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Pulmonary alveolar proteinosis

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Pulmonary alveolar proteinosis (PAP) (also called alveolar proteinosis, alveolar phospholipidosis, pulmonary alveolar lipoproteinosis, pulmonary alveolar phospholipoproteinosis) has been recognized for almost half a century, although descriptions of probable PAP cases can be found in the earlier medical literature. It is a rarely encountered disease reported to occur in a worldwide distribution [1], with an estimated incidence of 0.36 cases per million population and a prevalence of 3.7 per million of population [2].

At least three separate pathophysiologic mechanisms may lead to the characteristic feature of PAP: the excessive accumulation of surfactant lipoprotein in pulmonary alveoli, with associated disturbance of pulmonary gas exchange [1]. Congenital cases present in the neonatal period with life-threatening hypoxia (approximately 2% of total cases) and result from one of several genetic defects in the surfactant protein (SP)-B gene or the β_c molecule of the granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor [3,4]. Secondary PAP cases (accounting for approximately 5%–10% of total cases) are associated with various underlying diagnoses, especially hematopoietic and other malignancies [5]. The most common form of PAP (more than 90% of cases) is acquired by previously healthy adults. The prognosis for adult patients with PAP varies, but disease-specific survival rate exceeds 80% at 5 years. Clinical remission also may occur either spontaneously or after therapy, although such cases may represent a quiescent disease phase rather than complete spontaneous resolution. The survival rates for adult PAP patients seem to have increased progressively in the four decades since the initial clinical description of this condition [1]. Although an effective treatment using pulmonary lavage was described soon after the recognition of PAP as a distinct entity in 1958 [6], further insights into its pathogenesis were slow to be unraveled. Fortunately, the last decade has brought new advances in laboratory and clinical research that are lifting a veil not only on the rare condition PAP but also on general aspects of pulmonary surfactant biology and innate immune defense [7].

Clinical features

Medical history

Based on several analyses of published case reports and case series that together included more than 400 patients, the common clinical features of patients with PAP are relatively well established [1,8–11]. Although the diagnosis has been reported at all ages,

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a patient who has PAP is typically a previously healthy adult of median age 39 years who describes a history of slowly progressive dyspnea, with these symptoms being present for a median of 7 months. There is a wide range of possible clinical presentations, however, with 25% of patients having symptoms of 2 years or more; in up to one third of cases reported by Japanese investigators the patients were minimally symptomatic [8]. Less common symptoms include fatigue, weight loss, and low-grade fever. Cough is mostly nonproductive and may be absent in at least one fourth of patients, unless there is complicating pulmonary infection. Less than 20% of patients report hemoptysis or chest pain. Secondary pulmonary infections may occur in 13% of cases, with *Nocardia* spp. being reported most commonly [1].

There is a strong association between PAP and tobacco smoking. Overall, approximately three-fourths of patients are smokers at the onset of their disease. There is a more than a 2:1 preponderance of men among smokers, but this excess may be confounded by the often greater proportion of men who smoke in many societies, because there is no male bias among nonsmoking patients. African-American patients comprise 17% of the reported cases from North America.

Clinical examination

Physical examination is commonly remarkable for the absence of abnormal signs in the chest or elsewhere at rest. Crackles may be audible in 50% of cases, and clubbing and cyanosis have been described in approximately one fourth of some earlier case series but do not seem to be as common in recent decades [8,9,11–13].

Radiology

Chest radiography is the most useful radiologic screening test [14,15]. It reveals a pattern of bilateral diffuse alveolar densities in most patients, although interstitial, mixed, diffusely nodular, and focally dense patterns have been reported [9].

CT of the chest increases diagnostic accuracy [16] by confirming bilateral alveolar consolidation, which often appears in a ‘‘crazy-paving’’ pattern that consists of scattered or diffuse ground-glass attenuation with superimposed interlobular septal thickening and intralobular lines [17]. These radiologic appearances suggest PAP but are nonspecific, being found in a range of other diseases [18,19].

Pulmonary function tests

Hypoxemia caused by an increased alveolar-arterial oxygen gradient is nearly universal in symptomatic patients, and blood gas analysis together with measurement of the pulmonary diffusing capacity for carbon monoxide (D_LCO) are the most informative pulmonary function assessment tools. Among 410 published cases, the mean (± SD) P_aO_2 at diagnosis was 58.6 (15.8) mm Hg [1]. Consistent with this hypoxemia, measurements of D_LCO in a subset of patients were reported to be substantially reduced at 47% (12.7%) of the predicted normal values [9]. Lung function testing, including forced expiratory volume in 1 second (FEV_1), vital capacity (VC), and total lung capacity (TLC) may show evidence of a mild to moderate restrictive abnormality (TLC 74% ± 19.3%) [1,8,9]. These abnormalities are at least partially reversible with effective therapy, such as whole lung lavage, or during natural disease remissions [8].

Laboratory markers

Among several described abnormalities that involve raised serum levels of carcinoembryonic antigen, cytokeratin 19, and mucin KL-6 [1,11,20], the most common finding (82%) is a slight to moderate elevation of the serum level of lactate dehydrogenase (LDH). In addition to pulmonary function testing and gas exchange analysis, serial measurements of LDH in individual patients may provide an approximate index of disease activity because the LDH level and alveolar-arterial oxygen gradient show a moderate positive correlation. LDH levels may be found slightly elevated even in patients who are enjoying a clinical remission with arterial blood gas levels in the normal range. Also common in PAP are elevated serum levels of SP-A, -B and -D [1,21], the levels of which may correlate with disease severity. These findings are not specific, however, because elevated plasma levels of SPs occur in a wide range of other lung diseases [22–26]. Recently, monocyte chemoattractant protein (MCP-1) was reported to be increased in bronchoalveolar lavage fluid (BALF) from four patients [27].

Differential diagnosis

The differential diagnosis of an adult who has PAP is wide, because the clinical presentation of this syndrome is nonspecific [11,28]. Although the adult age group excludes congenital PAP as a possibility,

acquired PAP occasionally can be secondary to underlying hematopoietic malignancy [5,29], intrinsic or iatrogenic immunodeficiency disorders [30,31], lysinuric protein intolerance [32], acute silicosis, and other industrial inhalational syndromes [33].

On first presentation, acquired PAP may simulate an extensive number of conditions, including bacterial and viral pneumonia, *Pneumocystis carinii* pneumonia, acute respiratory distress syndrome, cardiogenic pulmonary edema, sarcoidosis, acute interstitial and organizing pneumonias of various etiologies, bronchiolitis obliterans organizing pneumonia, diffuse interstitial lung diseases, drug-induced pneumonitis, exogenous lipoid pneumonia, hypersensitivity pneumonitis, pulmonary hemorrhage syndromes, and bronchioalveolar carcinoma [16,18,19].

Algorithm for investigation

The diagnostic evaluation of a patient with possible PAP begins with a clinical and occupational history and physical examination that focuses on exercise tolerance and exclusion of cardiac disease or systemic disorders, such as sepsis, vasculitis, and malignancy. Blood and differential leukocyte counts help to detect any underlying hematopoietic malignancy, and blood chemistry tests, including renal and liver function assessment, help detect other systemic disorders. Moderately elevated LDH levels suggest PAP in a compatible clinical context. Plain radiography of the chest typically reveals bilateral diffuse alveolar densities; CT scans of the thorax somewhat improve the diagnostic accuracy for PAP but remain nonspecific. Lung function tests, including blood gases, lung volume measurement, and carbon monoxide gas transfer, are likely to reveal impaired gas transfer, hypoxemia, and a restrictive ventilatory defect.

Although future immunodiagnostic methods may be used to confirm a diagnosis of PAP rapidly and noninvasively (see later discussion of anti–GM-CSF antibody data), a definitive diagnosis still requires the demonstration of typical findings on either cytologic analysis of BALF or histologic examination of open or transbronchial lung biopsy specimens [34,35]. Previously, a lung biopsy was required to demonstrate alveolar filling, with excess surfactant material appearing as eosinophilic and periodic acid-Schiff positive lipoproteinaceous material with otherwise relatively preserved lung architecture and minor or absent evidence of inflammation. More recently, this approach has been supplanted in most cases by reliance on the macroscopic and microscopic appearance of returned fluid from a diagnostic fiberoptic bronchoalveolar lavage procedure [11,13,20,34,35]. PAP is associated with abundant milky bronchoalveolar lavage effluent, which on cytologic examination contains granular acellular eosinophilic proteinaceous material and foamy macrophages engorged with diastase-resistant periodic acid-Schiff positive intracellular inclusions, which also show characteristic features after Papanicolaou staining [36]. Electron microscopic demonstration of characteristic concentrically laminated phospholipid lamellar bodies can be confirmatory in cases of diagnostic doubt [1,20]. PAP rarely may be associated with considerable interstitial pulmonary fibrosis [37].

Therapeutic lung lavage

Physical removal of excess alveolar surfactant material by repeated segmental flooding with saline was first shown to be beneficial in 1960. Modern whole lung lavage using general anesthesia and single lung ventilation via a double lumen endotracheal tube has been standard therapy for several decades in symptomatic patients. This procedure usually is fairly well tolerated, with significant clinical, physiologic, and radiologic improvements expected in up to 84% of cases after the first lavage [1]. The median duration of freedom from recurrent symptoms after a single lavage treatment is 15 months, with repeat treatments commonly required [1]. Technical and patient safety aspects of whole lung lavage are well described elsewhere [11], with a total of 20 L to 40 L of saline required for the initially milky or turbid returning alveolar lavage fluid to become macroscopically clear. The rate of clearance of residual lavage fluid from the alveolar space is rapid [38], and some patients are suitable for bilateral sequential lung lavage during the same anesthetic session [11]. Recently, alternative techniques of lung lavage have been reported that use variations in fluid volume or other lavage protocols and fluid delivery directed by fiberoptic bronchoscopy [38–40].

A recommendation for whole-lung lavage therapy should be based on a skilled assessment of the relative risks and benefits of the procedure in each individual. Ultimately, however, most patients do require such treatment, with 63% of patients undergoing lavage within 5 years of diagnosis [1]. Lung lavage is associated with a 5-year survival rate from the time of PAP diagnosis somewhat superior to that of patients not afforded such treatment (± SD; 94% ± 2% versus 85% ± 5%) [1].

Outcome

Deaths directly related to PAP usually have involved respiratory failure (72%) or uncontrolled infection (18%), and most of these events occur within 1 year of diagnosis. Survival prospects for patients who have PAP seem to have improved consistently in the four decades since the condition was first described, such that survival rates approach 100% for cases reported within the last decade [1]. Spontaneous resolution has been reported in a small number of patients, and it seems likely that the disease process of PAP eventually enters a quiescent state in many other surviving patients. These observations are based on reported cases with relatively limited follow-up, however, and the true long-term outlook for these patients must be defined better.

Discovery of the role of granulocyte-macrophage colony-stimulating factor

GM-CSF was chemically purified in the late 1970s [41], and in 1984 it was one of the first human cytokines to be cloned [42]. It was an intense focus of clinical and laboratory investigation through the 1980s and 1990s because of its potent capacity to stimulate the proliferation and differentiation of neutrophilic and monocyte/macrophage lineage hematopoietic cells in vitro, an action that had remained unexplained since its recognition in 1964 [43]. This capacity provides the basis for its clinical use [44–46]. GM-CSF shares some, but not all, of its actions with granulocyte colony-stimulating factor, the other major neutrophilic hematopoietic regulator currently in clinical use [44,45]. In normal individuals, the pharmacologic administration of recombinant GM-CSF consistently leads to a dose-dependent stimulation of myeloid hematopoiesis, which results in peripheral blood neutrophilia, monocytosis, and eosinophilia [44].

Each of the previously mentioned actions requires engagement of GM-CSF with its high-affinity receptor complex. This receptor complex is a member of the cytokine class I receptor family and comprises a GM-CSF–specific α-chain and a common-β chain (β_c). The α-chain is the major binding site for the ligand and provides specificity [47,48]. The complex of the α-chain and β_c provides high affinity binding [49], and the β_c acts as the dominant signal transducer. In humans and mice, the β_c is also a component of the receptor complexes for interleukin-3 (IL-3) and IL-5 [50].

The intracellular signaling cascade induced by GM-CSF binding is complex, incompletely characterized, and shares several common components (particularly the Jak/stat pathway) with many other cytokines [51,52]. A specific conserved motif of the intracellular membrane proximal region of β_c, "box 1," is essential for Jak2 activation [53,54] and subsequent phosphorylation of stats 5a and b [55]. Although not essential for Jak2 activation, the α-chain also participates [56]. A second conserved domain of β_c is essential for the binding and activation of the adapter protein Shc, which results in the downstream activation of the GRB2/SOS/MAP kinase pathway [57]. The specific amino acid residues of β_c required for these separate functions have been characterized [58].

The capacity of hematopoietic growth factors, including GM-CSF, to influence the growth and function of hematopoietic cells in vitro has been known for more than 30 years, and these properties have been exploited in the pharmacologic administration of recombinant factors clinically [44–46]. These observations do not establish the physiologic role of these factors in vivo, however, because many factors, including cytokine concentration, temporal and spatial expression patterns, and the presence and control of effector cell populations, vary from either the experimental or pharmacologic setting. Other means of defining this physiologic role in the intact animal were required.

Gene-targeted mice that lack granulocyte-macrophage colony-stimulating factor

In 1994, two independent publications described the creation and analysis of animals that lack GM-CSF (GM-CSF$^{-/-}$) [59,60]. The phenotype was identical, with normal viability and apparently normal fertility [61] and normal steady-state hematopoiesis. The dominant abnormality, not found in GM-CSF$^{+/-}$ animals, was "alveolar-proteinosis"–like lung pathology with surfactant accumulation, which manifested as early as 4 to 8 weeks of age and was associated with an increased frequency of pulmonary infections [59,60]. Increased numbers of large, morphologically abnormal alveolar macrophages [62,63] also contained accumulated SPs and lipids. These findings suggested a critical role for GM-CSF in normal surfactant homeostasis, which was confirmed by an identical pulmonary phenotype in gene-targeted animals that lacked the signal transducing β_c chain of the GM-CSF receptor ($\beta_c^{-/-}$) [61,64,65].

Quantification of surfactant protein and lipid abnormalities

The healthy alveolar surface is lined by a thin liquid layer of surfactant, which lowers its surface tension and serves to stabilize alveoli against collapse [66]. Pulmonary surfactant is structurally heterogeneous complex lipid-protein mixture rich in phospholipids [67–69]. It is composed of 90% to 95% lipid by mass, predominantly di-saturated forms of phosphatidylcholine (SatPC) (approximately 45%), such as dipalmitoylphosphatidylcholine (DPPC), unsaturated PC (approximately 20%), and phosphatidylglycerol (approximately 5%–8%), and 5% to 10% protein [70,71]. The protein fraction contains four apoproteins, which modulate surfactant's biochemical properties and structure, and SP-A, -B, -C, and -D, named according to the chronologic order of their discovery [72]. SP-A and -D, members of Ca^{2+}-dependent carbohydrate binding collectin family, are large, water-soluble, ''collagen-like'' molecules [73,74], whereas SP-B and -C are small, highly lipid-soluble, hydrophobic molecules [4,75].

GM-CSF$^{-/-}$ mice have a 7.5-fold increase in bronchoalveolar lavage (BAL) total protein concentration [60] and more than tenfold increased levels of SP-A, -B, -D, and SatPC [76–80]. The total lung SatPC content of GM-CSF$^{-/-}$ mice was increased sixfold over controls, but the phospholipid composition was unaltered and the extracted surfactant functioned normally in vivo [81].

Surfactant metabolism and kinetic studies

The surfactant lipids and individual proteins are maintained in dynamic equilibrium through incompletely understood regulatory mechanisms that control synthesis, recycling, and catabolism in a coordinated fashion at the whole lung level [82–87]. Type II alveolar cells, with a large intracellular storage pool of surfactant within their lamellar bodies, are the sole source of surfactant lipid production, whereas type II cells and Clara cells can produce SPs [85,88]. In steady state, the half-life of surfactant lipids is approximately 4 to 10 hours, with approximately 50% recycled by type II cells [84,89]. Catabolism by alveolar macrophages and reuptake and catabolism by alveolar type II cells are the major routes of clearance. Historically, it was believed that the dominant catabolic pathway in most experimental animals was through type II cells, but recent studies using ''residualizing'' radiotracers in mice demonstrated that approximately 50% of surfactant lipids and SP-A are catabolized by alveolar macrophages [90]. Within alveolar macrophages, SP-A and DPPC are metabolized by separate pathways [91].

Detailed studies of surfactant metabolism have been conducted in GM-CSF$^{-/-}$ mice by Ikegami et al [77,79,81,92] and Reed et al [61]. The studies demonstrated no alteration in the secretion rate of surfactant lipids but impaired catabolism of SatPC [61,81]. GM-CSF$^{-/-}$ mice had gross impairment of the clearance of surfactant lipids and proteins [61,79,81]. The critical role of the macrophage in these defects was confirmed using isolated alveolar macrophages [93]. Degradation of SP-A and lipid metabolism was grossly impaired in GM-CSF$^{-/-}$ cells (approximately 10%–20% of wild-type) [93].

Phenotypic and functional studies of lung macrophages

Lung macrophages—alveolar and interstitial—are a critical component of immunologic defense of the lung and provide a pivotal link between the innate and acquired immune systems [7,94]. They are derived from bone marrow (BM) monocyte precursors [95,96] and have some limited local proliferative capacity [96], but they are responsive in vitro to M-CSF [97], GM-CSF [97–99], and IL-3 [100]. The effects of these cytokines vary, however, with differential gene expression and maturational and functional consequences [97,101]. Major functions of the alveolar macrophages include surfactant metabolism, phagocytosis of opsonized and unopsonized particles, killing of micro-organisms, and modulation of responses to local and systemic inflammatory mediators through secretion of cytokines [102,103].

A summary of the reported phenotypic and functional abnormalities of lung macrophages from GM-CSF$^{-/-}$ and $\beta_c^{-/-}$ mice and their reversibility with either local GM-CSF exposure or expression of the transcription factor PU.1 (discussed later) is provided in Table 1.

GM-CSF$^{-/-}$ cells had reduced expression of the β_2-integrins CD11a and CD11c [63] and integrins α_v and α_L [104], but CD11b and integrin α_M expression was unaltered [63,104]. The immunophenotypic profile of GM-CSF$^{-/-}$ alveolar macrophages is that of a less well-differentiated cell type, with persisting ER-MP20 and absent BM8 expression [104], which suggest incomplete terminal differentiation.

GM-CSF$^{-/-}$ alveolar macrophages have multiple defects in cytokine and mediator secretion in response to a range of stimuli. Summed leukotriene-C, -D, and -E_4 production in response to calcium ionophore

Table 1
Abnormalities described in alveolar macrophages derived from animals that lack granulocyte-macrophage colony-stimulating factor and animals that lack β_c and the capacity of either local GM-CSF expression or forced PU.1 expression to ameliorate these abnormalities

	Corrected by [reference]	
Abnormality [reference]	Local GM-CSF	Forced PU.1 expression
GM-CSF$^{-/-}$		
↑ cell diameter [62]		Yes [62]
↓ Surfactant catabolism (SP-A, DPPC, DPPE) [62,93]	Yes [93]	Partial [62]
↑ SP-A binding [93]	Yes [93]	
↓ PU.1 expression [62]	Yes [62]	
↓ BS-1 lectin binding [63]		
↓ Adhesion [62,63]	Yes [63]	Yes [62]
↓ Integrin expression [63,104]	Yes [63]	Yes [62]
↓ Mannose receptor expression [62]	Yes [62]	Yes [62]
↓ Toll-like receptor-4, and −2 expression [62]	Yes [62]	Yes [62]
↓ Fc_γ receptor expression [105]	Yes [105]	Yes [105]
↓ Phagocytosis of		
latex microspheres [62,63,104,105]	Yes [63,105]	Yes [62,104,105]
opsonized microspheres [105]	Yes [105]	Yes [105]
transferrin-coated microspheres [104]		Yes [104]
E. coli, *S. aureus*, zymosan [62]		Yes [62]
adenovirus [104]		Yes [104]
P. carinii [78]		
Abnormal cytoskeletal organization [104]		Yes [104]
↓ Leukotriene-C, -D, and E_4 production [63]	Partial [63]	
↓ PGE_2 production [106]		
↓ TNF-α secretion to LPS [62,63]	Yes [63]	Yes [62]
↓ IL-6 secretion to LPS [62]		Yes [62]
↓ IFN-γ secretion to LPS [105]		
↑ MCP-1 secretion to LPS [62]		
↓ Bactericidal activity against *E. coli*, *Streptococcus* [62]		Partial [62]
↓ IL-18 secretion to adenovirus [105]		Yes [105]
$\beta_c^{-/-}$		
↑ F4/80, CR3, and MHC class-II expression [107]		
↓ Adhesion [107]		
↓ Phagocytosis of colloidal carbon [107]		

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidy lethanolamine; IL, interleukin; MHC, major histocompatibility complex; PGE, prostaglandin E1; SP, surfactant protein; TNF-α, tumor necrosis factor-α.

was markedly suppressed, with reduced expression of the rate-limiting synthetic enzyme, 5-lipo-oxygenase activating protein [63]. Prostaglandin E_2 production also was impaired [106]. Tumor necrosis factor-α expression in response to lipopolysaccharide (LPS) exposure in vitro also was reduced [62,63]. MCP-1 was undetectable in the BAL of wild-type mice but was abundant in the BAL of GM-CSF$^{-/-}$ mice with GM-CSF$^{-/-}$ alveolar macrophages expressing MCP-1 in response to LPS, which suggests that this may contribute to the increased number of alveolar macrophages in GM-CSF$^{-/-}$ mice. The mRNA levels for the mannose receptor and Toll-like receptor-4 and -2 for LPS [108] also were reduced. Pro-inflammatory cytokine release (tumor necrosis factor-α and IL-6) in response to LPS and peptidoglycan, which is mediated via Toll-like receptor-4, Toll-like receptor-2, CD14, and MyD88, was reduced [62,63].

Numerous defects in phagocytosis have been described, including reduced uptake of fluorescein isothiocyanate (FITC)-labeled microspheres [62,63,104,105], whether exposed in vitro or in vivo, transferrin-labeled microspheres [104] and large diameter latex spheres [104]. Phagocytosis of adenovirus, *Escherichia coli*, *Staphylococcus aureus*, *P carinii*, and zymosan by GM-CSF$^{-/-}$ cells is impaired [62,78,104]. Although phagocytosis of group B Streptococci was normal [109], bacterial killing was

grossly impaired [62]. These defects may be attributable to abnormal subcellular cytoskeletal organization [104].

GM-CSF$^{-/-}$ cells also showed specific defects in Fcγ receptor-mediated phagocytosis of IgG coated particles (opsonophagocytosis) [105], which is controlled distinctly from phagocytosis using other means (complement or mannose receptor mediated), having a specific requirement for src-family kinase activity, specifically Syk [110]. It is known that GM-CSF can enhance FcγR expression and function on alveolar macrophages [111,112]. GM-CSF$^{-/-}$ mice have a blunted interferon-γ (IFN-γ) response to LPS in vivo [105], although isolated T cells produce normal levels of IFN-γ in vitro. Berclaz et al [105] postulated that this may be caused by an indirect defect, perhaps through regulation of an "IFN-γ-releasing factor," as proposed by Noguchi et al [113], with IL-12 and IL-18 candidates for such a factor.

FcγR-mediated phagocytosis and FcγR expression were markedly reduced in GM-CSF$^{-/-}$ cells [105]. Adenoviral infection failed to stimulate FcγR expression on GM-CSF$^{-/-}$ cells, as it did in controls, but in vitro exposure to IFN-γ restored this defect. This failure of stimulation was associated with low BAL levels of IFN-γ, IL-12, and IL-18. In vivo administration of IL-18 to GM-CSF$^{-/-}$ mice after adenoviral infection or forced PU.1 expression stimulated IFN-γ production but not to wild-type levels. Either manipulation has the ability to enhance IFN-γ production, but either alone seems to be inadequate to restore this defect fully. A proposed model for the observed defect in GM-CSF$^{-/-}$ alveolar macrophage function is through reduced IL-18 production, which leads to impaired T-cell IFN-γ production and inadequate stimulation of alveolar-macrophage FcγR expression and function. The critical role for IL-18, rather than IL-12, in pulmonary IFN-γ stimulation is supported by the normal response of IL-12$^{-/-}$ mice to experimental adenoviral infection and the adverse impact of IL-18 neutralizing antibodies [114].

Recent studies have explored the mechanism of GM-CSF actions on macrophage maturation and likely molecular mediators [62]. PU.1 is an *ets* family transcription factor that regulates myeloid and B-cell development [115] and is required for the expression of GM-CSF receptor and granulocyte colony-stimulating factor receptor and myeloid maturation [116]. Adherent GM-CSF$^{-/-}$ alveolar macrophages expressed low levels of PU.1, despite the presence of high in vivo levels of M-CSF. Normal PU.1 expression specifically requires GM-CSF, but low-level expression can be mediated via a GM-CSF-independent pathway. GM-CSF exposure stimulated PU.1 expression. The abnormal morphologic appearance of GM-CSF$^{-/-}$ alveolar macrophages could be corrected by retroviral expression of PU.1, which also restored all assessed functional defects, except for the only partial restoration of surfactant metabolism and bactericidal activity. These data demonstrate that GM-CSF regulates terminal differential of alveolar macrophages through PU.1. Although PU.1 was able to restore many functions, they were not completely normalized, so another transcription factor(s) still may be involved in GM-CSF–mediated signaling in these cells.

These data are consistent with the prior demonstration that GM-CSF and PU.1 lead to expression of many overlapping genes [117,118] and that M-CSF and GM-CSF resulted in similar but distinct gene expression patterns [117]. There is a graded effect of PU.1 levels on lineage commitment, with low levels leading to B-lineage differentiation and high levels leading to macrophage lineage differentiation [119]. Spi-B, another *ets* family transcription factor not normally expressed in myeloid cells, can substitute for PU.1 in PU.1$^{-/-}$ cells, which allows full myeloid maturation to proceed [120]. A direct comparison of the phenotype and function of GM-CSF$^{-/-}$ PU.1^{+} and PU.1$^{-/-}$ Spi-B$^{+/+}$ alveolar macrophages may reveal the degree of this apparent redundancy. Other known *ets* family members do not show such redundancy in double mutant animals [121]. Other unresolved issues relating to the degree of homology between the GM-CSF$^{-/-}$ cellular phenotype and PU.1 expression include whether there are other GM-CSF–independent factors, such as IL-3 [122], that are able to induce PU.1 expression in lung macrophages, whether PU.1 expression in other lineages, such as neutrophils [123] and eosinophils [118,124], also depends on GM-CSF, and whether there are additional cellular actions of GM-CSF on lung macrophages in addition to the induction of PU.1 expression, as has been suggested from recent cell line experiments [125].

Experimental approaches to correct the pulmonary pathology in individuals who lack granulocyte-macrophage colony-stimulating factor

Bone marrow transplantation

Transplantation of wild-type bone marrow cells into irradiated $\beta_c^{-/-}$ recipients resulted in morphologic clearance of surfactant [126,127]. BAL cellularity and macrophage morphology were normalized

and BAL protein levels were corrected. Several abnormalities persisted, however, including focal alveolar macrophage aggregates, periluminal lymphocytic infiltration, and limited areas of fibrosis. Even at 6 months after transplantation, dynamic pulmonary compliance, a measure of lung distensibility, remained at approximately 50% of normal, and airway conductance, a measure of ease of airflow, was unimproved at approximately 70% of normal [127]. Pooled preparations of alveolar macrophages contained wild-type donor cells, but the actual proportions were not determined. Cellular reconstitution using recombination activating gene (RAG)-2 donors similarly improved the lung disease [126].

Pulmonary granulocyte-macrophage colony-stimulating factor expression under surfactant protein-C promoter

The SP-C gene is only expressed by pulmonary epithelial type II cells, and its promoter sequences have been used to direct transgene expression exclusively to the lung [128,129]. GM-CSF$^{-/-}$ mice with constitutive pulmonary GM-CSF overexpression were produced (GM-CSF$^{-/-}$ SP-C-GM-CSF^{+}) [76]. With pulmonary GM-CSF expression, the levels of GM-CSF were high in BAL but undetectable in serum, with no systemic effects of GM-CSF evident [62]. GM-CSF$^{-/-}$ SP-C-GM-CSF^{+} mice developed no morphologic features of surfactant accumulation. Alveolar macrophages from GM-CSF$^{-/-}$ SP-C-GM-CSF^{+} mice were proliferating by proliferating cellular nuclear antigen (PCNA) staining, consistent with the mitogenic effects of GM-CSF, and had increased numbers of proliferating type II cells [130] with "alveolar wall hyperplasia."

Adenoviral granulocyte-macrophage colony-stimulating factor gene transfer

Adenovirus is a commonly used vector for therapeutic gene delivery [131]. The duration of expression has been limited by immune clearance of virally infected cells [132], with the dominant site of expression influenced by the route of delivery, cellular expression of the coxsackie-adenovirus receptor, and the degree of host immunosuppression [131,132]. The intratracheal (IT) administration of an adenoviral construct that contained the GM-CSF gene was studied in GM-CSF$^{-/-}$ mice after immunosuppression using an anti–T-cell receptor antibody [133]. Pulmonary GM-CSF expression was confirmed by ELISA of BAL at 1 week but declined to undetectable levels by 3 weeks. GM-CSF mRNA was still detected by reverse-transcriptase polymerase chain reaction (rtPCR) up to 5 weeks, however. Histologic improvement in surfactant accumulation was evident by 5 weeks.

Aerosolized administration of granulocyte-macrophage colony-stimulating factor

A human clinical study has shown aerosolized GM-CSF to be well tolerated and bioactive, which makes this delivery route clinically appealing for pulmonary-directed therapies [134]. When GM-CSF$^{-/-}$ mice received an aerosol of GM-CSF solution 5 days a week for 4 or 5 weeks they had marked

Table 2
Comparison of pathologic features of alveolar macrophages from mice that lack granulocyte-macrophage colony-stimulating factor and patients with acquired pulmonary alveolar proteinosis

Finding in GM-CSF$^{-/-}$ mice [reference]	Corresponding data in patients with acquired PAP [reference]
↑ Cell diameter [62]	Yes [27,135,136]
↓ PU.1 expression [62]	Yes [137,138]
↓ Adherence [62,63]	Yes [136]
↓ Phagocytosis of	
-latex microspheres [62,63,104,105]	Yes [139]
-*S. aureus* [62]	Yes [140,141]
↑ MCP-1 secretion to LPS [62]	High BAL: MCP-1 levels [27]
↓ Bactericidal activity against *Candida* [62]	Yes [136,140]
↓ αNAE staining [77,133]	Yes [136]
↓ Toll-like receptor-4, and −2 expression [62]	Yes [138]
↓ Mannose receptor expression [62]	Yes [138]
↓ Fc$_{\gamma}$ receptor expression [105]	Yes [138]
Normal phagocytosis of opsonized sheep red blood cells in $\beta_c^{-/-}$ mice [107,142]	Yes [143]

but incomplete improvement in surfactant accumulation. This improvement was reflected in BAL SP-B levels. Alveolar-macrophage morphology was improved.

Interpretation and overview

In aggregate, these studied provide major insights into the cell population responsible for the surfactant accumulation in GM-CSF$^{-/-}$ and $\beta_c^{-/-}$ mice. It is clear that local GM-CSF effects are adequate for normal surfactant homeostasis without requiring any systemic GM-CSF actions in the BM and that the effectors within the lung are nonlymphoid hematopoietic-derived cells, presumably myeloid cells and their progeny, including alveolar macrophages. The aerosol and adenoviral gene transfer data demonstrate that a significant time delay is required for this improvement to be manifest and that low levels of GM-CSF are probably adequate, which potentially explains the lack of abnormalities in GM-CSF$^{+/-}$ animals. It is unclear whether the observed delay implies a lag period necessary for macrophage maturation or the time required to degrade the large amount of accumulated surfactant. The transplant data clearly demonstrate that restoration of alveolar macrophage function is adequate for surfactant homeostasis; the other models reveal significant GM-CSF actions on type II cell number and function.

These studies clearly suggest strong functional similarities between the phenotype of GM-CSF$^{-/-}$ mice and the human condition of alveolar proteinosis. They demonstrate several abnormalities at a cellular and functional level that are present in GM-CSF$^{-/-}$ animals and suggest that analogous defects may exist in patients with alveolar proteinosis (Table 2).

GM-CSF$^{-/-}$ animals also have several relatively subtle systemic immunologic abnormalities. They manifest impaired stimulation of T cells to induce IFN-γ secretion [113,144], potentially caused by impaired IL-18 production. Generally, GM-CSF$^{-/-}$ mice have a reduced susceptibility to experimentally induced autoimmune disorders [145–148] despite an increased incidence of late spontaneous autoimmunity in some strain backgrounds [149]. There is less severe functional impairment of macrophages from sites other than the lung [149,150] and remarkably apparently normal neutrophil function [107,109,142]. The possible presence of such features has not been explored in patients with PAP.

These abnormalities manifest at the level of the whole animal as an impaired ability to resolve a range of infections under certain conditions, which usually implicates cellular effectors other than neutrophils, including *Listeria* [151], group B streptococcus [109], adenovirus [104], *P carinii* [78], and malaria [152].

Granulocyte-macrophage colony-stimulating factor antibodies and new diagnostic tests

Most reported serologic tests for PAP are based on small series of patients and lack specificity. No specific marker for the serologic diagnosis of PAP was available until the recent discovery of the autoantibody against GM-CSF. The discovery of this antibody provided the explanatory link between the pulmonary phenotype in GM-CSF$^{-/-}$ mice and patients with PAP.

History and assays for anti–granulocyte-macrophage colony-stimulating factor autoantibody

In 1999, the specific finding of neutralizing autoantibodies against GM-CSF in the BALF from 11 Japanese patients with idiopathic PAP was first reported [153]. Subsequently, examination of sera from 24 patients with "idiopathic" PAP from five different countries showed that the autoantibody was consistently and specifically present [154,155]. Detection of the autoantibody in sera can be used for the diagnosis of "idiopathic" PAP. To establish a simple and convenient methodology, several assays have been developed. The details of these assays have been published previously [154–156], and the pertinent aspects of their performance are briefly summarized. The assays are as follows:

1. Blot assay with ^{125}I-GM-CSF
2. Latex agglutination test using latex beads coupled with recombinant human GM-CSF
3. Bioassay of growth inhibition of a GM-CSF–dependent cell line by the autoantibody

Blot assay with ^{125}I-granulocyte-macrophage colony-stimulating factor

This assay is based on a technique of so-called "West-Western blotting." The serum sample to be tested for the presence of the autoantibody is loaded in an acrylamide gel followed by transfer onto a membrane, which is then incubated with radiolabeled GM-CSF. This procedure is relatively labor intensive and time consuming and requires gel-electrophoresis before protein transfer to the membrane, overnight incubation, and prolonged autoradiography (Fig. 1).

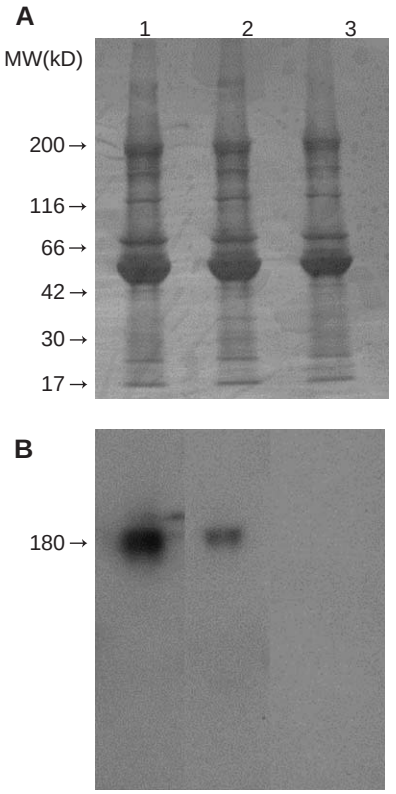

Fig. 1. Detection of the autoantibody using blot assay with 125I-GM-CSF. Serum problems are electrophoresed, transferred to membranes, stained with Coomassie brilliant blue (*A*), incubated with ^{125}I-GM-CSF, and autoradiographed (*B*). Serum proteins were incubated with ^{125}I-GM-CSF in the presence (Lane 2; 50-fold, Lane 3; 500-fold) or the absence (Lane 1) of non-radioactive GM-CSF.

The resulting autoradiograph shows the specific binding of the autoantibody, which can be reduced competitively by preincubation with excess nonradioactive GM-CSF.

Latex beads coupled with recombinant human granulocyte-macrophage colony-stimulating factor

This method is based on the passive agglutination of antibodies with their corresponding antigens, which is widely used for detection of various antibodies in serum, urine, or other biologic materials. Latex beads (1 μm diameter) are coupled with recombinant human GM-CSF. These coupled beads are transferred to a round bottom microplate and incubated for 24 hours. Agglutination is detected by visual inspection. The titer of the autoantibody can be defined using serial dilution.

Using this latex bead assay, sera from 110 persons, including 24 patients with idiopathic-PAP, 4 with secondary PAP, 2 with congenital PAP, 40 control patients with other lung diseases, and 40 normal control subjects were examined (Fig. 2). Agglutination was observed in all sera from patients with idiopathic PAP and 2 of 40 sera from normal subjects. It was negative in sera from 4 patients with secondary PAP, 2 patients with congenital PAP, and 40 patients with other lung diseases. The overall sensitivity was 100% and specificity was 98% (χ-square value = 99.177; $P<0.0001$). These data established that the latex agglutination test is a reliable method for the serologic diagnosis of idiopathic-PAP (Table 3).

Bioassay of growth inhibition of a granulocyte-macrophage colony-stimulating factor–dependent cell line

A GM-CSF, IL-3, and IL-5–dependent cell line, TF-1, provides a useful tool for determining the presence of biologically active concentrations of these cytokines. Using this cell line, the specific inhibition of GM-CSF bioactivity by the autoantibody can be estimated. This assay requires 3 days culture of TF-1 cells in medium-containing recombinant human GM-CSF either with or without diluted serum to be

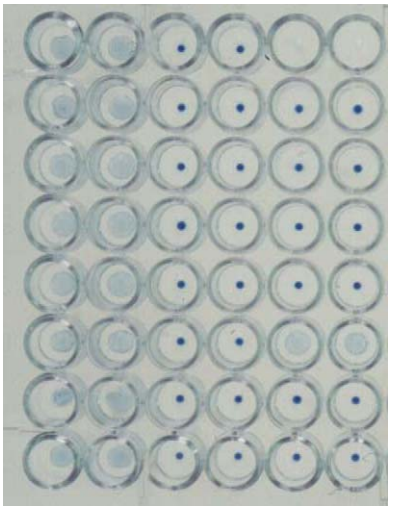

Fig. 2. Latex agglutination test for serologic diagnosis of I-PAP. Latex beads agglutinated by the autoantibodies fall to the bottom of the well in a fine mat. In contrast, beads that are not agglutinated fall to the bottom in a button. A distinct "dot" is observed as a negative result. Agglutination was observed in duplicate sera from eight patients with "idiopathic" PAP (2 left columns) but not in duplicate sera from eight patients with other lung diseases (2 middle columns) or in six of eight normal subjects (2 right columns).

Table 3
The latex agglutination for serologic diagnosis of idiopathic pulmonary alveolar proteinosis

	Latex agglutination test	
	Positive	Negative
I-PAP (n = 24)	24	0
S-PAP (n = 4)	0	4
C-PAP (n = 2)	0	2
Other lung diseases (n = 40)	0	40
Normal subjects (n = 40)	2	38

Abbreviation: S-PAP, secondary PAP.

assayed. TF-1 cell survival is then measured using the 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide assay, a chromogen that provides a visual readout of viable cell number (Fig. 3).

Determination of the serum autoantibody titer

The assays described are convenient and adequately sensitive for detection of the autoantibody, but they are semi-quantitative and imprecise in determining antibody titer. To improve this, an ELISA system using purified autoantibody as a standard was developed. Serum autoantibody against GM-CSF was assayed centrally in a blinded manner [155]. The concentration of purified autoantibody was determined by a sandwich-type ELISA with nonlabeled and peroxidase-labeled anti–human IgG antibodies, using human IgG as a standard for quantitative analysis. After appropriate dilution, incubation, and washing, any antibodies captured by recombinant GM-CSF are detected by peroxidase-labeled anti–human IgG $F(ab)_2$ antibody. The lower detection limit of the assay is 3 μg/mL.

Using this assay, the autoantibody was detected in sera from all patients with "idiopathic" PAP (n = 165) but not in any normal controls (n = 19) and other lung diseases (n = 10) consistent with the previous report (Fig. 4) [155]. This assay performs similarly well on BAL fluid, being positive in all patients with idiopathic PAP (n = 34) but negative in all normal controls (n = 18) and other lung diseases (n = 14). There was no significant difference between the serum levels of the autoantibody according to gender, age, smoking history, disease duration, or the presence of symptoms.

In contrast to the lack of correlation between serum autoantibody concentration and pulmonary findings, the serum levels of KL-6 and carcinoembryonic antigen (CEA) levels are clearly correlated with D_LCO in this cohort (Fig. 5).

Specificity and sensitivity of the autoantibody for serologic diagnosis of idiopathic pulmonary alveolar proteinosis

Fig. 6 shows the specificity and sensitivity of the titer of serum KL-6, CEA, SP-A, SP-D, MCP-1, and the autoantibody against GM-CSF for serologic diagnosis in 25, 5, 10, 9, 5, and 42 subjects with

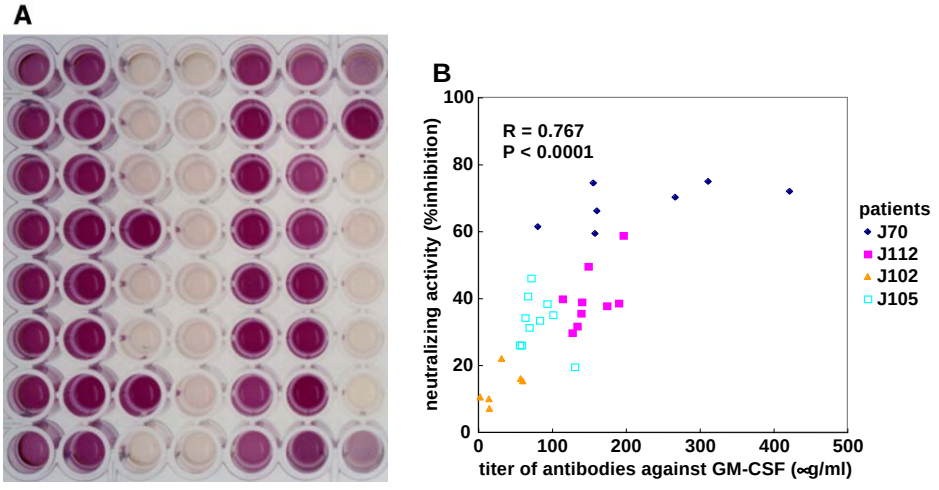

Fig. 3. (*A*) Formation of formazan by TF-1 cells after incubation with GM-CSF with or without diluted serum from a patient with idiopathic PAP. (*B*) Correlation between the neutralizing activities and titers of the autoantibodies in the serial sera formed clusters in each patient.

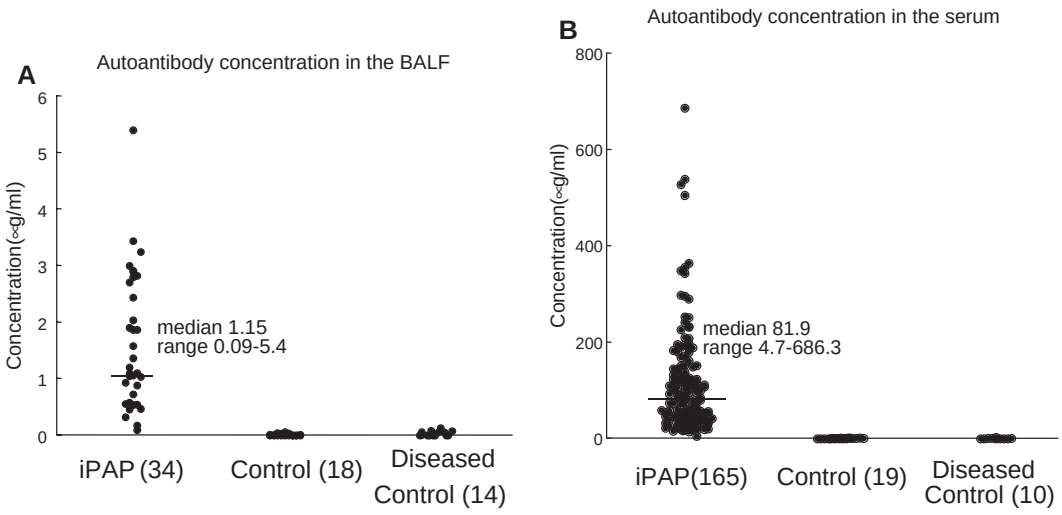

Fig. 4. (*A, B*) Titers of the autoantibody against GM-CSF in Japanese patients with idiopathic PAP.

idiopathic PAP, secondary PAP, idiopathic pulmonary fibrosis, sarcoidosis, collagen-vascular diseases, and normal controls, respectively. The receiver-operator characteristic curves indicate that regardless of level, the presence of the autoantibody achieves high specificity and sensitivity when it is applied for the serologic diagnosis of idiopathic PAP.

Role of granulocyte-macrophage colony-stimulating factor in alveolar macrophage development and function

Alveolar macrophages from GM-CSF$^{-/-}$ mice do not express the differentiation-inducing transcription factor, PU.1. Retroviral-mediated PU.1 expression in cultured GM-CSF$^{-/-}$ alveolar macrophages corrects most of the observed defects, including SP catabolism. GM-CSF plays a critical role in surfactant homeostasis by acting locally in the murine lung and stimulating alveolar macrophage terminal differentiation.

GM-CSF in the lung also is likely to be critical for surfactant homeostasis in humans, as supported by several observations. First, pathologic features of human PAP strongly resemble those of GM-CSF$^{-/-}$ mice. Second, alveolar macrophages in murine and human PAP share several similar morphologic and functional abnormalities (see Table 2). Third, BALF and serum from patients with idiopathic PAP contain high concentration of neutralizing autoantibodies against GM-CSF. Fourth, the presence of the autoantibody to GM-CSF is highly specific for idiopathic PAP. Because physiologic levels of GM-CSF protein

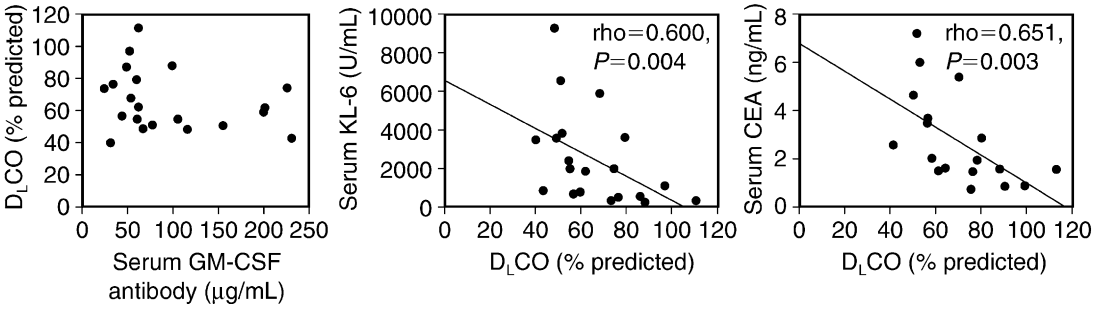

Fig. 5. Correlation between D_LCO (% predicted) and the titer of the autoantibody, serum KL-6, and between serum CEA in 25 patients with "idiopathic" PAP.

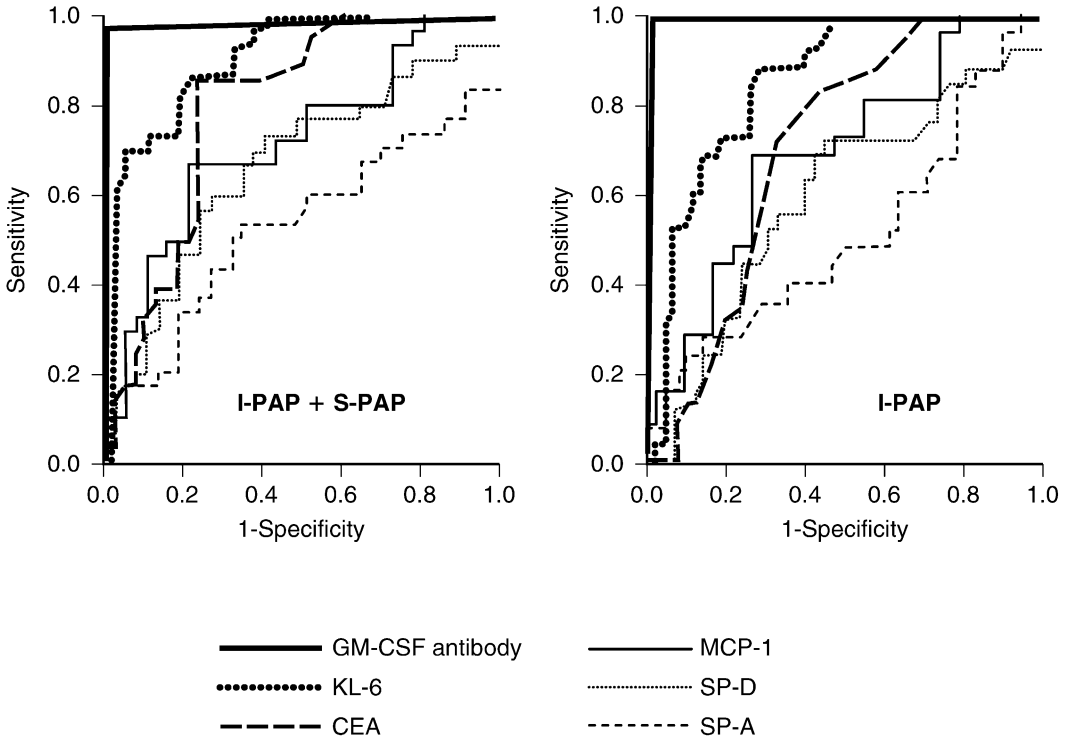

Fig. 6. Comparison of serum markers (GM-CSF antibody, KL-6, CEA, SP-A, SP-D, MCP-1) as diagnostic tests for PAP ("idiopathic", I-PAP; secondary, S-PAP) by receiver operator characteristic curves.

are normally low, the presence of neutralizing autoantibodies might have an important effect on GM-CSF bioactivity levels in the lung [156].

Analogous to murine PAP models, it is plausible that the autoantibodies reduce GM-CSF activity, which results in alveolar macrophage dysfunction and surfactant accumulation. Estimation of the neutralizing activity of the autoantibody in the lung of patients and characterization of their biologic properties revealed that GM-CSF bioactivity was completely abrogated in BALF of patients with idiopathic PAP but not in normal subjects. The autoantibodies were present in the alveoli in high concentrations and co-localized with GM-CSF. The autoantibody recognizes human GM-CSF with high avidity (K_{AV} = 20.0 ± 7.5 pM) and high specificity, reacting with its superstructure. Although target epitopes varied among patients, GM-CSF amino acids 78 to 94 were consistently recognized. The autoantibodies bind GM-CSF with high specificity and high affinity, abundantly exist in the lung, and effectively block GM-CSF binding to its receptor, which inhibits alveolar macrophage differentiation and function.

Granulocyte-macrophage colony-stimulating factor as therapy for pulmonary alveolar proteinosis

Although the enormous body of data that clarified the pathogenetic link between GM-CSF neutralizing antibodies and PAP was not known at the time, clinical trials that explored the therapeutic role of GM-CSF commenced in the mid 1990s. The first treated patient in the world responded favorably, with improved oxygenation, which prompted early reporting of this new phenomenon [157]. At the completion of this initial study using 5 μg/kg/day subcutaneous GM-CSF with the potential for later dose escalation, considering the initial treatment and the subsequent dose-escalation schedule, 6 of 14 evaluable patients responded (43% response rate; 95% confidence interval, 18%–71%), with responses lasting a median of 39 weeks (range, 6–67 weeks) [158]. Responses were generally slow to be manifest, with improvements in $[A - a]O_2$ not evident until 4 to 6 weeks, with maximal improvements not achieved until 6 to 10 weeks of therapy. Among treatment-related variables, only the peak eosinophil count predicted re-

sponse (median 0.63 versus 0.27 × 10^9/L; P=0.01). This study also noted the markedly attenuated hematopoietic response among treated patients [159], a phenomenon attributable to the neutralizing activity of the GM-CSF antibody. Subcutaneous GM-CSF treatment was easily deliverable, with only one patient ceasing treatment because of an adverse effect. Toxicities at 5 μg/kg were infrequent and mild, the most common being local erythema and induration in 36% of patients. Patients with PAP also seem to have a lower incidence of adverse effects than anticipated [44,45]. The presence of GM-CSF–neutralizing antibodies likely results in a reduced effective biologic dose.

Other independent studies have replicated many of these findings and support the therapeutic activity of GM-CSF in patients with acquired PAP. A prospective trial was run through the Cleveland Clinic, using 5 to 18 μg/kg/day for 10 to 48 weeks (median, 26 weeks). Using the criteria to define clinical benefit of a minimum 10 mm Hg improvement in [A–a]D_{O_2}, they have seen responses in 6 of 13 patients (46%) [160–162] that were accompanied by "symptomatic, physiologic, and radiographic improvement" [160]. Among responding patients, the mean arterial P_{O_2} improved from 69.1 ± 3.4 mm Hg at baseline to 84.0 ± 2.0 at the completion of therapy [162]. The overall response rate from these two prospective studies combined is 44% (95% confidence intervals, 26%–65%). There have been six published individual case reports of subcutaneous, GM-CSF using that they used 3 to 6 μg/kg/day [163–168], with five patients responding. The cumulative response rate among all published cases is 52% (17/33; 95% confidence intervals, 34%–69%), although there is likely to be publication bias among single case reports. This frequency of response to GM-CSF is significantly greater than the highest suggested rates of "spontaneous remission" in any series, and, together with the favorable responses to retreatment, clearly establish that SC GM-CSF has therapeutic activity in a proportion of patients with acquired PAP.

Given the detection of GM-CSF antibodies in all of the treated patients, it is not apparent why therapeutic responses were variable. Although limited data are available, the effective antibody titer, which reflects total GM-CSF–neutralizing capacity, may be lower in responders [21,162]. Improvements in oxygenation are achieved more rapidly after lavage than with GM-CSF; however, issues such as resource availability, comorbid conditions, and symptom severity also influence treatment considerations. Although it seems that within the dose range of 5 to 18 μg/kg/day of GM-CSF the proportion of patients responding to lavage may be higher, the magnitude of the therapeutic effect displayed by responding patients with either approach may be similar. From the available reports that provide specific data on pre- and post-lavage arterial P_{O_2} levels [1], the mean improvement in arterial P_{O_2} is 12 to 19 mm Hg with lavage (overall mean 14.5 mm Hg), whereas the mean improvements in arterial P_{O_2} for all patients who responded to GM-CSF were 23 mm Hg [158] and 14.9 mm Hg [162].

Although these studies have demonstrated the reproducible therapeutic activity of GM-CSF in patients with idiopathic acquired PAP, many aspects of this novel therapy require further investigation. Even within the limited number of patients treated to date, some patients required significant dose escalation to attain therapeutic effects. The sequential application of increasing doses seeking evidence of biologic activity, such as eosinophilia, may identify appropriate dose ranges for further evaluation. Although the subcutaneous route was used in most patients, based on its proven safety and efficacy in other settings, recent animal work suggests that inhaled GM-CSF may have greater pulmonary effects [77] but conversely it may not ameliorate any extrapulmonary aspects of the disease. Although aerosolized GM-CSF is well tolerated in humans [134,169] and this route of GM-CSF delivery has been used successfully in three patients with acquired PAP [170,171], the issues of pharmacokinetics and reliable drug delivery remain unresolved.

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