S1-Nuclease assay for SP-A, -B, and -C mRNA. SP-A, SP-B, and SP-C mRNAs were assessed in lungs from GM-/-, SP-C-GM-; GM+/-, SP-C-GM-; and GM-/-, SP-C-GM+ mice. Sizes of protected fragments are indicated on the right in nucleotides (nt). When normalized for loading using ribosomal L32 surfactant protein mRNA were not different in the various lines tested. There was no statistically significant difference found between the genotypic groups.  $n \geq 4$  by ANOVA.

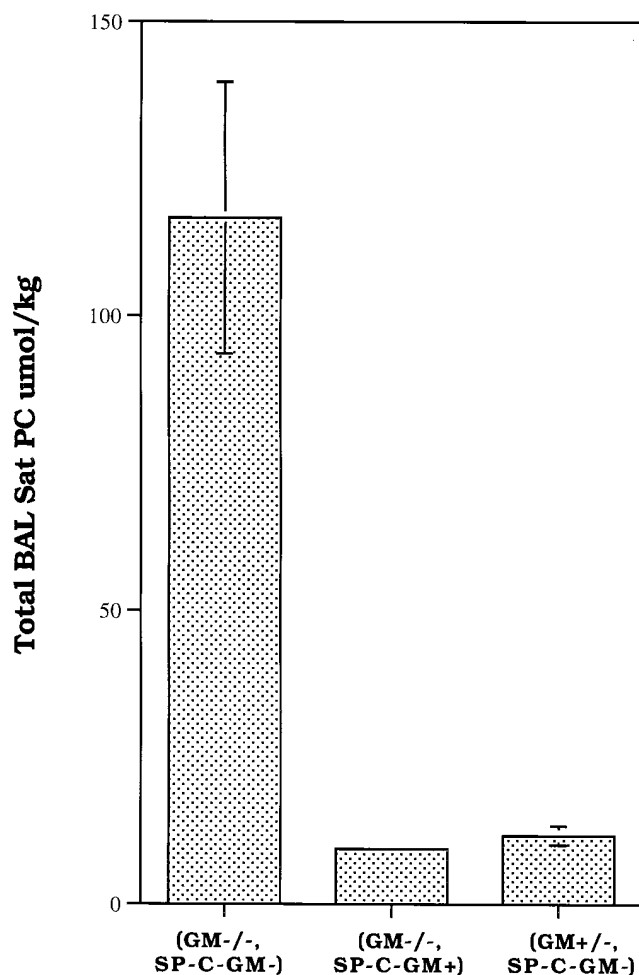


Figure 7. Phospholipid analysis of BAL from GM-deficient and -replete mice. Sat PC content in BAL from GM<sup>-/-</sup>, SP-C-GM<sup>-</sup> mice was > 10 fold higher than in GM<sup>-/-</sup>, SP-C-GM<sup>+</sup> or GM<sup>+/-</sup>, SP-C-GM<sup>-</sup> mice. Sat PC content in GM<sup>-/-</sup>, SP-C-GM<sup>+</sup> mice did not differ significantly from GM<sup>+/-</sup>, SP-C-GM<sup>-</sup> or GM<sup>+/-</sup> mice (not shown). Values are mean  $\pm$  SD,  $n \geq 4$  per group.

SP-C-GM<sup>+</sup> mice occurred independently of changes in surfactant protein mRNAs or intracellular SP-B staining. These findings suggest that altered surfactant metabolism, reuptake, or catabolism, rather than increased production of surfactant components, are involved in the PAP in GM<sup>-/-</sup> mice. The recent findings that surfactant phospholipid and <sup>125</sup>I-SP-A clearance was markedly decreased in the GM<sup>-/-</sup> mice and that rates of phospholipid synthesis and secretion were relatively unchanged provide further support for the role of GM-CSF in surfactant clearance pathways (27a). On the other hand, surfactant synthesis and surfactant protein RNA content were not altered in the GM<sup>-/-</sup> mice in spite of the marked accumulation of surfactant proteins and lipids. Thus, counterregulatory mechanisms failed to decrease steady state surfactant synthesis that might have ameliorated abnormalities of surfactant accumulation in the PAP mice. These findings may reflect disordered counterregulatory mechanisms or that steady state surfactant concentrations are maintained primarily by the regulation of recycling and catabolism rather than by pathways that modulate surfactant biosynthesis.

In the present study, the human SP-C promoter (3.7SP-C)

was used to direct synthesis of GM-CSF to alveolar type II cells and to distal bronchiolar epithelial cells in the lung. The h3.7SP-C promoter has been used extensively to express genes in a cell-specific manner in the lungs of transgenic mice. Expression of the exogenous cDNAs or minigenes is consistently restricted to type II epithelial cells and distal bronchiolar epithelial cells (for review see reference 30). We were unable to detect SP-C-GM-CSF mRNA by either RT-PCR or Northern blot analysis, even though GM-CSF protein was readily detected in the BAL fluid of the SP-C-GM<sup>+</sup> mice. The endogenous GM-CSF mRNA is frequently undetectable in biological samples, likely due to its short biological half-life (3). Instability of the GM-CSF mRNA may account for difficulty in detecting the transgenic GM-CSF mRNA. Nevertheless, restriction of pathological findings to the lung, increased concentrations of GM-CSF in the BAL fluid, and absence of extrapulmonary effects seen in the GM<sup>-/-</sup>, SP-C-GM<sup>+</sup> mice suggest that the SP-C-GM-CSF mRNA is produced in the lung and is translated into biologically active GM-CSF. The finding that GM-CSF mRNA was not readily detected in the transgenic mice might be related to loss of transgenic mice expressing high levels of GM-CSF mRNA, although we did not observe increased perinatal death of SP-C-GM founder mice.

GM-CSF was readily detected in BAL but not in peripheral blood from the bitransgenic mice, providing further support for the concept that GM-CSF is acting locally, rather than peripherally, to correct the alveolar proteinosis in the GM<sup>-/-</sup> model. Peripheral blood leukocytes, splenic histology, and the lack of changes in peritoneal macrophages support the likelihood that the effects of the SP-C-GM-CSF transgene were not mediated by extrapulmonary GM-CSF. In contrast, transgenic mice expressing GM-CSF under control of the Moloney murine leukemia virus promoter developed abnormalities of tissue macrophage cell populations that were not observed in the present studies (8). It remains possible, however, that the local production of GM-CSF in the lung influences cells of granulocytic and monocytic cell lineages at distant sites and that the production of GM-CSF in the lung has provided signals necessary for appropriate granulocytic-monocytic maturation in other organs that corrects the abnormalities of surfactant homeostasis seen in the lung.

While alveolar macrophage numbers were not substantially altered in the lungs of GM<sup>-/-</sup> mice (16), macrophages became increasingly foamy and laden with lipids and surfactant proteins. Marked perivascular and peribronchiolar lymphocytic infiltration were also noted in the lungs of GM<sup>-/-</sup> mice. Both of these histologic features of the GM<sup>-/-</sup> mice were resolved in the bitransgenic mice expressing GM-CSF in the lung. The mechanisms involved in the resolution of PAP and restoration of macrophage morphology have not been clarified in the present studies. While morphology of the macrophages appears to be normal in the bitransgenic mice, the numbers of alveolar macrophages isolated in the lungs by alveolar lavage were increased in the GM<sup>-/-</sup>, SP-C-GM<sup>+</sup> mice. These increased numbers of alveolar macrophages may contribute to more rapid clearance or catabolism in the corrected bitransgenic mice. Immunohistochemical staining for proliferating cell nuclear antigen was observed in a small percentage of macrophages in both wild-type and corrected mice, suggesting that local proliferation of macrophages may occur in the lungs of the GM<sup>-/-</sup> mice (data not shown). Since GM-CSF exerts proliferative, chemoattractant, and activating effects on alveo-



lar macrophages, the local proliferation of macrophages may not be the only mechanism resulting in the increased numbers and activity of alveolar macrophages in the corrected transgenic mice.

The present findings support a model by which local production of GM-CSF in the lung corrects PAP in the GM-/- mouse. However, the sites of expression and regulatory role of GM-CSF in normal lung physiology have not been clarified. While rat type II epithelial cells express GM-CSF in vitro (7), the normal in vivo sites and levels of GM-CSF protein or receptors in the lung remain to be elucidated. The relative instabilities of GM-CSF mRNA and protein complicate the analysis of potential signaling pathways that might be involved in regulation of surfactant homeostasis by GM-CSF and its receptors in the normal lung in vivo. It is therefore unclear whether surfactant homeostasis in the wild-type mouse and the abnormalities of surfactant homeostasis seen in GM-CSF mouse are mediated by interactions of GM-CSF with alveolar macrophages, type II epithelial cells, or both cell types.

Pulmonary alveolar proteinosis is a relatively uncommon, clinical syndrome with pathological features of surfactant lipid and protein accumulation similar to the findings of the GM-/- mouse. Pulmonary alveolar proteinosis has been associated with metabolic disease and acquired disorders of the hematopoietic systems (31). The present findings that the local production of GM-CSF corrects abnormalities of surfactant homeostasis in the bitransgenic GM-/-, SP-C-GM+ mice support the important role of GM-CSF in surfactant homeostasis and the hypothesis that abnormalities of GM-CSF or its receptors may be involved in the pathogenesis of PAP in humans.

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## References

- Burgess, A.W., J. Camakaris, and D. Metcalf. 1977. Purification and properties of colony-stimulating factor from mouse lung-conditioned medium. *J. Biol. Chem.* 252:1998-2003.
- Gough, N.M., D. Metcalf, J. Gough, D. Grail, and A.R. Dunn. 1985. Structure and expression of the mRNA for murine granulocyte-macrophage colony stimulating factor. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:645-653.
- Gasson, J.C. 1991. Molecular physiology of granulocyte-macrophage colony-stimulating factor. *Blood.* 77:1131-1145.
- Metcalf, D. 1993. Hematopoietic regulators: redundancy or subtlety? *Blood.* 82:3515-3523.
- Tazi, A., F. Bouchonnet, M. Grandsaigne, L. Boumsell, A.J. Hance, and P. Soler. 1993. Evidence that granulocyte-macrophage colony-stimulating factor regulates the distribution and differentiated state of dendritic cells/Langerhans cells in human lung and lung cancers. *J. Clin. Invest.* 91:566-576.
- Churchill, L., B. Friedman, R.P. Schleimer, and D. Proud. 1992. Production of granulocyte-macrophage colony-stimulating factor by cultured human tracheal epithelial cells. *Immunology.* 75:189-195.
- Blau, H., S. Riklis, V. Kravtsov, and M. Kalina. 1994. Secretion of cytokines by rat alveolar epithelial cells: possible regulatory role for SP-A. *Am. J. Physiol.* 266:L148-L155.
- Metcalf, D. 1991. Transgenic mice as models of hemopoiesis. *Cancer (Phila.)* 67(Suppl. 10):2695-2699.
- Mantel, C., Z. Luo, P. Hendrie, and H.E. Broxmeyer. 1993. Steel factor and granulocyte-macrophage colony stimulating factor act together to enhance choline-lipid turnover during synergistically stimulated proliferation of the human factor dependent cell line, MO7e. *Biochem. Biophys. Res. Commun.* 197:

978-984.

10. Dranoff, G., E. Jaffee, A. Lazenby, P. Golumbek, H. Levitsky, K. Brose, V. Jackson, H. Hamada, D. Pardoll, and R. Mulligan. 1993. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc. Natl. Acad. Sci. USA.* 90:3539-3543.
11. Bussolino, F., F. Colotta, E. Bocchietto, A. Guglielmetti, and A. Mantovani. 1993. Recent developments in the cell biology of granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor: activities on endothelial cells. *Int. J. Clin. & Lab. Res.* 23:8-12.
12. Dexter, T.M., J. Garland, D. Scott, E. Scolnick, and D. Metcalf. 1980. Growth of factor-dependent hemopoietic precursor cell lines. *J. Exp. Med.* 152:1036-1047.
13. Greenberger, J.S., M. Sakakeeny, R.K. Humphries, C.J. Eaves, and R. J. Eckner. 1983. Demonstration of permanent factor-dependent multipotential (erythroid/neutrophil/basophil) hematopoietic progenitor cells lines. *Proc. Natl. Acad. Sci. USA.* 80:2931-2935.
14. Donahue, R.E., E.A. Wang, D.K. Stone, R. Kamen, G.G. Wong, P.K. Sehgal, D.G. Nathan, and S.C. Clark. 1986. Stimulation of hematopoiesis in primates by continuous infusion of recombinant human GM-CSF. *Nature (Lond.)* 321:872-875.
15. Aglietta, M., W. Piacibello, P. Pasquino, F. Sanavio, A. Stacchini, C. Volta, A. Monteverde, L. Fubini, S. Morelli, and A. Severino. 1994. Rationale for the use of granulocyte-macrophage colony-stimulating factor in oncology. *Semin. Oncol.* 21(Suppl. 16):5-9.
16. Dranoff, G., A.D. Crawford, M. Sadelain, B. Ream, A. Rashid, R.T. Bronson, G.R. Dickersin, C.J. Bachurski, E.L. Mark, J.A. Whitsett, and R.C. Mulligan. 1994. Involvement of granulocyte-macrophage colony-stimulating factor in pulmonary homeostasis. *Science (Wash. DC)* 264:713-716.
17. Stanley, E., G.J. Lieschke, D. Grail, D. Metcalf, G. Hodgson, J.A.M. Gall, D.W. Maher, J. Cebon, V. Sinickas, and A.R. Dunn. 1994. Granulocyte/macrophage colony-stimulating factor-deficient mice show no major perturbation of hematopoiesis but develop a characteristic pulmonary pathology. *Proc. Natl. Acad. Sci. USA.* 91:5592-5596.
18. Lieschke, G.J., E. Stanley, D. Grail, G. Hodgson, V. Sinickas, J.A.M. Gall, R.A. Sinclair, and A. R. Dunn. 1994. Mice lacking both macrophage- and granulocyte-macrophage colony-stimulating factor have macrophages and co-existent osteopetrosis and severe lung disease. *Blood.* 84:27-35.
19. Weaver, T.E., and J.A. Whitsett. 1991. Function and regulation of expression of pulmonary surfactant-associated proteins. *Biochem. J.* 273:249-264.
20. Rider, D.D., M. Ikegami, and A.H. Jobe. 1992. Localization of alveolar surfactant clearance in rabbit lung cells. *Am. J. Physiol. (Lung Cell. Mol. Physiol.)* 8:L201-L209.
21. Glasser, S.W., T.R. Korfhagen, S.E. Wert, M.D. Bruno, K.M. McWilliams, D.K. Vorbroker, and J.A. Whitsett. 1991. Genetic element from human surfactant protein SP-C gene confers bronchiolar-alveolar cell specificity in transgenic mice. *Am. J. Physiol. (Lung Cell. Mol. Physiol.)* 261:L349-L356.
22. Korfhagen, T.R., S.W. Glasser, S.E. Wert, M.D. Bruno, C.C. Daugherty, J.D. McNeish, J.L. Stock, S.S. Potter, and J.A. Whitsett. 1990. Cis-acting sequences from a human surfactant protein gene confer pulmonary-specific gene expression in transgenic mice. *Proc. Natl. Acad. Sci. USA.* 87:6122-6126.
23. Wikenheiser, K.A., J.C. Clark, R.I. Linnola, M.T. Stahlman, and J.A. Whitsett. 1992. Simian virus 40 large T antigen directed by transcriptional elements of the human surfactant protein C gene produces pulmonary adenocarcinomas in transgenic mice. *Cancer Res.* 52:5342-5352.
24. Buckingham, K.W., and W.E. Wyder. 1981. Rapid tracheal infusion method for routine lung fixation using rat and guinea pig. *Toxicol. Pathol.* 9:17-20.
25. Clark, J.C., S.E. Wert, C.J. Bachurski, M.T. Stahlman, B.R. Stripp, T.E. Weaver, and J.A. Whitsett. 1995. Targeted disruption of the surfactant protein B gene disrupts surfactant homeostasis, causing respiratory failure in newborn mice. *Proc. Natl. Acad. Sci. USA.* 92:7794-7798.
26. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.
27. Lesur, O., R.A.W. Veldhuizen, J.A. Whitsett, W.M. Hull, F. Possmayer, A. Cantin, and R. Begin. 1993. Surfactant-associated proteins (SP-A, SP-B) are increased proportionally to alveolar phospholipids in sheep silicosis. *Lung.* 171:63-74.
- 27a. Ikegami, M., T. Ueda, W. Hull, J.A. Whitsett, R.C. Mulligan, G. Dranoff, and A.H. Jobe. 1996. Surfactant metabolism in transgenic mice after granulocyte macrophage-colony stimulating factor ablation. *Am. J. Physiol. (Lung Cell Mol. Biol.)*. In press.
28. Mitraka, B.M., and H.M. Rawnsley. 1981. Clinical biochemical and hematological reference values in normal experimental animals and normal humans. Masson Publishing USA, Inc., New York. 60-61.
29. Gearing, A. J., D. Metcalf, J.G. Moore, and N.A. Nicola. 1989. Elevated levels of GM-CSF and IL-1 in the serum, peritoneal and pleural cavities of GM-CSF transgenic mice. *Immunology.* 67:216-220.
30. Glasser, S.W., T.R. Korfhagen, S.E. Wert, and J.A. Whitsett. 1994. Transgenic models for study of pulmonary development and disease. *Am. J. Physiol.* 267 (Lung Cell. Mol. Physiol.)267:L489-L497.
31. Hildebrand, F.L., E.C. Rosenow, T.M. Habermann, and H.D. Tazelaar. 1990. Pulmonary complications of leukemia. *Chest.* 98:1233-1239.