Patient-derived Granulocyte/Macrophage Colony-Stimulating Factor Autoantibodies Reproduce Pulmonary Alveolar Proteinosis in Nonhuman Primates

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Rationale: Granulocyte/macrophage colony-stimulating factor (GM-CSF) autoantibodies (GMAb) are strongly associated with idiopathic pulmonary alveolar proteinosis (PAP) and are believed to be important in its pathogenesis. However, levels of GMAb do not correlate with disease severity and GMAb are also present at low levels in healthy individuals.

Objectives: Our primary objective was to determine whether human GMAb would reproduce PAP in healthy primates. A secondary objective was to determine the concentration of GMAb resulting in loss of GM-CSF signaling *in vivo* (i.e., critical threshold).

Methods: Nonhuman primates (*Macaca fascicularis*) were injected with highly purified, PAP patient-derived GMAb in dose-ranging (2.2–50 mg) single and multiple administration studies, and after blocking antihuman immunoglobulin immune responses, in chronic administration studies maintaining serum levels greater than 40 µg/ml for up to 11 months.

Measurements and Main Results: GMAb blocked GM-CSF signaling causing (1) a milky-appearing bronchoalveolar lavage fluid containing increased surfactant lipids and proteins; (2) enlarged, foamy, surfactant-filled alveolar macrophages with reduced PU.1 and PPAR γ mRNA, and reduced tumor necrosis factor- α secretion; (3) pulmonary leukocytosis; (4) increased serum surfactant protein-D; and (5) impaired neutrophil functions. GM-CSF signaling varied inversely with GMAb concentration below a critical threshold of 5 µg/ml, which was similar in lungs and blood and to the value observed in patients with PAP.

Conclusions: GMAb reproduced the molecular, cellular, and histopathologic features of PAP in healthy primates, demonstrating that GMAb directly cause PAP. These results have implications for therapy of PAP and help define the therapeutic window for potential use of GMAb to treat other disorders.

Keywords: alveolar macrophages; surfactant homeostasis; autoimmunity; neutrophils; anti-granulocyte/macrophage colony-stimulating factor therapy

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

High levels of granulocyte/macrophage colony-stimulating factor autoantibodies (GMAb) are present in patients with the common (idiopathic) clinical form of pulmonary alveolar proteinosis (PAP) and are presumed to be central to disease pathogenesis. However, levels of GMAb do not correlate with disease severity, they are present in most or all healthy people at low levels, and a definitive role in the pathogenesis of PAP has not been established.

What This Study Adds to the Field

Highly purified, PAP patient-derived GMAb reproduced the biochemical, cellular, and histopathologic features of idiopathic PAP after transfer into healthy nonhuman primates.

Pulmonary alveolar proteinosis (PAP) is a rare syndrome characterized by the progressive accumulation of surfactant lipids and proteins in alveolar macrophages and alveoli, and a clinical course ranging in severity from indolent subclinical disease to progressive respiratory failure and death (1–3). Disease onset is insidious but poorly studied because patients usually present late when surfactant accumulation in alveoli is sufficient to impair oxygen uptake and cause dyspnea. Pulmonary lesions typically occur in a diffuse geographic pattern (i.e., abnormal regions juxtaposed to normal-appearing regions) throughout all lung zones and are characterized histopathologically by well-preserved alveoli filled with enlarged, surfactant-engorged macrophages, and intraalveolar surfactant. Pulmonary leukocytosis also occurs (4). Serum levels of surfactant protein (SP)-A, SP-B, and SP-D are increased in patients with PAP and serve as useful biomarkers of disease activity (5–7).

Normally, the level of alveolar surfactant is tightly regulated by the balanced production of surfactant in alveolar epithelial cells and its clearance by recycling and catabolism in these cells and by catabolism in alveolar macrophages (8). The serendipitous discovery that PAP develops in mice deficient in granulocyte/macrophage colony-stimulating factor (GM-CSF) (9, 10) or its receptor (11) provided an important pathogenic clue. Surfactant production, uptake, and clearance by alveolar epithelial cells and uptake by alveolar macrophages were unaffected by the lack of GM-CSF, whereas catabolism in alveolar macrophages was reduced (9, 12, 13). The coordinate regulation of this and many other macrophage functions by

(Received in original form January 4, 2010; accepted in final form March 10, 2010)

Supported by National Heart Lung and Blood Institute grants HL0085453 (B.C.T.) and HL085610 (J.A.W.), the National Center for Research Resources, and National Institutes of Health Office of Rare Diseases grant RR019498 (B.C.T.).

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 182, pp 49–61, 2010

Originally Published in Press as DOI: 10.1164/rccm.201001-0008OC on March 11, 2010
Internet address: www.atsjournals.org

GM-CSF by the “master” myeloid transcription factor PU.1 led to the conclusion that GM-CSF regulates the terminal differentiation of alveolar macrophages in the lungs of mice (3, 14–20). GM-CSF deficiency has not been reported in patients with PAP (21). However, the discovery that high levels of GM-CSF autoantibodies (GMAb) are strongly associated with the common clinical form known as “idiopathic” PAP provided a second critical pathogenic clue (22). These autoantibodies are polyclonal (23), comprise neutralizing and nonneutralizing IgG (24), primarily IgG₁ and IgG₂ (15, 23), and eliminate GM-CSF bioactivity in the serum and lungs of patients with idiopathic PAP (23, 25). *Ex vivo*, purified GMAb reproduced the impairment of GM-CSF–stimulated leukocyte CD11b levels (i.e., the CD11b stimulation index) and other neutrophil abnormalities observed in patients with PAP (25). Notwithstanding, serum GMAb levels in patients with PAP do not correlate with disease severity (5, 7). Further, GMAb are present in most or all healthy individuals (15), albeit at lower levels than in patients with PAP, and GMAb are routinely present and comprise the dominate anticytokine activity in pharmaceutical immunoglobulin prepared from healthy human individuals (26). Thus, a causal role for GMAb in the pathogenesis of PAP has not been definitively established.

Based on this background, we hypothesized that prolonged exposure to GMAb at levels sufficient completely to neutralize GM-CSF bioactivity *in vivo* (i.e., the critical threshold level) would cause PAP by blocking GM-CSF signaling to alveolar macrophages impairing their ability to clear surfactant (15). We injected healthy, nonhuman primates with highly purified, PAP patient-derived GMAb and monitored for the development of PAP for up to 11 months using serum, blood leukocyte, and lung lavage biomarkers of PAP and lung histopathology. These results, of which some were recently reported in brief (27–29), demonstrate that GMAb are central to the pathogenesis of idiopathic PAP.

METHODS

Details of the experimental methods are provided in the online supplement and described briefly next.

Human GMAb

GMAb were purified (25) from a patient with biopsy-proven PAP (30) and high levels of GMAb (3). GMAb were measured by ELISA (15), assessed for purity using gel electrophoresis (25), neutralization of GM-CSF using TF-1 cells (15), and endotoxin content using the Limulus Amebocyte assay (Cambrex, Walkersville, MD).

TABLE 1. TIME AND EVENTS SCHEDULE FOR THE CLINICAL STUDIES EVALUATING PASSIVE IMMUNIZATION OF NONHUMAN PRIMATES WITH HIGHLY PURIFIED HUMAN PAP PATIENT-DERIVED GMAb

Study Parameter	Clinical Study						
	1	2	3	4	5	6	7
Nonhuman primates							
Passively immunized subject	C18588	C18588	C18588	C18588	C18585	C27820	C27842
Nonimmunized subject	C18585	C18585	C18585	C18585	C18588	C18585	C18588
Start date, dd/mm/yy*	20/3/06	19/4/06	19/6/06	11/06/07	28/08/06	5/12/07	1/07/08
End date, dd/mm/yy*	10/4/06	27/4/06	30/6/06	18/7/07	17/10/6	17/11/08	17/11/08
Duration, d	22	9	12	38	52	349	140
B-lymphocyte depletion†	No	No	No	No	No	Yes	Yes
Neutrophil function evaluated	Yes	Yes	No	Yes	Yes	Yes	Yes
Serum biomarkers evaluated	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Chest radiograph	Yes	Yes	No	No	Yes	Yes	Yes
Chest high-resolution chest CT scan	No	No	No	No	Yes	Yes	No
Bronchoalveolar lavage, no.	No	No	No	Yes (1)	No	Yes (4)	Yes (2)
GMAb‡							
Number of administrations	1	1	1	8	3	13	5
Total amount, mg/subject	2.2	2.7	14.8	160	60	350	100
Doses administered, mg	2.2	2.7	14.8	20	20	50, 20	20
Peak serum concentration, µg/ml	—	29	162	137	210	702	243
Mean peak concentration, µg/ml	—	(29)	(162)	106 ± 9	204.5	283 ± 43	211 ± 12
Trough serum concentration, µg/ml	<1 (0.4)	<1 (0.02)	<1 (0.14)	<1 (0.5)	2.1	24	22
Mean trough concentration, µg/ml	—	—	—	9 ± 4.6	5.6	49 ± 3.4	46 ± 5.6
Initial half-life, d§	6.1	—	—	—	6.9	14.5	10
Subsequent half-life, d§	—	0.5	1.2	2 ± 0.08	2.3	18 ± 2.1	16.8 ± 2.7
Duration above serum level, d							
100 µg/ml	0	0	1	2	3	112	26
80 µg/ml	0	0	1	4	4	168	50
60 µg/ml	0	0	2	7	9	250	82
40 µg/ml	0	0	3	11	16	327	110
30 µg/ml	0	0	3	15	20	339	123
20 µg/ml	1	0	4	19	27	349	133
15 µg/ml	3	0	4	21	32	349	140
10 µg/ml	5	0	5	23	39	349	140
5 µg/ml	8	0	6	25	45	349	140
1 µg/ml	15	2	9	27	52	349	140

Abbreviations: GMAb = granulocyte/macrophage colony–stimulating factor autoantibodies; PAP = pulmonary alveolar proteinosis.

* The clinical study start date is calendar date on which the first GMAb dose was given and the study end date is the calendar date when study-related data collection was discontinued.

† Using rituximab and cyclophosphamide as described in the Methods section.

‡ Isolated from human patients with PAP, administered to primates, and measured as described in the Methods section. Dates of administration and amount given are indicated: clinical study 1–5 (see Figure E1); clinical study 6 and 7 (Figure 2).

§ Initial GMAb half-life was that measured after the first dose in a naive subject; all others were subsequent GMAb half-life measurements.

|| Number of study days that the serum GMAb level was above the indicated serum concentration.

Primates

This study was conducted with institutional approval using four female primates (*Macaca fascicularis*, Covance, TX) in the Cincinnati Children's vivarium. The demographics, health screening, vaccination and treatment records, housing, feeding, care, and assessment of the primates are described in the online supplement. All were in good health and free of infection.

Rationale, Study Design, and Study-related Procedures

We tested the hypothesis that human GMAb directly cause the pathologic manifestations of PAP. Seven clinical protocols evaluated administration of highly purified GMAb to healthy primates, including three single- and two multiple-administration safety and dose-finding studies, and two chronic administration studies (Table 1). Results from each study informed subsequent study designs. Preliminary studies (protocols 1–5) established the conditions necessary for long-term studies (protocols 6 and 7) evaluating chronic exposure to human GMAb (passive immunization). Xenographic immune responses were blocked in protocols 6 and 7 by depleting B lymphocytes using anti-CD20 monoclonal antibody (Rituxan, Genentech, San Francisco, CA) and cyclophosphamide (Cytoxan, Baxter, Deerfield, IL). Control primates received this same treatment and phosphate-buffered saline without GMAb. GMAb were administered intravenously and serum levels were monitored to plan repeated administrations.

Study-related procedures, including clinical assessments, phlebotomy, B-lymphocyte depletion, pharmacokinetic analysis, radiologic assessments, bronchoscopy and bronchoalveolar lavage (BAL), cytology including immunohistochemistry (20, 31–33), surfactant lipid and protein analysis (12, 34, 35), open lung biopsies, histopathologic and ultrastructural analysis of lung tissues, and alveolar macrophages are described in the online supplement. GMAb were measured by ELISA (15) in serum and lung epithelial lining fluid (ELF) (by the urea-dilution method [36]).

GM-CSF neutralization was measured with the CD11b stimulation index (25). The development of PAP was monitored by measuring serum SP-D weekly. Weight, vital signs, behavior, activity, blood leukocyte counts, and chest radiographs were used to assess safety.

BAL was performed using three 10-ml aliquots per site in the right middle and lower lobes and lingula. The volume (21 ± 1.3 vs. 19.6 ± 1.4 ml) and efficiency (74.2 ± 2.6 vs. $68.9 \pm 3.5\%$) of BAL fluid recovery was similar in GMAb-injected and control primates ($n = 16$ or 15 samples, respectively; $P = 0.343, 0.242$, respectively). Cell viability was routinely greater than 95%. Adherent alveolar macrophages were used to measure PU.1 and PPAR γ mRNA levels by quantitative polymerase reaction amplification with Taq-Man primers (AB Biosystems, Carlsbad, CA) and endotoxin-stimulated tumor necrosis factor (TNF)- α release as described (16).

Statistical Analyses

Numerical data were tested for normality using the Kolmogorov-Smirnov test and for equal variance using the Levene median test. Parametric data are presented as the mean $\pm$ SEM. Comparisons of parametric and nonparametric data used Student t test and Mann-Whitney rank-sum test as appropriate. P values less than 0.05 indicated statistical significance; P values of less than 0.01 and less than 0.001 are indicated with single and double asterisks. All experiments were done at least twice with similar results.

RESULTS

Preliminary Safety, Pharmacokinetic, and Pharmacodynamic Studies of GMAb

PAP patient-derived, GM-CSF affinity-purified GMAb had an electrophoretic pattern identical to that of purified human IgG

Figure 1. Effects of granulocyte/macrophage colony-stimulating factor (GM-CSF) affinity-purified, pulmonary alveolar proteinosis (PAP) patient-derived GM-CSF autoantibodies (GMAb) (23, 25) on GM-CSF signaling in nonhuman primates *in vitro* and *in vivo*. (A) GMAb blocked GM-CSF-dependent growth of TF-1 cells *in vitro*. Results of four separate experiments were fit by nonlinear regression to a four parameter logistic curve ($y = \text{min} + [\text{max} - \text{min}] / (1 + [x/\text{EC}_{50}]^{\text{Hillslope}})$, where $\text{min} = 0 \pm 2.26$, $\text{max} = 102 \pm 1.77$, $\text{EC}_{50} = 0.208 \pm 0.008$, and $\text{Hillslope} = 4.8159 \pm 0.691$; $P = 0.0001$). The correlation coefficient is indicated. (B) GMAb blocked GM-CSF-stimulated phosphorylation of STAT5 in blood leukocytes in heparinized whole blood (*top*) and in alveolar macrophages (without removing from bronchoalveolar lavage [BAL] fluid) obtained from passively immunized (+ GMAb) or control (no GMAb) primates. Clinical specimens were obtained in Study 6 on Day 125 and incubated without or with GM-CSF (10 ng/ml, 15 min) followed by Western blotting to antibodies against phosphorylated or total STAT5 (phosphoSTAT5 and STAT5, respectively). (C) Reversible inhibitory effects of GMAb on GM-CSF signaling in blood neutrophils *in vivo*. Heparinized blood samples from passively immunized (filled circle) and control (open circle) primates were incubated in the absence or presence of various concentrations of GM-CSF and the increase in leukocyte CD11b levels (i.e., CD11b stimulation index) was measured by flow cytometry (25). Specimens

were obtained in Study 6 on Day 295, at which time the levels of GMAb in GMAb-injected and control primate were $60.9 \mu\text{g/ml}$ and zero, respectively. Similar results were obtained with samples obtained for five other study dates. The GM-CSF concentration used in the standard assay throughout this study is indicated by the dashed line. (D) Effect of GMAb dose on serum levels 30 minutes after intravenous administration. The regression line and 95% confidence interval are shown. (E) Serum half-life of GMAb in passively immunized primates without (open circle) or with (filled circle) B-cell depletion, clinical studies 1–5 or 6–7, respectively.

as we previously reported (25), and blocked GM-CSF signaling *in vitro* as demonstrated by the inhibition of GM-CSF-dependent proliferation of TF-1 cells (Figure 1A). The amounts of GMAB required to inhibit the activity of GM-CSF by 50% (IC₅₀) was 4.13 ± 0.273 mol of GMAB per mole of GM-CSF, similar to previously reported results (23, 25). GMAB blocked GM-CSF signaling *in vivo* as shown by the inhibition of GM-CSF receptor-mediated STAT5 phosphorylation in blood leukocytes (Figure 1B) and alveolar macrophages (Figure 1B). Disruption in GM-CSF signaling was reversible because the reduction in the CD11b stimulation index by GMAB was normalized by increasing the concentration of GM-CSF used (Figure 1C).

A series of five clinical studies were used to evaluate the dosing and administration of GMAB in nonhuman primates (Table 1). The serum level of GMAB was proportional to the dose administered (Figure 1D) and doses of 20 and 50 mg/ primate resulted in high serum GMAB concentrations, comparable with those in patients with PAP (3). Following the initial dose of GMAB in naive primates, the serum half-life of GMAB (~6–8 d) was shorter than expected, rapidly decreased further, and was short (~1–2 d) after subsequent doses (*see* Figure 1E in the online supplement, Figure 2, and Table 1). In clinical studies 1 and 2, the CD11b stimulation index increased (*see* Figure 1E)

rather than decreasing as expected [25] because of trace amounts of endotoxin in the GMAB used (*see* Figure E2). Endotoxin was removed from the GMAB used in clinical studies 3–7. Despite improvement in GMAB purity, knowledge of the GMAB dose–response curve, use of higher doses, reduction of the dosing interval, and evaluation in a naive primate, the dynamic reduction in serum GMAB half-life persisted and an adequate serum GMAB concentration could not be maintained (*see* Figure E1 and clinical studies 3–5), suggesting an antihuman immunoglobulin immune response against GMAB. Subsequent use of rituximab and cyclophosphamide to deplete B lymphocytes and block the antihuman GMAB immune response (Table 1 and clinical studies 6 and 7) resulted in a consistently prolonged half-life of GMAB in serum of 17.1 ± 1.6 days in 18 repeated administrations (*see* Figure 1E). These studies established the safety and conditions of passive immunization with GMAB required for prolonged blockade of GM-CSF signaling in healthy nonhuman primates.

Chronic Passive Immunization of Healthy Nonhuman Primates with GMAB

Informed by preliminary studies, two chronic administration protocols were conducted: clinical studies 6 and 7 (Table 1 and Figure 2). In both, the dose of GMAB used was based on

Figure 2. Effects of granulocyte/macrophage colony-stimulating factor autoantibodies (GMAB) on serum and leukocyte biomarkers of pulmonary alveolar proteinosis (PAP) and homeostatic B-cell expansion following pharmacologic B-cell depletion. Healthy nonhuman primates received rituximab (open diamond) and cyclophosphamide (open triangle) to block antihuman immunoglobulin immune responses and GMAB (50 [filled diamonds] or 20 [filled triangles] mg/dose) as indicated continuously to maintain serum levels above 40 $\mu\text{g/ml}$ for 327 days in Study 6 (left), or 110 days in Study 7 (right). Serum levels of GMAB, whole-blood CD11b stimulation index (CD11b SI), serum surfactant protein (SP)-D, and B-cell counts were measured in GMAB-exposed (filled circle) and control (open circle) primates as indicated. In Study 6, serum SP-D levels were fit by polynomial regression ($R^2 = 0.52$, GMAB-exposed primate). Serum SP-D concentration in untreated primates ($n = 10$) is indicated (hatched region). Bronchoalveolar lavage (BAL) (open square) and lung biopsies (open circle)

were performed on GMAB-exposed and control primates on the days indicated in parentheses. Measurements obtained before first injection of GMAB are indicated (asterisk). Following the last dose of GMAB in Study 6, the serum concentrations of GMAB were 44, 39, and 24 $\mu\text{g/ml}$ on Days 321, 328, and 348, respectively. Similarly, in Study 7 serum concentrations of GMAB were 47, 28, and 12 $\mu\text{g/ml}$ on Days 105, 119, and 147, respectively.

a previously reported minimum serum GMAb level of approximately 10.4–19 $\mu\text{g/ml}$ present in patients with PAP with active lung disease (15). A target value of twice the upper limit of this range was chosen to ensure adequate GM-CSF blockade and was maintained by regular monitoring and readministering GMAb as required continuously to maintain serum levels greater than 40 $\mu\text{g/ml}$ (Figure 2). A marked reduction in the CD11b stimulation index demonstrated continuous pharmacodynamic effects of GMAb on GM-CSF signaling *in vivo* (Figure 2). In both studies, chronic GMAb exposure resulted in a progressive, time-dependent increase in serum SP-D concentration (Figure 2). B-lymphocyte depletion was used in both studies and resulted in the expected rapid reduction in B-cell counts (Figure 2). An incidental finding was that GMAb blocked homeostatic reexpansion of B cells following pharmacologic depletion (Figure 2). Hematologic indices in GMAb-exposed and control primates, respectively, also included total leukocytes ($7,646 \pm 335$, $8,515 \pm 351$; $P = 0.76$); neutrophils ($3,338 \pm 252$, $3,241 \pm 180$; $P = 0.757$); monocytes (696 ± 44 , 494 ± 22 ; $P = 0.001$); lymphocytes ($3,228 \pm 161$, $4,304 \pm 176$; $P < 0.001$); and CD3+ cells ($2,679 \pm 133$, $3,131 \pm 145$; $P = 0.024$) ($\times 1000$ per mm^3 ; $n = 67$ determinations each).

Safety of GMAb Administration

Three single-administration acute exposure studies of escalating GMAb doses ranging from 2.2–14.8 mg per subject (studies 1–3) and two multiple administration studies (20 mg/dose) ranging from 3 to 8 doses/subject were well-tolerated and without any effects on appetite, growth, or behavior. Similarly, B-lymphocyte depletion and chronic exposure to repeated doses of GMAb (20 or 50 mg/dose) for up to 11 months (studies 6 and 7) had no effect on appetite, growth, or behavior. No adverse events were observed in any subject in any of the seven clinical studies, thus

establishing the short-term safety of administering GMAb to healthy primates.

Effects of GMAb on Lung Histopathology, Cytology, and Alveolar Macrophage Structure and Function

Examination of surgical lung biopsies from GMAb-exposed primates revealed a patchy distribution of histopathologic lesions comprised of well-preserved alveoli filled with alveolar macrophages and extracellular eosinophilic debris after exposure to GMAb for 3.5 or 11 months (Figures 3A–3C and Figure 3D, respectively) compared with controls, which were normal in appearance (not shown). Alveolar macrophages in GMAb-exposed primates were large and foamy in appearance (Figure 3B) and had increased oil red O staining of neutral lipids (Figure 3E) compared with controls (Figure 3F). The intra-alveolar material in GMAb-exposed primates had increased SP-A, SP-B, and SP-D immunostaining compared with controls (Figures 3G, 3I, and 3K and Figures 3H, 3J, and 3L, respectively).

Cytologic examination of BAL cells from GMAb-exposed primates revealed that alveolar macrophages were foamy (Figures 4A and 4B) and morphometric analysis revealed they were 1.8-fold larger than alveolar macrophages from controls (Figure 4C). Alveolar macrophages from GMAb-exposed primates also had increased staining with periodic acid–Schiff reagent (Figures 4D–4F), oil red O (Figures 4G–4I), and abnormal immunostaining for SP-B (Figures 4J–4K). Enumeration BAL cells revealed increased numbers of alveolar macrophages, neutrophils, and lymphocytes in GMAb-exposed primates compared with controls (Figure 4L), similar to results in patients with PAP (23, 37) and GM-CSF-deficient mice (9, 10).

Ultrastructural analysis done after GMAb-exposure for 3.5 months revealed that the foamy appearance of alveolar macro-

Figure 3. Effects of granulocyte/macrophage colony-stimulating factor autoantibodies (GMAb) on lung histopathology. Primates were those described in the legend to Figure 2; lung specimens were obtained at 112 days (A–C, E–L) or 321 days (D). (A–C) Typical histopathologic lesions in the lung of a primate exposed to GMAb for 112 days showing alveoli with well-preserved wall architecture (solid arrows) that are filled with foamy alveolar macrophages (asterisks) and intraalveolar eosinophilic debris (open arrows). The lung histology of the corresponding phosphate-buffered saline (PBS)-injected primate was normal (not shown). Hematoxylin and eosin stain. (D) Typical histopathologic lesion in the lung of a primate exposed to GMAb for 321 days. Note that alveoli have well-preserved walls (arrows) and are filled with foamy alveolar macrophages (asterisk). (E and F) Histochemical staining of neutral lipids in frozen lung sections from a GMAb-exposed (E) or PBS-exposed (F) primate. The dark red color indicates neutral lipid accumulation in alveolar macrophages.

Oil red O staining with hematoxylin counterstaining. (G–L) Lung biopsy specimens from GMAb-injected (G, I, and K) or PBS-injected (H, J, and L) primates were immunostained to detect surfactant protein (SP)-A (G and H), SP-B (I and J), or SP-D (K and L) and counterstained with nuclear fast red. Original photomicrographs were obtained at magnifications of $\times 20$ (A, C, and G–L), $\times 100$ (B and D), or $\times 10$ (E and F). The bar represents 20 μm (A–D, G–L) or 50 μm (E and F).

Figure 4. Effects of granulocyte/macrophage colony-stimulating factor autoantibodies (GMAb) on bronchoalveolar lavage (BAL) cytology. Cytology was evaluated in BAL cell preparations from GMAb-exposed (A, D, G, J, and L) or control (B, E, H, K, and L) primates obtained in Study 6 on Day 225. (Similar results were obtained in Study 6 on Days 125, 202, and 348, and in Study 7 on Days 99 and 139.) Original magnification $\times 20$. Scale bar = 50 μ m. (A and B) Diff-Quick staining. (C) Alveolar macrophage size relative to the control mean, determined morphometrically in 600 cells from each BAL sample evaluated ($n = 3$ per primate). Alveolar macrophages were larger in GMAb-exposed primates ($P < 0.001$). (D and E) Periodic acid-Schiff (PAS) staining. (F) Visual grading scale used to quantify PAS staining of alveolar macrophages (left) and the results of staining of alveolar macrophages from passively immunized (+ GMAb) and control (- GMAb) primates (right). A total of 600 alveolar macrophages were evaluated for BAL specimen ($n = 3$ per primate). Each cell was assigned a number (0, 1, 2, or 3) reflecting the degree of PAS staining (0, +, ++, or +++) as indicated. The number of cells at each grade was multiplied by the numerical grade and products added

and divided by 600 to obtain the PAS staining score. The mean ($\pm$ SEM) score of three separate BAL specimens was increased in the GMAb-exposed compared with control primate ($P < 0.001$). (G and H) Oil red O staining. (I) Quantification of oil red O staining in alveolar macrophages was analogous to that used for PAS staining and was increased in the GMAb-exposed compared with control primate ($P = 0.008$). (J and K) surfactant protein-B immunostaining. (L) Numbers of alveolar macrophages (AM), neutrophils (PMN), and lymphocytes (Lym) in BAL fluid from GMAb ($n = 12$) and control ($n = 11$) primates. Cell differentials were determined by examination of 500 cells per sample using Diff-Quick-stained slides (20). Neutrophils were not detected (ND) in controls. Asterisks indicate a $P < 0.001$ for the comparison between GMAb-exposed and controls.

phages in GMAb-exposed primates was caused by accumulation of lamellar bodies and lipid droplet inclusions (Figures 5A–5H). Compared with controls, the lipid droplets in alveolar macrophages from GMAb-exposed primates were 1.3-fold larger than those in controls (Figure 5K). The increased size of alveolar macrophages was proportional to the number of inclusions (Figure 5L).

Alveolar macrophages from GMAb-exposed primates contained reduced numbers of mRNA transcripts for the transcription factors PU.1 (Figure 6A) and PPAR γ (Figure 6B), and reduced endotoxin-stimulated TNF- α release (Figure 6C) compared with controls, similar to results for patients with PAP (17, 38) and GM-CSF-deficient mice (20).

These abnormal histologic, cytologic, and molecular findings are similar to those of patients with PAP associated with GMAb

(2, 3) or mutations in the GM-CSF receptor α chain gene (*CSF2RA*) (6), and to those of mice deficient in GM-CSF (9, 10) or its receptor (11).

Effects of GMAb on Lung Levels of Surfactant Lipids and Proteins

Passive immunization disrupted surfactant homeostasis as demonstrated by analysis of BAL fluid (Figure 7). Compared with controls, GMAb exposure caused the BAL fluid to be milky-appearing (Figure 7A) and to have a greater than twofold increase in turbidity (Figure 7B), total phospholipids (Figure 7C), and saturated phosphatidylcholine (Figure 7D). Cholesterol levels in the BAL fluid were unchanged (14 ± 3 , 11 ± 2 μ g/ml, respectively; $n = 10$ each; $P = 0.494$) and triglycerides were not detected (assay sensitivity = 1 pmol/ml, not shown).

Figure 5. Effects of granulocyte/macrophage colony-stimulating factor autoantibodies (GMAb) on alveolar macrophage ultrastructure. (A and B) Lung ultrastructure after exposure to phosphate-buffered saline (PBS) (A) or GMAb (B) for 321 days. Alveoli in GMAb-exposed primates have well-preserved walls (double arrows) and contain alveolar macrophages (arrows) that are enlarged and foamy. (Epon-embedded semithin lung sections stained with toluidine blue, original magnification $\times 100$). Bar = 10 μm . Electron micrographs of alveolar macrophages obtained by bronchoalveolar lavage (BAL) from primates exposed to PBS (C) or GMAb (D–H) for 112 days. Alveolar macrophages from GMAb-exposed primates had a characteristic, foamy appearance caused by numerous lamellar body (arrows) and lipid droplet inclusions (asterisks). Some alveolar macrophages were normal in size but filled nearly completely

with lamellar bodies (D), whereas others were enlarged and had varying numbers of lamellar bodies and lipid droplets (E and F) and some were very large and were filled completely with lipid droplets (G). Examination at higher magnification revealed that lamellar inclusions had a visible limiting membrane (arrows), whereas lipid droplets did not (asterisks). (H) (Epon-embedded thin sections stained with Uranyl acetate/lead citrate, original magnification $\times 7,000$). Bar = 2 μm (C–G) or 0.5 μm (H). The numbers of lamellar bodies (I) and lipid droplets (J) were significantly increased in alveolar macrophages after only 112 days of GMAb exposure compared with alveolar macrophages from the control primate. Compared with controls, the mean diameter of lipid droplets in alveolar macrophages was greater in GMAb-exposed primates (K). For each comparison (I–K), 100 cells were evaluated per primate ($P < 0.01$). (L) Number of inclusions (lamellar plus lipid) per cell versus cell size alveolar macrophages from the GMAb-exposed primate. Alveolar macrophage size was the area of the cell in pixels (divided by 10^6) determined using electronic digital photomicrograph files of 100 cells using Axiophot (Leica) software. Linear regression analysis was performed using SigmaPlot software.

GMAb did not change the relative fractional composition of surfactant phospholipids as measured by thin-layer chromatography (not shown). Compared with controls, GMAb-exposed primates had increased total protein (Figure 7E) and levels of SP-A, -B, -C, and -D in BAL fluid (Figure 7F). In summary, GMAb-exposure increased pulmonary surfactant pool size without significantly altering its composition.

A Critical Threshold of GMAb for Myeloid Cell Dysfunction in Blood and Lungs

Serum and leukocyte biomarkers of PAP were used to measure the critical threshold of GMAb in blood. GMAb reduced the CD11b stimulation index (Figure 8A) and phagocytic capacity of neutrophils in whole blood (Figure 8B), similar to results for patients with PAP (25). The CD11b stimulation index varied inversely with serum GMAb concentrations below 5 $\mu\text{g}/\text{ml}$ and was zero at higher values (Figure 8C), similar to results for the CD11b stimulation index and endogenous GMAb levels in healthy individuals and patients with PAP (15). These results indicated the critical threshold of GMAb is 5 $\mu\text{g}/\text{ml}$ in serum.

Biomarkers of PAP in serum and lung were used to estimate the critical threshold of GMAb in the lung ELF (measured by

the urea-dilution method [36]). Repeated measurements of GMAb simultaneously in serum and lung ELF demonstrated that lung levels were $10.2 \pm 2.1\%$ that of serum levels in passively immunized primates (Figure 8D) and that levels were negligible in controls (not shown). Based on the minimum serum GMAb concentration of 40 $\mu\text{g}/\text{ml}$ in immunized primates in Studies 6 and 7 and this value for the serum to lung ratio, the minimum GMAb concentration in lung ELF was calculated to be 3.8 to 4 $\mu\text{g}/\text{ml}$. Because both immunized primates in these studies developed PAP, the critical threshold of GMAb in lung ELF was estimated to be 4 $\mu\text{g}/\text{ml}$ or less (assuming development of PAP requires levels of GMAb to remain continuously above the critical threshold).

Because serum SP-D is elevated in proportion to lung disease severity in patients with PAP (7) and was elevated in GMAb-exposed primates compared with controls (Figure 8D), we used this biomarker to monitor for the development of PAP. Serum SP-D concentration increased progressively over time in GMAb-exposed primates and remained unchanged and within the normal range in control primates in both chronic GMAb administration studies (Figure 2). The earliest time at which serum SP-D was clearly increased above the normal range was after 2 to 3 months after initiating GMAb exposure. From a plot of time versus GMAb concentration (Figure 8F), the critical

Figure 6. Effects of granulocyte/macrophage colony-stimulating factor autoantibodies (GMAb) on alveolar macrophage function. Compared with controls, alveolar macrophages from GMAb-exposed primates had significantly decreased PU.1 (A) and PPAR γ (B) mRNA transcript levels, and secreted a reduced amount of tumor necrosis factor- α in response to lipopolysaccharide stimulation (C) ($n = 3$ determinations per condition; $P < 0.001$ for all comparisons with control). Results are for samples obtained in Study 6 on Day 225, and are similar to results on three or one other determinations in Study 6 and 7, respectively (A and B).

threshold of GMAb in lung ELF was estimated to be between 4 and 6 $\mu\text{g/ml}$. This result is consistent with pharmacokinetic data from clinical study 7, which showed that the GMAb concentration in lung ELF was never greater than 15 $\mu\text{g/ml}$, greater than 10 $\mu\text{g/ml}$ for only 7 days, and greater than 5 $\mu\text{g/ml}$ for 80 of 140 days (Figure 8F).

DISCUSSION

In this study, we found that prolonged exposure to a high level of highly purified, PAP patient-derived GMAb reproduced the typical pathologic manifestations of PAP in healthy, nonhuman primates, including the disruption of GM-CSF signaling *in vivo* in blood leukocytes and alveolar macrophages; reduced PU.1 and PPAR γ mRNA levels and impaired proinflammatory cytokine signaling in alveolar macrophages; surfactant accumulation in alveolar macrophages and alveoli; a progressive increase in serum SP-D concentration; pulmonary leukocytosis; and reduced neutrophil function. These results provide strong

Figure 7. Effects of granulocyte/macrophage colony-stimulating factor autoantibodies (GMAb) on pulmonary surfactant homeostasis. Primates were those described in the legend to Figure 2 and bronchoalveolar lavage (BAL) specimens ($n = 16$, +GMAb; $n = 15$, -GMAb) were obtained as illustrated. (A) Gross appearance of BAL fluid. Specimens were from Study 6, Day 308, similar to results on five other occasions. (B) Turbidity of the BAL fluid from GMAb-exposed ($n = 16$) and control ($n = 15$) primates. (C) Total phospholipid concentration in BAL fluid ($n = 10$ each). (D) Saturated phosphatidylcholine concentration in BAL fluid ($n = 10$ each). (E) Total protein in BAL fluid ($n = 6$ samples per group; $P = 0.088$). (F) Surfactant protein-A, -B, -C, and -D levels in BAL fluid relative to respective mean values in controls. Bars represent the mean of triplicate (GMAb-exposed) or duplicate (control) determinations. Double asterisks indicate P value < 0.001 for the comparison of results from GMAb-exposed (black bars) and phosphate-buffered saline-exposed (gray bars) primates.

evidence that GMAb directly cause the pathogenesis of idiopathic PAP.

Implications for the Biologic Role of GM-CSF and Pathogenesis of Idiopathic PAP

We propose a mechanism in which GMAb reduce GM-CSF bioactivity *in vivo* in rheostatic fashion at concentrations below a critical threshold level and completely block it at higher levels thereby impairing GM-CSF-dependent myeloid cell functions (Figure 9). In GMAb-injected healthy primates, GMAb impaired alveolar macrophage surfactant clearance, PU.1 and PPAR γ expression, and TLR4 signaling. These observations are similar to results in patients with idiopathic PAP (15, 17, 38) or PAP caused by GM-CSF receptor dysfunction caused by *CSF2RA* mutations (6), and mice deficient in GM-CSF (20) or its receptor (11). In each, interruption of GM-CSF signaling causes PAP and similar morphologic changes and functional impairment in alveolar macrophages. Although the exact mechanism by which GMAb impair surfactant clearance by alveolar macrophages was not identified in our study, several lines of evidence suggest that the reduction of PU.1 in alveolar macrophages is critical to the pathogenesis of PAP. In mice,

Figure 8. Measurement of the critical threshold of granulocyte/macrophage colony-stimulating factor autoantibodies (GMAb) in blood and lung. (A) CD11b stimulation index. Specimens ($n = 57$) were from Studies 6 and 7. (B) Phagocytic capacity (PC) of neutrophils in whole blood. Specimens ($n = 7$, evaluated in triplicate) were from Study 6 on Days 210, 238, 245, and 252, and from Study 7 on Days 43, 57, and 63. (C) Effect of serum GMAb concentration on the CD11b stimulation index in passively immunized primates. At concentrations of GMAb above the putative critical threshold (solid line), the CD11b stimulation index was reduced to zero (dashed line). Specimens are from Study 3 (single 14.7-mg GMAb dose; open circle; $n = 8$ determinations); Studies 4 and 5 (multiple 20-mg GMAb doses; triangle up, triangle down; $n = 6$ and 15 determinations, respectively); and Studies 6 and 7 (multiple 50-mg or 20-mg GMAb doses with B-cell depletion; open diamond, open square; $n = 52$ and 39 determinations, respectively). (D) GMAb in serum and lung (ELF) from GMAb-exposed primates. Specimens ($n = 12$) were from Study 6, Days 125, 225, and 308; Study 7, Day 99. (E) Serum surfactant protein-D concentration. Specimens ($n = 65$) were from Studies 6 and 7. (F) Number of days that the GMAb concentration in lung epithelial lining fluid (ELF) was maintained above specified levels in Studies 6 (open diamond) and 7 (open square).

pulmonary GM-CSF is required for PU.1 expression (20) and surfactant catabolism (12) in alveolar macrophages. Importantly, forced expression of PU.1 in alveolar macrophages from GM-CSF-deficient mice restored surfactant catabolism (and

other functions) (20). In humans, GM-CSF is also required for PU.1 expression in alveolar macrophages (17). PU.1 levels and activity are reduced in alveolar macrophages in patients with idiopathic PAP and are restored by GM-CSF therapy (17). We propose that pulmonary GM-CSF is required to stimulate the terminal differentiation of alveolar macrophages in humans based on observations that GM-CSF, by PU.1, stimulates the terminal differentiation of alveolar macrophages in mice (20), and a similar pattern of macrophage abnormalities is seen in patients with idiopathic PAP (14, 15, 17), patients with hereditary PAP (6, 39), GM-CSF-deficient mice (16, 18–20), GM-CSF receptor-deficient mice (11, 40), and GMAb-injected nonhuman primates (Figures 3–6). We further propose that GMAb cause PAP by blocking pulmonary GM-CSF signaling *in vivo*, thereby reducing PU.1 levels in alveolar macrophages impairing surfactant catabolism in alveolar macrophages. The presence of small alveolar macrophages engorged with only lamellar bodies and large cells containing only intracytoplasmic lipid droplets staining with oil red O (indicating neutral lipids), and the lack of any increase in neutral lipids in BAL fluid is consistent with the conclusion that alveolar macrophages internalize lamellar bodies that are broken down to neutral lipids, which cannot be further processed or properly excreted. Further studies are required to confirm these observations and determine the precise defects in surfactant lipid metabolism caused by the loss of GM-CSF stimulation.

Our observation that neutrophil functions were impaired in GMAb-injected primates is consistent with results for patients with PAP and GM-CSF-deficient mice, in which neutrophils have functional reductions in phagocytosis, respiratory burst, cellular adhesion, and bacterial killing (25). Further, purified GMAb rheostatically reduced GM-CSF-stimulated functions of neutrophils from healthy human individuals in *ex vivo* studies (25). In contrast to alveolar macrophages, however, neither PU.1 levels nor any markers of terminal differentiation were altered in neutrophils from human patients with PAP or GM-CSF-deficient mice, indicating that GM-CSF is not required for the terminal differentiation of blood neutrophils (25).

In summary, the striking similarity pattern of differential impairment of myeloid cell functions in mice, humans, and monkeys caused by interruption of GM-CSF signaling (9, 10, 15, 25, 41) suggests that GM-CSF may regulate myeloid cell functions in all three species by common mechanisms. These results and published reports (3, 22, 24, 25, 29, 42) suggest the term “autoimmune PAP” may now be used instead of “idiopathic PAP” in reference to the common clinical form of PAP associated with GMAb.

Clinical Implications for the Diagnosis, Natural History, and Monitoring of PAP

The conclusion that GMAb are central to the pathogenesis of PAP supports the value of an increased serum level of GMAb in establishing a diagnosis of PAP. This is further supported by a report that the both the sensitivity and specificity of the currently available blood test (15, 23) approach 100% (41). Notwithstanding, it will be important to establish universally accepted methods, calibration standards, and normal and abnormal ranges in certified laboratories to enable routine clinical use.

The progressive increase in serum SP-D concentration caused by exposure to GMAb suggests this simple, noninvasive blood test may be useful in monitoring lung disease severity in patients with PAP. In support of this are results from patients with autoimmune PAP (7), hereditary PAP (6), and mice with disruption of GM-CSF signaling (6), in each, the serum SP-D is increased. Because serum SP levels are increased in other lung

Figure 9. Proposed mechanism by which granulocyte/macrophage colony-stimulating factor (GM-CSF) autoantibodies cause pulmonary alveolar proteinosis. High levels of GM-CSF autoantibodies (GMAb) block GM-CSF signaling to alveolar macrophages and their precursors, thereby arresting terminal differentiation (*dashed line*) and impairing surfactant catabolism (*open arrow*) and host defense functions (*dotted line*) of alveolar macrophages. Surfactant homeostasis is disrupted by the slow, progressive accumulation of surfactant, resulting in eventual respiratory dysfunction of insidious onset with impaired oxygen diffusion, hypoxemia, and dyspnea. Loss of GM-CSF signaling does not alter basal neutrophil blood counts, cellular morphology, or expression of PU.1 and multiple differentiation markers. GMAb do reduce the capacity of some neutrophil functions in rheostatic fashion (*dotted line*) [25] contributing to an increased risk of infection but without affecting neutrophil differentiation. GM-CSF also blocks the homeostatic expansion of B lymphocytes after acute B lymphopenia (not shown).

disorders, an increased serum SP-D level that is nonspecific is of limited use in the diagnosis of PAP.

The observation that the critical threshold GMAb concentration associated with loss of GM-CSF signaling was similar in the blood and lung ELF suggests a clinical outcome measure (e.g., serum GMAb concentration) and target value (4–6 $\mu\text{g}/\text{ml}$). Although GMAb levels do not correlate with PAP disease severity, a high level of GMAb is diagnostic of PAP (41). Thus, our data predict that a decrease in serum GMAb concentration below the critical threshold may be associated with resolution of PAP. This may be useful for predicting clinical responses to therapies that target reduction in GMAb levels (i.e., plasmapheresis [30, 43] or rituximab-mediated depletion of antibody-producing B lymphocytes [44]). Further, these results predict that spontaneous resolution of PAP may be associated with a fall in GMAb levels. Because the critical threshold will vary with the IC_{50} and GMAb are polyclonal, it will be important to determine if this value varies significantly among patients with PAP or among patients with differing degrees of disease severity.

Our results inform the natural history of PAP at disease onset, which is poorly studied because patients with PAP usually present late. Our conclusion that GMAb reproduced early manifestations of PAP in nonhuman primates is supported by the presence of neutrophil and alveolar macrophage abnormalities; a mild (greater than twofold) increase in surfactant pool size (in contrast to greater than eightfold increases in patients with advanced PAP [45]); a small increase in serum SP-D; and the absence of radiologic, behavioral, or symptomatic manifestations. We propose the following model (and timing) of events in the initiation and progression of autoimmune PAP: an increase in GMAb (disease initiation) $\rightarrow$ loss of GM-CSF signaling (seconds) $\rightarrow$ altered GM-CSF transcriptome (minutes) $\rightarrow$ altered macrophage biochemistry (i.e., decreased surfactant catabolism) (hours) $\rightarrow$ surfactant accumulation (days) $\rightarrow$ radiologic manifestations (months to years) $\rightarrow$ symptoms (years). Several lines of evidence support this model. First, GMAb disrupted GM-CSF signaling in myeloid cells immediately and at similar concentrations in the blood and lungs. Second, purified GMAb instantly blocked the binding of GM-CSF to cognate receptors (22), GM-CSF signaling, and functions of normal neutrophils *ex vivo* (25), and disrupted GM-CSF-stimulated proliferation of monocytes in short-term culture (46). Third, the delay preceding an increase in

serum SP-D levels suggests that disruption of surfactant homeostasis at the biochemical and cellular levels is delayed. Fourth, surfactant pool size seems to increase progressively over time in GM-CSF-deficient mice (9, 12). Fifth, because 31% of autoimmune patients with PAP with significant radiologic manifestations were asymptomatic, radiologic manifestations occur before the development of symptoms (7). Sixth, in two children with hereditary PAP caused by *CSF2RA* mutations resulting in loss of GM-CSF signaling from birth, one became symptomatic at 4 years old and the other was asymptomatic and had mild disease (i.e., mild radiologic changes and a moderately reduced diffusing capacity of carbon monoxide) at age 8 years (6). These two results indicate that factors in addition to loss of GM-CSF signaling are important in determining lung disease severity in PAP.

Pharmacokinetic and Pharmacodynamic Implications for the Potential Therapeutic Use of GMAb to Treat Other Diseases

GMAb reduced $\text{TNF-}\alpha$ release by endotoxin-stimulated macrophages and basal neutrophil functions indicating that GM-CSF regulates the inflammatory responses of myeloid cells *in vivo*. This is supported by observations in GM-CSF-deficient mice (16, 47), GM-CSF receptor-deficient mice (11, 40), wild-type mice injected with antimurine GM-CSF antibodies (48), patients with autoimmune PAP (15), and patients with hereditary PAP (6). In each, disruption of GM-CSF signaling, regardless of the mechanism involved, similarly reduced macrophage-mediated proinflammatory cytokine signaling or neutrophil reactivity. Importantly, observations potentially implicating GM-CSF in the pathogenesis of rheumatoid arthritis (49) and multiple sclerosis (50) have led to interest in the potential use of neutralizing antihuman GM-CSF antibodies to treat serious inflammatory and autoimmune diseases (51). Our results suggest that PAP may potentially complicate such therapies depending on GM-CSF antibody affinity, dosing, and administration. The minimum concentration of GMAb in lung ELF associated with PAP was approximately 4 $\mu\text{g}/\text{ml}$, thus providing an estimate of the concentration required to disrupt surfactant homeostasis. The level required to block GM-CSF signaling in lung macrophages and blood leukocytes was similar, agrees well with prior *ex vivo* human studies (25), and is close to the endogenous levels of GMAb associated with active lung disease in patients with PAP (15). Complete blockade of GM-

CSF signaling for as little as 2 to 3 months may result in initiation of PAP. Thus, our results help to define the therapeutic window for the use of GM-CSF autoantibodies to treat other diseases (15). The potential occurrence of iatrogenic PAP will likely be reversible because GM-CSF administration is therapeutic in patients with autoimmune PAP (52) and reverses the PAP phenotype in GM-CSF-deficient mice (53). Several biomarkers may be useful in monitoring for the development and resolution of PAP in this clinical setting, including the level of GMAb and SP-D in serum, and the CD11b stimulation index and STAT5 phosphorylation in blood leukocytes.

An interesting observation was the unexpected increase in the CD11b stimulation index in the early preliminary studies, which provided a clue about the trace endotoxin contamination of GMAb. The use of endotoxin-free GMAb in subsequent clinical studies resulted in the expected reduction in the CD11b stimulation index (15, 25). Notwithstanding, the rapid decline in serum half-life of GMAb indicated human immunoglobulins (*i.e.*, GMAb) elicited a xenographic immune response in *Macaca fascicularis*. We confirmed this by conducted clinical studies without or with B-lymphocyte depletion using rituximab and cyclophosphamide. This drug combination had been shown to be safe and induce beneficial immunomodulatory effects in prior reports of adoptive immunotherapy (54, 55). Although considered a limitation of our study, B-lymphocyte depletion had no effect on GM-CSF signaling in blood neutrophils or alveolar macrophages (Figure 1B), surfactant clearance by alveolar macrophages (Figures 3–5), or surfactant homeostasis (Figure 7).

GMAb blocked the homeostatic expansion B lymphocytes following pharmacologically induced B lymphocytopenia. Because homeostatic lymphocyte expansion (56) and GM-CSF (51) are both important in autoimmune disorders, this observation suggests GMAb may be useful in immunotherapeutic strategies targeting B lymphocytes. Because immune globulin is effective as therapy of autoimmune and systemic inflammatory disorders (57) and it consistently contains GM-CSF autoantibodies as the dominant anticytokine activity (26), our observation suggests the mechanism of action of immune globulin therapy of autoimmune diseases may involve disruption of GM-CSF signaling. An effective dose of immune globulin (2 g/kg body weight) would result in serum GMAb levels of 0.5 to 1 µg/ml (15), which is within the range associated with negative regulation of myeloid cell functions in primates (Figure 8C) and healthy humans (15). Interestingly, in renal transplant candidates, immune globulin and rituximab reduced anti-HLA antibody levels, the wait-time before transplantation (from 144 ± 89 to 5 ± 6 mo), and increased the number of patients transplanted (58). Further studies to evaluate this potential therapeutic role of GMAb are needed.

Conflict of Interest Statement: T.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. D.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.U. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.C.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.N. received more than \$100,001 from the Ministry of Health, Labor, and Welfare of Japan, more than \$100,001 from the Japanese Society for the Promotion of Science, and more than \$100,001 from the Ministry of Education, Culture, Sports, Science, and Technology of Japan in sponsored grants. G.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.E.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.E.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.I. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.A.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.L. received \$1,001–

\$5,000 from Talecris Biotherapeutics in advisory board fees and more than \$100,001 from Talecris Biotherapeutics in industry-sponsored grants. S.D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.P.K. received more than \$100,001 from Genzyme in industry-sponsored grants in clinical trial to the institution and more than \$100,001 from the National Institutes of Health in multiple grants. A.B. received \$10,001–\$50,000 from Bioclinica in consultancy fees for CT scan reading for research trial, up to \$1,000 from PTC Therapeutics for speaking at study initiation meeting, \$5,001–\$10,000 from PTC Therapeutics in sponsored grants, \$10,001–\$50,000 from the National Institutes of Health in sponsored grants, and \$50,001–\$100,000 from the Cystic Fibrosis Foundation in sponsored grants. F.R. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.C.T. serves as a consultant to Boehringer Ingelheim (no money is paid to B.C.T., time is compensated by payment to Cincinnati Children's Hospital Medical Center), \$1,001–\$5,000 from MorphoSys in consultancy fees as a lung disease specialist for trial design, and \$1,001–\$5,000 from MedImmune in consultancy fees as a lung disease specialist for trial design, \$1,001–\$5,000 from Lilly as a board member (product discontinued, board disbanded), and \$10,001–\$50,000 from the Alpha 1 Foundation as a grant to support the role of scientific director.

Acknowledgment: This work was supported in part by grants from the National Heart, Lung and Blood Institute (HL0085453 to B.C.T. and HL085610 to J.A.W.), and from the National Center for Research Resources and the National Institutes of Health Office of Rare Diseases (RR019498 to B.C.T.). We are indebted to Diane Black, Claudia Chalk, Paula Blair, Dave Loudy, and Gail Macke for technical support; to Jasmine Hales and Jefferson Childs for help with care and management of the primates; and Drs. Christopher Karp, Division of Immunobiology, Cincinnati Children's Hospital Research Foundation, Lisa Young, Division of Pulmonary Medicine, Cincinnati Children's Hospital, and Frank McCormack, Division of Pulmonary, Critical Care and Sleep Medicine, University of Cincinnati Medical Center, for critical reading of the manuscript.

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