

Elevated gelatinase activity in pulmonary alveolar proteinosis: role of macrophage-colony stimulating factor

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Abstract: Pulmonary alveolar proteinosis (PAP) is an anti-granulocyte macrophage-colony stimulating factor (GM-CSF) autoimmune disease resulting in the accumulation of phospholipids in the alveoli. GM-CSF knockout (KO) mice exhibit a strikingly similar lung pathology to patients with PAP. The lack of functionally active GM-CSF correlates with highly elevated concentrations of M-CSF in the lungs of PAP patients and GM-CSF KO mice. M-CSF has been associated with alternative macrophage activation, and in models of pulmonary fibrosis, M-CSF also contributes to tissue resorption and fibrosis. Matrix metalloproteinase-2 (MMP-2) and MMP-9 have been implicated in extracellular matrix degradation in animal models of fibrosis and asthma. We show for the first time that the lungs of PAP patients contain highly elevated levels of MMP-2 and MMP-9. PAP broncholaveolar lavage (BAL) cells but not bronchial epithelial cells expressed increased MMP-2 and MMP-9 mRNA relative to healthy controls. Both MMPs were detectable as pro and active proteins by gelatin zymography; and by fluorometric global assay, PAP-MMP activity was elevated. BAL cells/fluids from GM-CSF KO mice also demonstrated significantly elevated MMP-2 and MMP-9 gene expression, protein, and activity. Finally, PAP patients undergoing GM-CSF therapy exhibited significantly reduced MMPs and M-CSF. These data suggest that in the absence of GM-CSF, excess M-CSF in PAP may redirect alveolar macrophage activation, thus potentially contributing to elevated MMP expression in the lung. *J. Leukoc. Biol.* 79: 133–139; 2006.

Key Words: alveolar macrophages · matrix metalloproteinases · surfactant · GM-CSF

INTRODUCTION

Matrix metalloproteinases (MMPs) are neutral proteinases that participate in the degradation of the extracellular matrix (ECM) [1]. Extracellular macromolecules are important for creating the cellular environments required during development, morphogenesis, inflammation, and wound heal-

ing. A major function of the MMPs is thought to be the removal of ECM in the tissue resorption component of wound healing [2]. The regulation of MMP activity by active cleavage or through binding to natural inhibitors is believed to play an active role in lung fibrosis [1, 3]. MMP-9 activity and expression have been associated with the airway remodeling found in emphysema, acute respiratory distress syndrome, idiopathic pulmonary fibrosis, and asthma, consistent with the breakdown of collagen type IV in the ECM [4–8]. MMP-2 activity and expression have been linked with airway smooth muscle hyperplasia and hypertrophy, which are also important pathogenic aspects of asthma, as well as in bronchiolitis obliterans, organizing pneumonia-based, idiopathic, pulmonary fibrosis [9, 10].

Pulmonary alveolar proteinosis (PAP) is a rare, anti-granulocyte macrophage-colony stimulating factor (GM-CSF) autoimmune lung disease, characterized by inefficient surfactant catabolism by alveolar macrophages [11, 12]. The association of GM-CSF deficiency with PAP lung disease resulted from observations in the GM-CSF knockout (KO) mouse. These mice develop a PAP-like lung disease after 6–8 weeks with seemingly no hematopoietic abnormalities [13, 14]. The lung disease can be corrected by the site-directed expression of GM-CSF in the murine lung [15]. This was the first evidence to suggest that GM-CSF is essential for normal lung homeostasis. Alveolar macrophages in human PAP have no intrinsic defect in their ability to secrete GM-CSF or respond to GM-CSF [16]. Observations in human PAP [17] and the GM-CSF KO mouse [18] have shown elevated levels of M-CSF, which is thought to be a compensatory mechanism for the lack of GM-CSF [18]. The process of switching to M-CSF may be a result of a redirection of macrophage classical activation, which ultimately results in the expression of proteins associated with alternative macrophage activation [19]. It is interesting that M-CSF has been correlated with enhanced fibrosis in murine models of interstitial pulmonary fibrosis [20, 21] and has the capacity to up-regulate MMP-2 and MMP-9 expression in epithelial tumor cell lines [18, 22, 23].

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The possible involvement of MMP-2 and MMP-9 in PAP pathology has not been investigated. Based on the many reports of elevated MMPs in various lung diseases, we hypothesized that in PAP, the deficiency of functional GM-CSF together with overproduction of M-CSF might be associated with overexpression of MMP-2 and MMP-9. To examine this hypothesis, we studied MMP expression and activity in bronchoalveolar lavage (BAL) cells and fluids from PAP patients and GM-CSF KO mice.

MATERIALS AND METHODS

Study population

The Cleveland Clinic Foundation Institutional Review Board (OH) approved this protocol, and written informed consent was obtained from all subjects. Healthy control individuals had no history of lung disease and were not on medication. The diagnosis of idiopathic PAP was established by histopathological examination of material from open lung or transbronchial biopsies, showing the characteristic filling of the alveoli with eosinophilic amorphous material with preserved lung architecture and absence of inflammation, and exclusion of secondary etiologies by negative lung cultures or occupational history [24]. All PAP patients were symptomatic with dyspnea, were hypoxemic on room air, and had typical alveolar infiltrates on radiographs. Patients participated in a prospective clinical trial of recombinant human GM-CSF (Leukine, Berlex, Seattle, WA) as described previously [25]. Treatment consisted of 250 µg/day by subcutaneous administration with an increased dose every 2 weeks and a maximum daily dose of 18 µg/kg/day by 8 weeks. Median duration of therapy was 25.5 weeks [26]. Patients were evaluated at baseline prior to initiation of GM-CSF therapy and during therapy.

Cell collection

Alveolar macrophages were derived from BAL obtained by fiberoptic bronchoscopy as described previously [16]. Differential cell counts were obtained from cytopspins stained with a modified Wright's stain. Mean viability of lavage cells was greater than 95% as determined by trypan blue dye exclusion. Bronchial epithelial cell preparations were obtained as described previously and characterized by Wright stain and immunocytochemistry for cytokeratin content [27].

GM-CSF KO model

Dr. Glenn Dranoff (Dana-Farber Institute, Boston, MA) [13], who gave permission to obtain the mice, generated the GM-CSF mice, and Dr. Robert Paine (University of Michigan, Ann Arbor, MI) subsequently provided the mice, which having been backcrossed eight generations to C57Bl/6 wild-type mice, were obtained from the Jackson Laboratory (Bar Harbor, ME). BAL macrophages were obtained from GM-CSF KO mice and age and gender-matched wild-type C57Bl/6. For BAL cell harvest, mice were injected intraperitoneally with ketamine (80 mg/kg) and xylazine (10 mg/kg). The thoracic cavity was opened, and the lungs were exposed; after cannulating the trachea, a tube was inserted, and BAL was carried out with 37°C phosphate-buffered saline (PBS) in 1 ml aliquots. For all experiments, three sets of pooled BAL from three to five mice were used.

MMP zymography

Gelatin zymography was used for detection of MMP-2 and MMP-9 activity. The gel consisted of 7.5% acrylamide containing 2 mg/ml gelatin (Gelatin, type A, from pork skin, electrophoresis reagent, Sigma Chemical Co., St. Louis, MO, Cat. #G-8150). PAP and healthy control BAL protein were loaded on the gelatin gel along with molecular weight markers and positive control (lipopolysaccharide-stimulated monocytes). For gelatin zymography, after electrophoresis, gels were soaked in 2.5% Triton X-100 (in 50 mM Tris, pH 7.6) with gentle shaking at room temperature for 60 min with one change. Gels were rinsed three times in activation buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 5 mM

CaCl₂) and then incubated at 37°C for 16–20 h. The gels were stained with Coomassie blue for 1 h and destained in a solution of 45% methanol and 10% acetic acid. Gelatinase activities appeared as clear bands against a blue background. Human MMP-2/MMP-9 and mouse MMP-9 zymography standards were used as positive controls (Chemicon International, Temecula, CA). In addition, molecular weights of gelatinolytic bands were estimated using the kaleidoscope-prestained protein standards (Bio-Rad, Hercules, CA). Gelatinase activity was defined by the presence of clear bands contrasting against the blue-stained gel background. Sensitivity of this procedure ranges from 50 to 100 pg.

RNA purification and analysis

Total RNA was extracted from BAL cells and peripheral blood mononuclear cells from PAX tubes (Becton Dickinson, San Jose, CA) by RNAeasy protocol (Qiagen, Valencia, CA). Expression of mRNA was determined by real-time reverse transcriptase-polymerase chain reaction (PCR) using the ABI Prism 7000 detection system (TaqMan; Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. RNA specimens were analyzed in duplicate using human or murine primer sets for a housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and MMP-2, MMP-9, tissue inhibitor of MMP (TIMP)-1, TIMP-2, TIMP-3, and α₂-macroglobulin (ABI, Applied Biosystems, Gaithersburg, MD). Threshold cycle values for genes of interest were normalized to GAPDH and used to calculate the relative quantity of mRNA expression in PAP or GM-CSF KO samples relative to untreated or healthy control or wild-type control values.

Monocyte/macrophage preparation

Human peripheral blood monocytes were derived as described previously [28, 29]. Briefly, monocytes were purified from Nycodenz gradients and cultured for 6 days. Cells were then treated with M-CSF (1000 U/ml) or medium alone, and after 48 h, mRNA was extracted and analyzed by real-time PCR.

MMP protein quantitation

The microparticle-based assay for the detection of soluble MMPs was purchased from R&D Systems (Minneapolis, MN) and performed as per the manufacturer's specifications. Briefly, BAL specimens were obtained from PAP patients (n=6) and healthy controls (n=6). Microparticles with proprietary fluorescence for MMP-2 and -9 were allowed to incubate with samples, followed by detection with a streptavidin/biotin reaction using the Luminex platform. Data were compared with standard curves for each MMP and expressed as mean pg/ml ± SEM.

Global MMP activity assay

We used a microtiter assay format with the fluorogenic peptide substrate I (R&D Systems, ES001). The peptide substrate (Mca-P-L-G-L-Dpa-A-R-NH₂) contains a highly fluorescent 7-methoxycoumarin group, which is quenched efficiently by resonance energy transfer to the 2, 4-dinitrophenyl group. Briefly, 10 µM substrate/100 µl reaction mixture containing BAL fluid from healthy controls or PAP patients was allowed to incubate for 30 min up to 4 h. Enzymatic reactivity was determined at 320 nm excitation and 405 nm emission. The enzymatic activity was compared with background PBS with peptide and a positive control (*Clostridium* collagenase). The same assay was used to assess MMP functional activity in the BAL obtained from the GM-CSF KO mouse and wild-type controls. Data were expressed as mean fluorescent intensity (MFI) ± SEM.

MMP-9- and MMP-2-specific activity assays

BAL fluids from PAP patients and healthy controls were evaluated for MMP-2- and MMP-9-specific activity as defined in the product information booklet using the Biotrak assay system (Amersham, Piscataway, NJ). Briefly, BAL fluid was treated with p-aminophenylmercuric acetate (APMA) to detect the presence of the pro forms of MMP-2 and MMP-9. The activity was measured by conversion of APMA and color change. Samples were evaluated at 405 nm in duplicate, and activity was expressed in mg/ml.

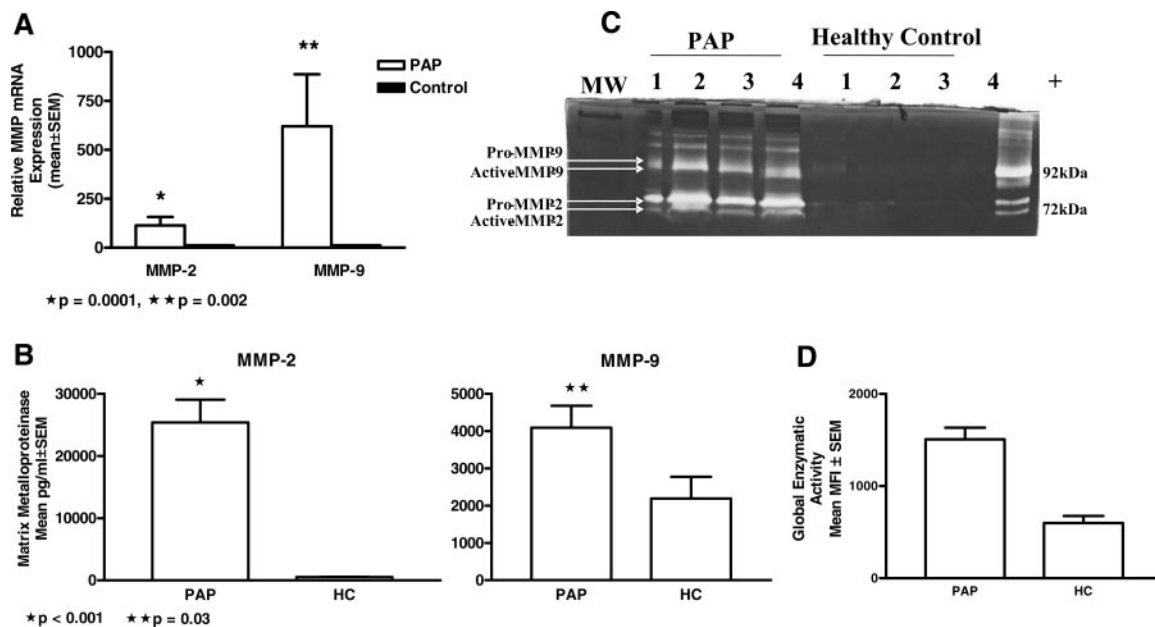


Fig. 1. MMPs are elevated in PAP BAL fluid and cells. MMP-2 and MMP-9 mRNA are increased in PAP BAL cells ($n=3$) compared with controls ($n=3$; a). BAL fluids from PAP patients ($n=6$) and healthy controls ($n=6$) were evaluated for MMP proteins and compared with standard curves for each MMP and background. MMP-2 ($P<0.001$) and MMP-9 ($P=0.03$) were increased in PAP BAL fluid relative to healthy control (HC; b). (c) Gelatin zymography was performed on PAP and healthy control BAL fluid. PAP BAL fluid (lanes 1–4, left, $n=4$, 15 μ l unconcentrated BAL) and healthy control (lanes 1–4, right, $n=4$, controlled for protein concentration) were run together with molecular weight (MW) standards and positive control (+). The pro and active forms of MMP-9 and MMP-2 were highly expressed in PAP BAL relative to healthy controls. (d) The presence of functional activity was verified using a global fluorometric MMP assay. PAP BAL had significantly higher MMP activity ($n=6$, $P=0.001$) than healthy controls ($n=6$).

Statistics

Data were analyzed by one-way ANOVA, Mann-Whitney, and Student's *t*-test using Prism software (GraphPad, Inc., San Diego, CA.). Significance was defined as $P \leq 0.05$.

RESULTS

MMP-2 and -9 expression is elevated in the BAL fluid and cells of PAP patients

To determine whether MMP levels were elevated in PAP lung, we examined mRNA expression by real-time PCR. MMP-2 (95 ± 37 -fold increase relative to healthy controls, $P<0.0001$, $n=3$) and MMP-9 (44 ± 10 , $P=0.002$, $n=3$) mRNA was increased in the PAP BAL cells (**Fig. 1a**). In contrast, MMP-2 and MMP-9 mRNA expression of PAP bronchial epithelial cells did not differ from controls (data not shown). We next determined whether MMP protein was elevated in BAL fluid. MMP-2 and -9 (Fig. 1b) were significantly elevated in BAL from PAP as compared with the healthy controls using a soluble multiplex-microparticle assay ($P=0.002$). Gelatin zymography demonstrated that PAP BAL fluids had strikingly elevated levels of pro and active MMP-2 and MMP-9 proteins compared with healthy controls (Fig. 1c). Furthermore, high levels of MMP functional activity were detected by the global MMP activity assay in PAP BAL compared with control ($P=0.001$; Fig. 1d). A MMP-2- and MMP-9-specific activity assay confirmed PAP BAL elevation of MMP-2 (14.0 ± 0.51 PAP vs. 2.2 ± 0.39 mg/ml control, $P<0.0001$) and MMP-9 activity (1.2 ± 0.28 vs. 0.3 ± 0.13 mg/ml).

TIMP-1, TIMP-2, and $\alpha 2$ -macroglobulin expression are not deficient in cells from PAP patients

As TIMP-1, TIMP-2, and $\alpha 2$ -macroglobulin are the main inhibitors of MMP-9 and MMP-2, we investigated if there was a relative decrease in the mRNA expression of these MMP inhibitors by alveolar macrophages or bronchial epithelial cells. Real-time PCR of healthy control ($n=3$) and PAP ($n=3$) BAL cells showed no difference of TIMP-1, TIMP-2, and $\alpha 2$ -macroglobulin mRNA expression (data not shown). It is interesting that there was a statistically significant elevation of TIMP-3 expression in PAP BAL (16 ± 1.2 -fold, $n=3$, $P=0.02$). PAP bronchial epithelial cells were not different from healthy controls for any of the inhibitors.

M-CSF is elevated in PAP and up-regulates MMP-2 and MMP-9 expression in human monocyte-derived macrophages

We previously reported that the BAL fluids of PAP patients contained increased M-CSF [17]. To determine the source of M-CSF in the lung, we examined alveolar macrophages and bronchial epithelial cells from PAP patients and healthy controls. M-CSF secretion by PAP alveolar macrophages was higher than that of healthy controls (**Fig. 2a**). PAP epithelial cell secretion of M-CSF was ten- to 20-fold less than that of alveolar macrophages and was not higher than controls (data not shown). M-CSF mRNA was also elevated in PAP alveolar macrophages ($n=4$, Fig. 2b, $P=0.01$). To determine if M-CSF could up-regulate MMP-2 and MMP-9, we cultured monocyte-derived macrophages with and without M-CSF. MMP-2 ($P=0.04$)

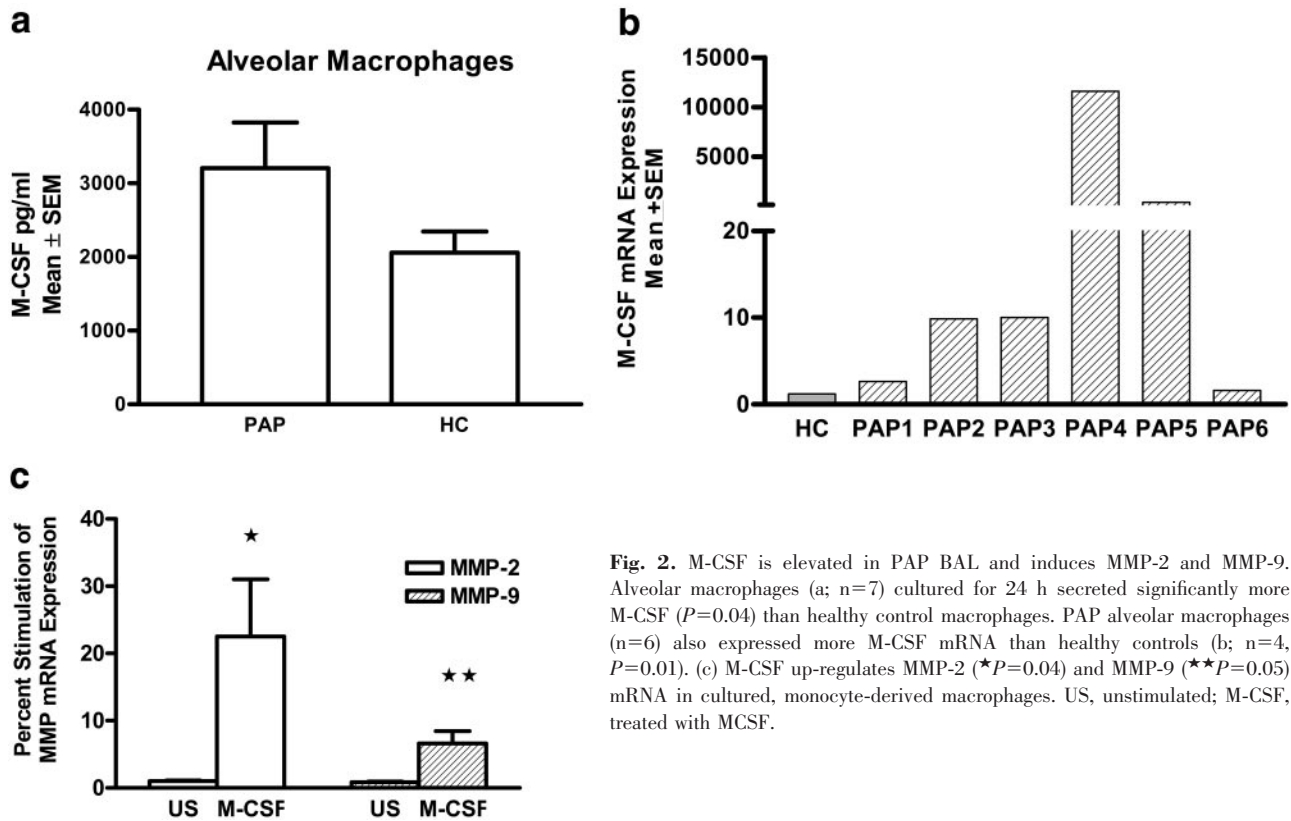


Fig. 2. M-CSF is elevated in PAP BAL and induces MMP-2 and MMP-9. Alveolar macrophages (a; n=7) cultured for 24 h secreted significantly more M-CSF ($P=0.04$) than healthy control macrophages. PAP alveolar macrophages (n=6) also expressed more M-CSF mRNA than healthy controls (b; n=4, $P=0.01$). (c) M-CSF up-regulates MMP-2 ($*P=0.04$) and MMP-9 ($**P=0.05$) mRNA in cultured, monocyte-derived macrophages. US, unstimulated; M-CSF, treated with MCSF.

and MMP-9 ($P=0.05$) mRNA expression was increased in M-CSF-stimulated compared with baseline monocytes (Fig. 2c, n=3).

GM-CSF KO mice exhibit increased MMP-2 and MMP-9 mRNA expression

Shibata et al. [18] reported that BAL fluid from GM-CSF KO mice contained elevated M-CSF. To determine if MMP-2 and MMP-9 were elevated, we evaluated BAL cells and fluid from GM-CSF KO mice and wild-type controls. MMP-2 (Fig. 3A, n=3, $P=0.004$) and MMP-9 (n=3, $P=0.001$) mRNA expression was up-regulated in GM-CSF KO BAL cells relative to wild-type controls. MMPs were also functionally active in the global activity assay (Fig. 3B, $P=0.003$).

GM-CSF therapy reduces MMPs and M-CSF in PAP

Patients who received GM-CSF therapeutically underwent bronchoscopy at baseline and 6 months later. Global MMP activity of PAP BAL fluid was decreased significantly by GM-CSF therapy (Fig. 4, n=6, $P=0.001$). Further, M-CSF levels also decreased significantly in PAP BAL fluid by 50 ± 6% (n=4, $P<0.001$) after GM-CSF therapy.

DISCUSSION

These studies are the first to show that MMP-2 and MMP-9 are elevated in the BAL fluid of PAP patients relative to the

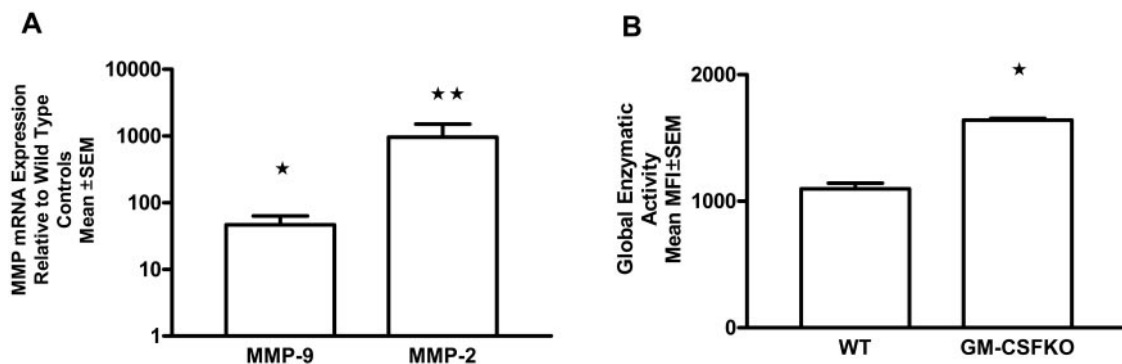


Fig. 3. MMP-2 and MMP-9 are up-regulated in GM-CSF KO mice. BAL cells and fluid from GM-CSF KO (n=3) and wild-type control mice (n=3) were evaluated for MMPs. (A) MMP-2 ($**P=0.004$) and MMP-9 ($*P=0.001$) mRNA expression was elevated significantly in GM-CSF KO relative to wild-type BAL cells. (B) MMP global activity was significantly higher in BAL fluid of GM-CSF KO mice than wild-type (WT) controls ($*P=0.003$).

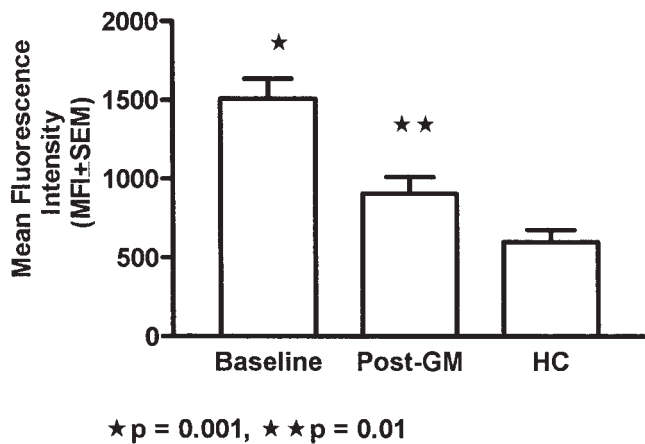


Fig. 4. GM-CSF therapy reduces MMPs in PAP. MMP global activity was significantly higher in BAL fluid of PAP patients than controls ($n=6$; $P=0.001$). PAP patients receiving GM-CSF (Post-Gm) therapy exhibited a significant decrease in MMP-global activity compared with baseline prior to start of therapy ($n=6$, $P=0.01$).

healthy controls. Furthermore, MMP-2 and MMP-9 are functionally active as determined by a global MMP fluorometric assay and an activity assay specific for MMP-2 and MMP-9. Activity was also confirmed by conventional zymography, showing pro and active forms of gelatinases A (MMP-2) and B (MMP-9), suggesting that there is activation of the zymogen pro-MMP into its active form in PAP lungs. The data presented here also indicate that alveolar macrophages are the probable source of MMPs and not bronchial epithelial cells. It is interesting that even with the elevated macrophage expression of MMPs and the increased MMP activity in the lung, there appeared to be no increase in the expression of MMP inhibitors TIMP-1, TIMP-2, and α 2-macroglobulin in the lungs of PAP patients. The only exception was TIMP-3 gene expression, which was higher in PAP alveolar macrophages. These data suggest that there is a generalized, disproportionate presence of active MMP-2 and MMP-9 relative to their inhibitors. Observations in the GM-CSF KO mouse also showed a significant elevation in alveolar macrophage MMP-2 and MMP-9 protein, functional activity, and gene expression relative to wild-type controls. Finally, our data suggest that there is a distinct, inverse relationship between MMP/M-CSF expression and GM-CSF, as MMPs and M-CSF levels decreased in response to GM-CSF therapy.

PAP is an anti-GM-CSF autoimmune disease in which lipoproteinaceous material accumulates within the alveoli of the lung [30]. We and others [16, 17, 31, 32] have reported that the lungs of PAP patients are prototypical of chronic inflammation with elevated levels of monocyte chemotactic proteins (MCP-1, MCP-2, MCP-3), interleukin (IL)-8, IL-10, and M-CSF. This observation occurs in the absence of robust changes in BAL differential cell count [31] or evidence of tissue remodeling and fibrosis [33, 34]. The destruction of the subepithelial basement membrane by MMP-2 and MMP-9 has been proposed to precede the intra-alveolar fibrotic process, resulting in ECM remodeling [2]. These MMPs have also been implicated in the morphological and inflammatory changes observed in asthma, chronic obstructive pulmonary disease, emphysema, and idio-

pathic pulmonary fibrosis [4–7]. This evidence suggests that the surfactant-filled environment present in the PAP lung may prevent the progression of ECM degradation, cellular recruitment, and fibrosis.

We have previously shown that MCP-1, through the down-regulation of its own receptor (CC chemokine receptor 2), may be one mechanism by which cellular recruitment to the lung is prevented [31]. In addition, some MMPs (MMP-9) have the capacity to process matrix proteins, cytokines, and adhesion molecules to generate fragments with enhanced or reduced biological effects. For example, the proteolytic degradation of chemokines by MMP-9 results in decreased chemotactic activity [35]. The observation that PAP lungs have extremely elevated levels of MMP-2 and MMP-9 suggests that this might be one mechanism contributing to the lack of cellular recruitment in response to the elevated BAL chemokines.

In addition to inducing MMP production, multiple cytokines may act synergistically to alter monocyte/macrophage differentiation, thus influencing adaptive T cell responses, which might also influence the process of tissue resorption and fibrosis [36–39]. M-CSF in conjunction with IL-10 has been shown to decrease macrophage/dendritic cell differentiation creating a “subdued” antigen-presenting cell, which is inefficient at mediating T cell activation and proliferation [40]. M-CSF and IL-10 are elevated in PAP [17, 41]. Further, surfactant itself has been shown to decrease fibroblast proliferation, T cell proliferation, and macrophage differentiation [42–45]. The potential contribution of these processes to the pathophysiology of the PAP lung is unknown.

The idiopathic human PAP disease and the GM-CSF KO mouse have elevated M-CSF, which is thought to be a compensatory mechanism for the lack of functional GM-CSF [17, 18]. M-CSF is an important cytokine for macrophage differentiation [46, 47], survival [48], and activation [49, 50]. M-CSF is also a product of macrophage alternative activation [19]. This suggests that in PAP, where M-CSF is elevated, and GM-CSF is deficient, macrophages may have undergone alternative activation. In addition, studies using microglia report that M-CSF may up-regulate MMP-2 and MMP-9 [23], findings that we have confirmed with monocyte-derived macrophages in the present study. Thus, in PAP lung, excess M-CSF may maintain an alternatively activated macrophage phenotype, which includes overproduction of MMPs.

Alveolar macrophages from M-CSF KO mice constitutively expressed MMP-2, MMP-9, and MMP-12 [51]. It is interesting that emphysematous changes have been described in aged GM-CSF KO mice, suggesting MMP activity [52], although this has not been documented in human PAP. Further, animals that are deficient in GM-CSF and M-CSF have a more pronounced alveolar proteinosis with elevated IL-3 and increased incidence of bacterial acute pneumonia and mortality [53]. It is possible that imbalances in M-CSF and GM-CSF may be responsible for increased MMP expression, as MMPs are elevated in M-CSF [51] and GM-CSF KO models. This implicates the important regulatory role of M-CSF and GM-CSF in defining the state of macrophage differentiation and activation.

In summary, in the normal lung, GM-CSF induces maturation of alveolar macrophages with efficient surfactant degradation, resulting in healthy lung homeostasis. In PAP, reduced

levels of functional GM-CSF may contribute to enhanced production of M-CSF, ultimately promoting enhanced MMP expression through redirecting macrophage activation. Even with elevated MMP activity in the lungs of PAP patients, there is no observable ECM degradation or fibrosis. These data suggest that in GM-CSF deficiency, M-CSF may regulate the activation phenotype of alveolar macrophages in PAP and potentially contribute to localized elevation of MMP in the lung.

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