

ORIGINAL ARTICLE

# GM-CSF Autoantibodies and Neutrophil Dysfunction in Pulmonary Alveolar Proteinosis

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## ABSTRACT

### BACKGROUND

Increased mortality from infection in patients with pulmonary alveolar proteinosis occurs in association with high levels of autoantibodies against granulocyte–macrophage colony-stimulating factor (GM-CSF). We tested the hypothesis that neutrophil functions are impaired in patients with pulmonary alveolar proteinosis and that GM-CSF autoantibodies cause the dysfunction.

### METHODS

We studied 12 subjects with pulmonary alveolar proteinosis, 61 healthy control subjects, and 12 control subjects with either cystic fibrosis or end-stage liver disease. We also studied GM-CSF<sup>-/-</sup> mice and wild-type mice. We evaluated basal neutrophil functions, neutrophil functions after priming by GM-CSF to augment antimicrobial functions, and the effects of highly purified GM-CSF autoantibodies on neutrophil functions in vitro and in vivo.

### RESULTS

Neutrophils from subjects with pulmonary alveolar proteinosis had normal ultrastructure and differentiation markers but impaired basal functions and antimicrobial functions after GM-CSF priming. GM-CSF<sup>-/-</sup> mice also had reduced basal neutrophil functions, but functions after GM-CSF priming were unimpaired. The neutrophil dysfunction characteristic of pulmonary alveolar proteinosis was reproduced in a dose-dependent fashion in blood specimens from healthy control subjects after incubation with affinity-purified GM-CSF autoantibodies isolated from patients with pulmonary alveolar proteinosis. The injection of mouse GM-CSF antibodies into wild-type mice also caused neutrophil dysfunction.

### CONCLUSIONS

The antimicrobial functions of neutrophils are impaired in patients with pulmonary alveolar proteinosis, owing to the presence of GM-CSF autoantibodies. The effects of these autoantibodies show that GM-CSF is an essential regulator of neutrophil functions.

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**P**ULMONARY ALVEOLAR PROTEINOSIS<sup>1</sup> IS A rare disorder in which surfactant accumulates within pulmonary alveoli, causing respiratory insufficiency.<sup>2,3</sup> The disease is specifically associated with high levels of autoantibodies against granulocyte-macrophage colony-stimulating factor (GM-CSF) in blood and tissues, including pulmonary alveoli.<sup>4</sup> These autoantibodies neutralize the biologic activity of GM-CSF.<sup>5</sup> In mice, GM-CSF stimulates the terminal differentiation of alveolar macrophages, primarily through the action of the transcription factor PU.1.<sup>6</sup> The homozygous deletion of GM-CSF genes causes pulmonary alveolar proteinosis in mice<sup>7,8</sup> by impairing the clearance of pulmonary surfactant by alveolar macrophages that are dependent on GM-CSF.<sup>9</sup> In patients with pulmonary alveolar proteinosis, PU.1 levels are reduced in alveolar macrophages, but levels increase in response to GM-CSF therapy.<sup>10</sup> There is evidence that the regulation of alveolar macrophages by GM-CSF is similar in humans and mice.<sup>3,11</sup>

Infections, which are caused predominantly by opportunistic pathogens, account for 18% of reported deaths attributable to pulmonary alveolar proteinosis.<sup>2</sup> Some of these infections occur at extrapulmonary sites, an indication that the predisposition to infection is systemic, rather than confined to the lungs.<sup>2</sup> Similarly, in GM-CSF<sup>-/-</sup> mice, mortality from infection and susceptibility to bacterial, fungal, and mycobacterial pathogens are increased.<sup>11-13</sup> These findings suggest that pulmonary alveolar proteinosis is characterized by defective immune function, especially because GM-CSF is important during infection.<sup>14</sup>

GM-CSF augments the antimicrobial functions of neutrophils by means of a mechanism known as priming.<sup>15,16</sup> GM-CSF priming increases levels of the adhesion molecule CD11b, which promotes the adhesion of neutrophils to the vascular endothelium, a critical event in the recruitment of neutrophils into infected tissues.<sup>17</sup> GM-CSF also primes phagocytosis, oxidative burst, and bactericidal activity in neutrophils. Since GM-CSF autoantibodies may impair these functions, we studied neutrophils from patients with pulmonary alveolar proteinosis and from GM-CSF<sup>-/-</sup> mice. We also evaluated the functions of normal human or mouse neutrophils after incubation with highly purified GM-CSF autoantibodies.

## METHODS

### PARTICIPANTS

The institutional review board of the Cincinnati Children's Hospital Medical Center approved the study. All participants or their legal guardians gave written informed consent, and minors gave assent. Between July 24, 2004, and June 9, 2006, all 12 subjects with pulmonary alveolar proteinosis who were referred to our center for evaluation or treatment were enrolled in the study. We also studied 61 healthy control subjects, 6 control subjects with cystic fibrosis, and 6 control subjects with end-stage liver disease. (The case histories of the subjects with pulmonary alveolar proteinosis are given in the Supplementary Appendix, available with the full text of this article at [www.nejm.org](http://www.nejm.org).) GM-CSF autoantibodies were quantified in serum with the use of an enzyme-linked immunosorbent assay (ELISA), as previously described.<sup>5</sup> Granulocyte colony-stimulating factor (G-CSF) was quantified in serum by means of an ELISA (Quantikine Kit, R&D Systems).

### HUMAN NEUTROPHILS

The isolation of human neutrophils in blood on Ficoll (MP Biomedicals) gradients was initiated within 1 hour after phlebotomy. After the lysis of red cells, neutrophils were resuspended in phosphate-buffered saline containing 10 mM D-glucose.<sup>18</sup> Mean ( $\pm$ SE) viability, determined immediately after isolation by means of trypan-blue staining, was  $97.5 \pm 1.2\%$  in neutrophils from subjects with pulmonary alveolar proteinosis and  $97.9 \pm 0.6\%$  in neutrophils from healthy control subjects. In some experiments, neutrophils were isolated for evaluation with the use of Mono-Poly resolving medium (MP Biomedicals), washed in phosphate-buffered saline (without hypotonic red-cell lysis), and resuspended in Hank's balanced salt solution, 20% fetal bovine serum, and 25 mM HEPES buffer (pH 7.4); these neutrophils are referred to as washed neutrophils. Cell-surface markers of apoptosis were assessed by flow cytometry (Apoptosis Detection Kit I, Pharmingen).

Neutrophil differentiation was evaluated with the use of cell-surface differentiation markers and flow cytometry.<sup>6,19</sup> We measured PU.1 messenger RNA levels in neutrophils using reverse transcription and quantitative polymerase-chain-reaction

amplification<sup>6</sup> and evaluated PU.1 protein using Western-blot analysis.<sup>6</sup> Neutrophil ultrastructure was evaluated in 20 neutrophils from each of two subjects with pulmonary alveolar proteinosis (Patients 6 and 7) and from each of two healthy control subjects.<sup>20</sup>

The basal capacity of isolated neutrophils to phagocytose opsonized fluorescent microspheres was evaluated as previously described for macrophages,<sup>21</sup> except that internalization of the microspheres was quantified with the use of confocal microscopy at a magnification of 63 $\times$ .<sup>19</sup> The phagocytic index was calculated as the percentage of neutrophils that had phagocytosed microspheres multiplied by the number of microspheres per cell. For each human (or mouse) studied, 400 neutrophils were evaluated.

Phagocytosis by neutrophils in whole blood (hereafter called the phagocytic capacity) was measured by flow cytometry<sup>21</sup> to eliminate the potential influence of reduced adhesion. Triplicate samples of heparinized human blood (200  $\mu$ l) were incubated (at 37°C for 60 minutes) with IgG-opsonized fluorescent microspheres (7.5 $\times$ 10<sup>6</sup>, prepared as previously described<sup>21</sup>) in capped, siliconized microcentrifuge tubes with gentle orbital rotation (Thermomixer, Eppendorf). After red-cell lysis, flow cytometry was performed to evaluate neutrophils. Phagocytic capacity was calculated as the percentage of neutrophils containing internalized microspheres multiplied by the mean fluorescence intensity of phagocytic neutrophils (both determined with the use of flow cytometry) and multiplied by the neutrophil count in the blood.

Cellular adhesion was evaluated by seeding isolated neutrophils into low-adhesion plastic dishes (Corning, catalog no. 3471). After 2 hours, the plates were washed twice with phosphate-buffered saline, and adherent cells were counted with the use of a microscope.<sup>6</sup> The production of hydrogen peroxide was measured in neutrophils in whole blood as previously described.<sup>22</sup> All data for the basal phagocytic index, phagocytic capacity, oxidative burst, and cellular adhesion were normalized by dividing by the mean value for the healthy control group and multiplying by 100.

The amount of *Staphylococcus aureus* killed (American Type Culture Collection no. 49476) in 1 hour by neutrophils in whole blood or by isolated neutrophils was determined as previously

described,<sup>18,23</sup> except that interferon- $\gamma$  and lyso-staphin, respectively, were omitted.

We studied neutrophil functions after GM-CSF priming by incubating heparinized whole blood for 30 minutes in the absence or presence of 10 ng of human GM-CSF per milliliter (Leukine, Berlex) or 10 ng of mouse GM-CSF per milliliter (R&D Systems). The increase in CD11b levels on neutrophils after GM-CSF priming (CD11b stimulation index) was calculated as the mean fluorescence intensity of CD11b on neutrophils primed by GM-CSF minus that of CD11b on nonprimed neutrophils, divided by the mean fluorescence intensity of nonprimed neutrophils and multiplied by 100. Phagocytic capacity after GM-CSF priming was calculated as the phagocytic capacity of neutrophils primed by GM-CSF minus that of nonprimed neutrophils.

#### MOUSE NEUTROPHILS

Experiments in mice were conducted according to protocols approved by the local institutional animal care and use committee. GM-CSF<sup>-/-</sup> mice were backcrossed for more than 10 generations into C57BL/6J mice, which served as the wild-type control mice. Mouse neutrophils were isolated and evaluated as described for human neutrophils, except as follows. Neutrophils were isolated from bone marrow on Percoll (Amersham) gradients<sup>24</sup>; more than 90% of the isolated cells were neutrophils, and more than 95% were viable. Phagocytic capacity was determined with the use of 100  $\mu$ l of blood, 30-minute periods of incubation, and immunostaining of neutrophils with Ly6G.

#### EFFECTS OF GM-CSF ANTIBODY

We made two GM-CSF autoantibody preparations using GM-CSF affinity chromatography as previously described<sup>5</sup>: one from a single subject with pulmonary alveolar proteinosis (Patient 6) and one from serum pooled from 11 subjects with the disease (purified GM-CSF autoantibodies and purified pooled GM-CSF autoantibodies, respectively). Affinity-purified autoantibodies were concentrated with the use of ultrafiltration (Microcon 30 K, Amicon), resuspended in phosphate-buffered saline, and assessed for purity by means of electrophoresis on polyacrylamide gels. We measured the ability of GM-CSF autoantibodies to neutralize GM-CSF, using TF1 cells as previously described.<sup>5</sup>

For *in vitro* studies, purified GM-CSF autoantibody or human immune globulin (Gammagard, Baxter) was incubated with heparinized blood or washed neutrophils (0.5  $\mu\text{g}$  per milliliter, except as noted) before CD11b levels or phagocytic capacity was assessed. Since human GM-CSF and mouse GM-CSF are not immunologically cross-reactive, for *in vivo* studies, monoclonal anti-mouse GM-CSF antibody (22E9, Endogen) or isotype-control antibody (rat IgG2a, Pharmingen; 200  $\mu\text{g}$  per mouse) was injected intraperitoneally into C57BL/6J mice (five in each group). The phagocytic capacity of neutrophils was assessed 3 days after injection.

#### STATISTICAL ANALYSIS

We evaluated the numerical data for a normal distribution using the Kolmogorov–Smirnov test and for equal variance using the Levene median test; parametric data are presented as means ( $\pm$ SE) and nonparametric data are presented as medians and interquartile ranges. Statistical comparisons of parametric data were made with Student's *t*-test for two-group comparisons and with one-way analysis of variance with post hoc analysis according to the Holm–Sidak method for multiple-group comparisons. Nonparametric data were compared with the use of the Mann–Whitney rank-sum test. *P* values of less than 0.05 were considered to indicate statistical significance. All experiments were repeated at least twice, with similar results.

## RESULTS

#### NEUTROPHIL COUNTS AND DIFFERENTIATION MARKERS

Neutrophil counts in blood and G-CSF levels in serum were normal in the 12 subjects with pulmonary alveolar proteinosis (Table 1). Neutrophils from subjects with pulmonary alveolar proteinosis and those from healthy control subjects had similar ultrastructure (Fig. 1A), cell-surface differentiation markers (Table 2), and levels of PU.1 expression (data not shown). Differentiation markers on neutrophils from GM-CSF<sup>-/-</sup> and wild-type mice were also similar (Table 2).

#### NEUTROPHIL FUNCTION

##### *Patients*

The basal phagocytic functions of isolated neutrophils, evaluated with the use of confocal micros-

copy, were reduced in subjects with pulmonary alveolar proteinosis as compared with healthy control subjects (Fig. 1B and Table 2). In whole blood, the percentage of phagocytic neutrophils and the number of phagocytosed microspheres per neutrophil were also reduced in subjects with pulmonary alveolar proteinosis (Fig. 1C and Table 2). Basal cellular adhesion, oxidative burst, and bactericidal activity were reduced in subjects with pulmonary alveolar proteinosis as compared with healthy control subjects (Table 2).

The phagocytic capacity of washed neutrophils incubated in Hank's balanced salt solution without added GM-CSF declined during a 3-hour period (100.0 $\pm$ 0.6 at 0 minutes, 92.3 $\pm$ 0.3 at 90 minutes, and 71.8 $\pm$ 5.0 at 180 minutes; 3 determinations per time point) (90 minutes vs. 0 minutes, *P*=0.04; 180 minutes vs. 0 minutes, *P*<0.001). The decline was not due to apoptosis, which occurred in less than 2% of neutrophils at each time point. In Patient 6, who had pulmonary alveolar proteinosis and received GM-CSF therapy for 13 weeks, the basal phagocytic capacity of neutrophils (normalized to the capacity in 20 healthy control subjects) improved from 44.1 $\pm$ 2.2% before therapy to 104.5 $\pm$ 1.9% after therapy. Although we studied only three children with pulmonary alveolar proteinosis (Patients 2, 3, and 5), their pattern of neutrophil impairment was similar to that of the adult subjects.

GM-CSF priming *in vitro* increased CD11b levels on neutrophils in the blood of healthy control subjects to maximum levels at low levels of GM-CSF (Fig. 1E and Table 2). In contrast, GM-CSF priming of CD11b levels on neutrophils from subjects with pulmonary alveolar proteinosis was severely impaired, with levels increasing by only modest amounts at high GM-CSF levels. The phagocytic capacity of neutrophils after GM-CSF priming was also severely impaired in the subjects with pulmonary alveolar proteinosis (Fig. 1F and Table 2).

We also examined basal and GM-CSF-primed neutrophil functions in control subjects with cystic fibrosis or end-stage liver disease, neither of which is associated with GM-CSF autoantibodies. The basal phagocytic capacity and GM-CSF priming of CD11b levels of neutrophils was normal in both disorders (Table 3). Thus, neutrophil dysfunction is not a characteristic feature of these chronic diseases.

**Table 1.** Clinical Characteristics of Subjects with Pulmonary Alveolar Proteinosis.\*

Patient No.	Sex	Smoking Status†	Presentation	Microbial Isolate‡	Age at Diagnosis yr	Age at Enrollment yr	Duration of Symptoms mo	Serum GM-CSF Autoantibody§ µg/ml	Serum G-CSF¶ ng/ml	Neutrophils in Blood   cells/mm <sup>3</sup>
1	M	Former smoker	Brain abscess, lung infiltrates	<i>Staphylococcus epidermidis</i>	37	41	46	227	0.0	2688
2	M	Never smoked	Pneumonia, lung abscess	<i>Haemophilus influenzae</i>	12	12	20	482	4.2	1039
3	F	Never smoked	Pneumonia	Influenza B	14	15	21	86	15.4	2968
4	F	Never smoked	Dyspnea	None identified	25	25	18	289	16.2	5184
5	F	Never smoked	Dyspnea	None identified	15	15	27	39	3.3	3564
6	M	Former smoker	Pneumonia	None identified	32	36	42	676	0.0	3396
7	M	Never smoked	Dyspnea	MAC	52	52	13	306	12.8	1686
8	F	Current smoker	Dyspnea	None identified	39	39	35	760	36.1	3909
9	F	Current smoker	Asymptomatic, lung infiltrates	<i>Nocardia asteroides</i>	39	43	77	297	0.0	2074
10	M	Never smoked	Pneumonia	<i>N. asteroides</i> , MAC	43	48	69	159	0.0	2744
11	M	Former smoker	Pneumonia	MAC	65	67	91	451	0.0	3872
12	F	Former smoker	Pneumonia	<i>Mycobacterium tuberculosis</i>	58	60	29	118	0.0	3434

\* Details of the case histories are provided in the Supplementary Appendix.

† Former smokers had discontinued smoking before evaluation; current smokers were smoking at the time of evaluation.

‡ Pathogens were isolated from a culture of cerebrospinal fluid (Patient 1), lung-lavage fluid (Patients 2, 3, 7, 10, 11, and 12), or chest-wall–biopsy specimen (Patient 9). MAC denotes *Mycobacterium avium* complex.

§ The upper limit of the confidence interval (99th percentile) for the serum GM-CSF autoantibody level in 61 healthy control subjects was 3.52 µg per milliliter.

¶ The median serum G-CSF level in 59 healthy control subjects was 3.8 (interquartile range, 0.0 to 16.8), which did not differ significantly from that in the subjects with pulmonary alveolar proteinosis (P=0.48).

|| The mean (±SE) neutrophil count in blood specimens from 61 healthy control subjects was 3876±221, which did not differ significantly from that in the subjects with pulmonary alveolar proteinosis (P=0.11).

*GM-CSF<sup>-/-</sup> Mice*

Basal neutrophil functions were reduced in GM-CSF<sup>-/-</sup> mice in a pattern similar to that among subjects with pulmonary alveolar proteinosis. Phagocytosis was reduced the most, followed by adhesion, oxidative burst, and bactericidal activity ( $r^2=0.94$ ,  $P=0.03$ ) (Fig. 1D and Table 2).

In contrast to basal functions, GM-CSF-primed neutrophil functions — including CD11b levels and phagocytic capacity — were not impaired in GM-CSF<sup>-/-</sup> mice (Table 2). This finding contrasts with that in subjects with pulmonary alveolar proteinosis in the absence of GM-CSF priming, but the mechanism underlying the disruption of GM-CSF functions differs: in GM-CSF<sup>-/-</sup> mice, it is the absence of GM-CSF production; in humans, it is the high levels of neutralizing GM-CSF autoantibodies.<sup>5</sup>

*Effect of GM-CSF Autoantibodies*

Affinity-purified GM-CSF autoantibodies produced a single band similar in size to purified human IgG on polyacrylamide gels under nonreducing conditions and two bands corresponding to the expected sizes of immunoglobulin heavy and light chains under reducing conditions (Fig. 2A). The amounts of autoantibodies required to inhibit the activity of GM-CSF by 50% were similar with purified GM-CSF autoantibodies from a single patient and with purified pooled GM-CSF autoantibodies (10.3 and 10.6 mol of IgG per mole of GM-CSF, respectively); these values were also similar to previously reported values.<sup>5</sup> In vitro incubation of the GM-CSF autoantibodies with whole blood from healthy control subjects or from wild-type mice blocked GM-CSF priming of CD11b levels on human neutrophils but not mouse neutrophils (Fig. 2B). Phagocytic capacity primed by GM-CSF was also reduced in a dose-dependent fashion by the presence of GM-CSF autoantibodies (Fig. 2C). A low GM-CSF level (10 pg per milliliter) maintained normal neutrophil phagocytic capacity at 90 minutes as compared with 0 minutes ( $P=0.11$ ) (Fig. 2D). The addition of GM-CSF autoantibodies reduced the phagocytic capacity of washed neutrophils and blocked the ability of GM-CSF to maintain phagocytic functions in neutrophils during short-term, ex vivo incubation (Fig. 2D). Purified GM-CSF autoantibodies from a single patient and purified pooled GM-CSF autoantibodies had similar inhibitory effects on CD11b levels primed with GM-CSF (mean of three replicates,

**Figure 1. Ultrastructure and Functions of Neutrophils from Subjects with Pulmonary Alveolar Proteinosis (PAP), Healthy Control Subjects, GM-CSF<sup>-/-</sup> Mice, and Wild-Type Mice.**

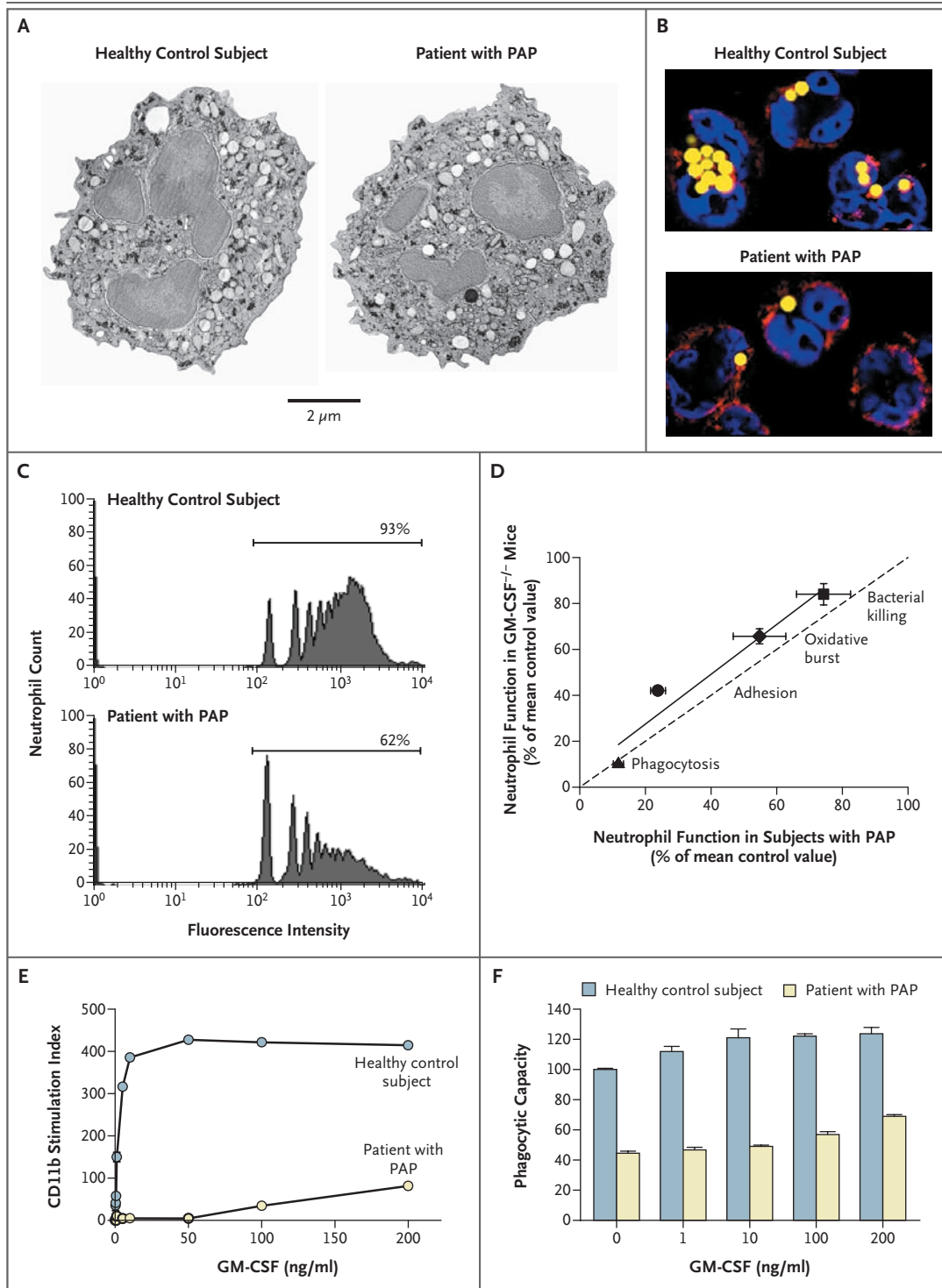
Electron photomicrographs of neutrophils show their ultrastructure (Panel A). The scale bar represents 2  $\mu\text{m}$ . Confocal photomicrographs (Panel B) show neutrophil phagocytosis of microspheres (shown in yellow); F-actin is shown in red and nuclei in blue. Panel C shows flow cytometry histograms of phagocytosis by neutrophils in whole blood, as well as the percentage of phagocytic neutrophils (calculated over the area indicated by the horizontal black bar). Each successive peak represents the number of cells with an increased number of microspheres per cell (e.g., the first peak after  $10^2$  is for 1 microsphere per cell; and the second peak is for 2 microspheres per cell). Panel D shows the similarity of impairment in various corresponding neutrophil functions in GM-CSF<sup>-/-</sup> mice and in subjects with PAP. The dashed line indicates equal impairment; the data are from Table 2. Panel E shows the CD11b stimulation index for neutrophils in a blood specimen from a healthy control subject and one from a subject with PAP after GM-CSF priming. The CD11b stimulation index was calculated as the mean fluorescence intensity of CD11b on neutrophils primed by GM-CSF minus that of CD11b on nonprimed neutrophils, divided by the mean fluorescence intensity of nonprimed neutrophils and multiplied by 100. Panel F shows the phagocytic capacity of neutrophils from a subject with PAP and a healthy control subject before and after GM-CSF priming. Phagocytic capacity was calculated as the percentage of neutrophils containing internalized microspheres multiplied by the mean fluorescence intensity of phagocytic neutrophils (both determined with the use of flow cytometry) and multiplied by the neutrophil count in the blood. In Panels D and F, I bars and T bars indicate standard errors.

$9.61\pm 4.90$  and  $0.00\pm 4.83$ , respectively;  $P=0.24$ ) and on GM-CSF-primed phagocytic capacity (mean of three replicates,  $-0.84\pm 6.17$  and  $4.21\pm 0.53$ , respectively;  $P=0.46$ ).

Injection of a monoclonal mouse GM-CSF antibody into wild-type mice reproduced the impaired neutrophil phagocytic capacity observed in GM-CSF<sup>-/-</sup> mice (Fig. 2E). The mean serum GM-CSF antibody level at the time of evaluation was  $69.3\pm 8.1$   $\mu\text{g}$  per milliliter.

## DISCUSSION

Our study showed impairment of basal phagocytosis, adhesion, oxidative burst, and bactericidal activity of neutrophils from subjects with pulmonary alveolar proteinosis and neutrophils from GM-CSF<sup>-/-</sup> mice. Neutrophils from the subjects also had impaired responses to GM-CSF priming.



The dysfunction was reproduced in normal human neutrophils by incubating them with highly purified GM-CSF antibodies from subjects with pulmonary alveolar proteinosis and also by injecting mouse GM-CSF autoantibodies into nor-

mal mice. The observed constellation of neutrophil abnormalities can explain the increased risk of infection, especially infection with opportunistic organisms, that is associated with pulmonary alveolar proteinosis<sup>2,3</sup> and with other phagocyte

**Table 2.** Differentiation Markers and Functions of Neutrophils from Healthy Control Subjects, Subjects with Pulmonary Alveolar Proteinosis (PAP), Wild-Type Mice, and GM-CSF<sup>-/-</sup> Mice.\*

Marker or Function	Control Value <sup>†</sup>	Value for GM-CSF Deficiency <sup>‡</sup>	P Value
<b>Expression of CD antigen (% of cells positive for antigen)</b>			
Human			
CD11b			
No. evaluated	13	4	
Median	99.0	99.9	0.06
Interquartile range	98.3 to 99.7	99.4 to 100.0	
CD16			
No. evaluated	17	4	
Median	99.0	99.9	0.28
Interquartile range	99.0 to 99.7	98.9 to 99.9	
CD18			
No. evaluated	17	4	
Median	94.0	99.0	0.24
Interquartile range	90.0 to 98.5	87.9 to 99.9	
CD114			
No. evaluated	17	4	
Median	97.0	98.9	0.18
Interquartile range	94.3 to 99.1	98.4 to 99.5	
CD116			
No. evaluated	17	4	
Median	75.0	80.3	0.30
Interquartile range	47.0 to 80.3	65.5 to 94.1	
Mouse			
CD11b			
No. evaluated	4	4	
Median	100	100	0.69
Interquartile range	92.4 to 100	100 to 100	
CD16 or CD32			
No. evaluated	3	3	
Median	98.1	99.1	0.40
Interquartile range	97.5 to 98.4	98.1 to 99.2	
Ly6G			
No. evaluated	4	4	
Median	94.7	98.3	0.03
Interquartile range	90.1 to 96.2	97.9 to 98.7	
<b>Basal phagocytic index<sup>§</sup></b>			
Human			
No. evaluated	4	5	
Mean ±SE	100.0±8.2	11.7±1.6	<0.001
Mouse			
No. evaluated	4	3	
Mean ±SE	100.0±4.7	10.0±0.9	<0.001

<b>Table 2. (Continued.)</b>			
<b>Marker or Function</b>	<b>Control Value†</b>	<b>Value for GM-CSF Deficiency‡</b>	<b>P Value</b>
<b>Basal phagocytic capacity¶</b>			
Human			
No. evaluated	40	9	
Mean ±SE	100.0±3.4	69.4±7.2	<0.001
Mouse			
No. evaluated	5	5	
Mean ±SE	100.0±6.6	55.8±7.0	0.002
<b>Adhesion of isolated neutrophils (no. of adherent cells/plate)</b>			
Human			
No. evaluated	4	3	
Mean ±SE	100.0±2.1	23.8±2.3	<0.001
Mouse			
No. evaluated	4	3	
Mean ±SE	100.0±1.5	42.0±1.2	<0.001
<b>Oxidative burst in whole blood (% of mean control value)</b>			
Human			
No. evaluated	14	4	
Mean ±SE	100.0±4.3	54.8±8.0	<0.001
Mouse			
No. evaluated	5	5	
Mean ±SE	100.0±1.6	65.6±3.3	<0.001
<b>Bacterial killing in whole blood (% of inoculum)  </b>			
Human			
No. evaluated	8	4	
Mean ±SE	82.6±3.0	61.4±6.8	0.007
Mouse			
No. evaluated	9	10	
Mean ±SE	87.7±1.8	73.6±4.1	0.008
<b>Bacterial killing time of isolated neutrophils (min)**</b>			
Human			
No. evaluated	5	4	
Mean ±SE	21.3±2.6	35.7±4.8	0.03
<b>CD11b stimulation index††</b>			
Human			
No. evaluated	10	8	
Median	429	-0.9	<0.001
Interquartile range	320 to 572	-7.5 to 4.8	
Mouse			
No. evaluated	4	4	
Median	17.1	13.6	0.69
Interquartile range	12.7 to 22.2	8.73 to 22.5	

Function	Control Value <sup>†</sup>	Value for GM-CSF Deficiency <sup>‡</sup>	P Value
<b>Phagocytic capacity after GM-CSF priming<sup>‡‡</sup></b>			
Human			
No. evaluated	3	3	
Mean ±SE	37.1±8.4	1.1±2.1	0.01
Mouse			
No. evaluated	5	5	
Mean ±SE	22.1±9.3	13.1±5.3	0.43

\* Data for the basal phagocytosis of isolated neutrophils (basal phagocytic index), the basal phagocytosis by neutrophils in whole blood (basal phagocytic capacity), adhesion of isolated neutrophils, and oxidative burst in whole blood (hydrogen peroxide production) were normalized by dividing by the mean of the control group and multiplying by 100.

† Control values are given for either healthy control subjects (for comparison with subjects with PAP) or wild-type mice (for comparison with GM-CSF<sup>-/-</sup> mice).

‡ Values for GM-CSF deficiency are given for either subjects with PAP or GM-CSF<sup>-/-</sup> mice.

§ The phagocytic index was calculated as the percentage of neutrophils that had phagocytosed microspheres multiplied by the number of microspheres per cell.

¶ Phagocytic capacity was calculated as the percentage of neutrophils containing internalized microspheres multiplied by the mean fluorescence intensity of phagocytic neutrophils and multiplied by the neutrophil count in the blood.

|| Bacterial killing in whole blood was defined as the percentage of *Staphylococcus aureus* killed in 1 hour by neutrophils in whole blood.

\*\* Bacterial killing time of isolated neutrophils was defined as the time required to kill 50% of the *S. aureus* inoculum.

†† The CD11b stimulation index was calculated as the mean fluorescence intensity of CD11b on neutrophils primed by GM-CSF minus that of CD11b on nonprimed neutrophils, divided by the mean fluorescence intensity of nonprimed neutrophils and multiplied by 100.

‡‡ Phagocytic capacity after GM-CSF priming was calculated as the phagocytic capacity of GM-CSF-primed neutrophils minus that of nonprimed neutrophils.

Neutrophil Function	Mean Value (±SE)	P Value
Basal phagocytic capacity <sup>†</sup>		
Subjects with cystic fibrosis	91.1±4.0	0.33
Subjects with end-stage liver disease	90.1±14.3	0.34
Healthy subjects	100.0±3.4	
CD11b stimulation index <sup>‡</sup>		
Subjects with cystic fibrosis	292±179	0.30
Subjects with end-stage liver disease	400±101	0.59
Healthy subjects	460±61	

\* We studied 6 control subjects with cystic fibrosis, 6 with end-stage liver disease, and 40 healthy control subjects for phagocytic capacity and 10 for CD11b level.

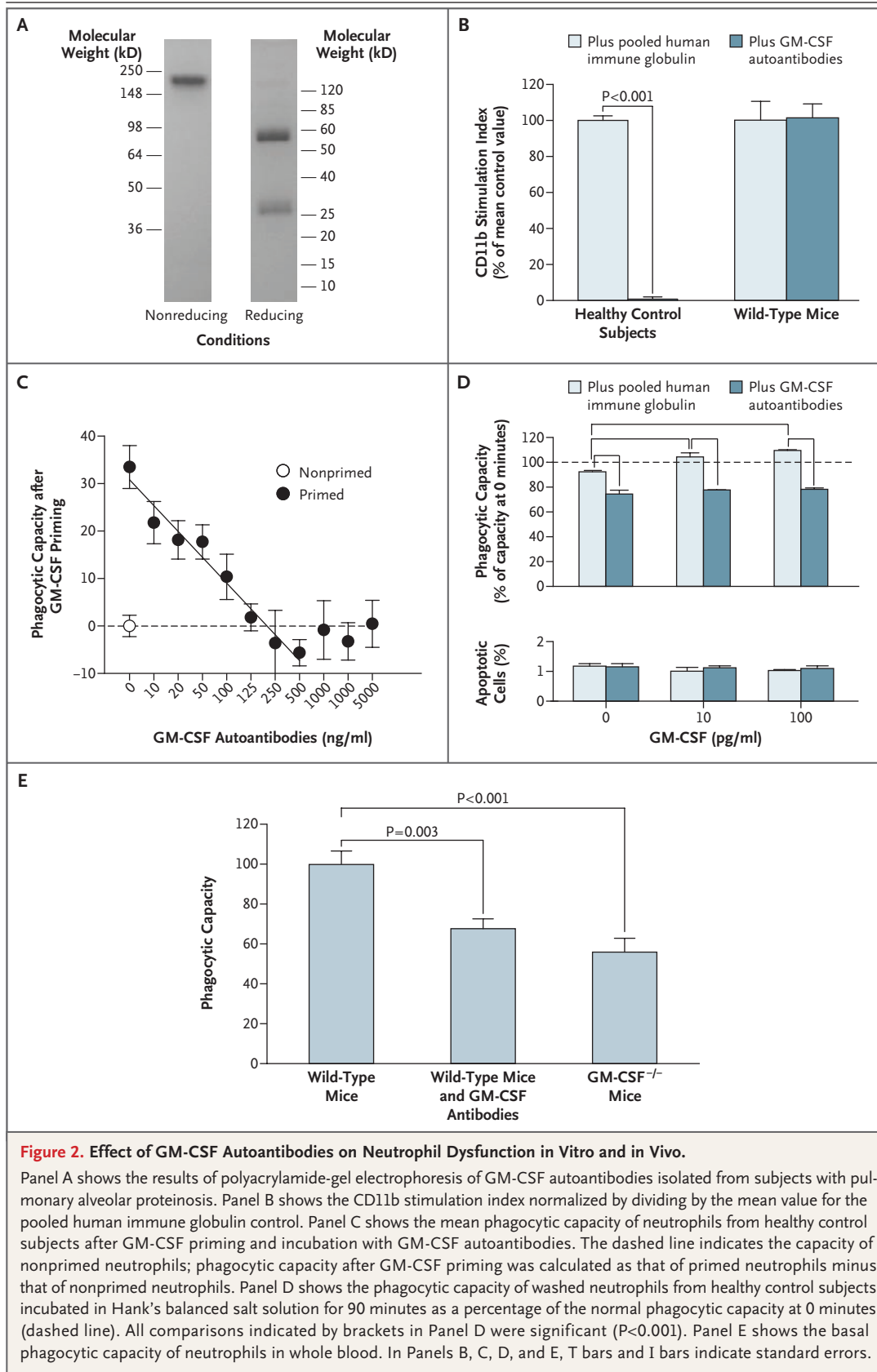
† Phagocytic capacity was calculated as the percentage of neutrophils containing internalized microspheres multiplied by the mean fluorescence intensity of phagocytic neutrophils and multiplied by the neutrophil count in the blood.

‡ The CD11b stimulation index was calculated as the mean fluorescence intensity of CD11b on neutrophils primed by GM-CSF minus that of CD11b on nonprimed neutrophils, divided by the mean fluorescence intensity of nonprimed neutrophils and multiplied by 100.

immunodeficiencies,<sup>25</sup> as well as the increased mortality from infection in GM-CSF<sup>-/-</sup> mice.<sup>12</sup>

Neutrophil dysfunction in patients with pulmonary alveolar proteinosis was associated with high levels of GM-CSF autoantibodies and could not be attributed to a nonspecific effect of chronic illness. Neutralizing GM-CSF autoantibodies have been reported in patients with myasthenia gravis,<sup>26</sup> but the antibody levels are relatively low, and pulmonary alveolar proteinosis has not been reported in this setting.

G-CSF can also prime the antimicrobial function of neutrophils<sup>27</sup> by means of a mechanism distinct from that in GM-CSF priming.<sup>28</sup> In our study, however, serum G-CSF levels and blood neutrophil counts, which are regulated by G-CSF,<sup>27</sup> were similar in subjects with pulmonary alveolar proteinosis and in healthy subjects. In some patients with Felty's syndrome, G-CSF autoantibodies are thought to cause neutropenia by neutralizing G-CSF activity<sup>29</sup>; however, pulmonary alveolar proteinosis has not been reported in these pa-



tients. Therefore, G-CSF and GM-CSF have distinct effects on neutrophil functions. Levels of macrophage colony-stimulating factor (M-CSF), which has regulatory effects on myeloid cells that overlap with the regulatory effects of GM-CSF, are increased in patients with pulmonary alveolar proteinosis and in GM-CSF<sup>-/-</sup> mice.<sup>3,6,10</sup> However, since M-CSF receptors are not present on neutrophils, elevated M-CSF levels in patients with pulmonary alveolar proteinosis are unlikely to influence neutrophil functions.

We found that GM-CSF priming of neutrophil functions was blocked in patients with pulmonary alveolar proteinosis but not in GM-CSF<sup>-/-</sup> mice, although the pattern of basal neutrophil dysfunction was similar between the two. In humans and mice, abrogation of GM-CSF signaling (by means of gene knockout in mice and by means of antibodies in patients with pulmonary alveolar proteinosis) reduces, but does not abolish, multiple neutrophil functions. Moreover, GM-CSF appears to be unnecessary for neutrophil differentiation: neutrophil morphology, cell-surface differentiation markers, and levels of expression of PU.1 (a transcription factor that is critical for neutrophil differentiation<sup>30</sup>) were similar in subjects with pulmonary alveolar proteinosis and healthy control subjects. This evidence contrasts with the fact that GM-CSF stimulates the terminal differentiation of alveolar macrophages, primarily by increasing levels of PU.1 expression.<sup>6</sup>

The GM-CSF receptor mediates the dose-dependent effects of GM-CSF in a mutually exclusive, reciprocal fashion through two  $\beta$ -chain residues: Ser585 at low GM-CSF levels (in general, <300 pg per milliliter) and Tyr577 at high levels (>300 pg per milliliter).<sup>31</sup> Thus, the GM-CSF receptor acts as a binary switch that promotes cell survival at low GM-CSF levels but also stimulates proliferation and antimicrobial functions (including an increased CD11b level on the cell surface) at high levels. This arrangement of the GM-CSF receptor places the level of GM-CSF bioactivity at the cen-

ter of the mechanism that regulates neutrophil functions.

Our findings, together with those in previously published reports,<sup>13,32</sup> provide support for the feasibility of GM-CSF therapy to augment innate immune defenses in patients with serious infections. Conversely, therapy with a humanized monoclonal GM-CSF antibody<sup>33</sup> could be of use in reducing neutrophil priming (and functional capacity) in patients with chronic inflammatory disorders characterized by increased numbers of activated neutrophils, such as severe neutrophilic asthma,<sup>34</sup> cystic fibrosis,<sup>35</sup> and the adult respiratory distress syndrome.<sup>36</sup> It is relevant that GM-CSF antibodies reduce neutrophilic pulmonary inflammation in a dose-dependent fashion in endotoxin-exposed mice.<sup>37</sup> Finally, assessment of blood neutrophil functions may provide convenient, functional surrogate-outcome measures for use in clinical trials evaluating new therapies for pulmonary alveolar proteinosis.

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#### REFERENCES

- Rosen SH, Castleman B, Liebow AA. Pulmonary alveolar proteinosis. *N Engl J Med* 1958;258:1123-42.
- Seymour JF, Presneill JJ. Pulmonary alveolar proteinosis: progress in the first 44 years. *Am J Respir Crit Care Med* 2002;166:215-35.
- Trapnell BC, Whitsett JA, Nakata K. Pulmonary alveolar proteinosis. *N Engl J Med* 2003;349:2527-39.
- Kitamura T, Tanaka N, Watanabe J, et al. Idiopathic pulmonary alveolar proteinosis as an autoimmune disease with neutralizing antibody against granulocyte/macrophage colony-stimulating factor. *J Exp Med* 1999;190:875-80.
- Uchida K, Nakata K, Trapnell BC, et al. High-affinity autoantibodies specifically eliminate granulocyte-macrophage colony-stimulating factor activity in the lungs of patients with idiopathic pulmonary alveolar proteinosis. *Blood* 2004;103:1089-98.
- Shibata Y, Berclaz PY, Chroneos ZC, Yoshida M, Whitsett JA, Trapnell BC. GM-CSF regulates alveolar macrophage differentiation and innate immunity in the lung through PU.1. *Immunity* 2001;15:557-67.
- Stanley E, Lieschke GJ, Grail D, et al. Granulocyte/macrophage colony-stimulating factor-deficient mice show no major

- perturbation of hematopoiesis but development of a characteristic pulmonary pathology. *Proc Natl Acad Sci U S A* 1994;91:5592-6.
8. Dranoff G, Crawford AD, Sadelain M, et al. Involvement of granulocyte-macrophage colony-stimulating factor in pulmonary homeostasis. *Science* 1994;264:713-6.
  9. Ikegami M, Ueda T, Hull W, et al. Surfactant metabolism in transgenic mice after granulocyte macrophage-colony stimulating factor ablation. *Am J Physiol* 1996;270:L650-L658.
  10. Bonfield TL, Raychaudhuri B, Malur A, et al. PU.1 regulation of human alveolar macrophage differentiation requires granulocyte-macrophage colony-stimulating factor. *Am J Physiol Lung Cell Mol Physiol* 2003;285:L1132-L1136.
  11. Seymour JF, Presneill JJ. Pulmonary alveolar proteinosis: what is the role of GM-CSF in disease pathogenesis and treatment? *Treat Respir Med* 2004;3:229-34.
  12. Seymour JF, Lieschke GJ, Grail D, Quilici C, Hodgson G, Dunn AR. Mice lacking both granulocyte colony-stimulating factor (CSF) and granulocyte-macrophage CSF have impaired reproductive capacity, perturbed neonatal granulopoiesis, lung disease, amyloidosis, and reduced long-term survival. *Blood* 1997;90:3037-49.
  13. Gonzalez-Juarrero M, Hattler JM, Izzo A, et al. Disruption of granulocyte macrophage colony stimulating factor production in the lungs severely affects the ability of mice to control *Mycobacterium tuberculosis* infection. *J Leukoc Biol* 2005;77:914-22.
  14. Zhan Y, Lieschke GJ, Grail D, Dunn AR, Cheers C. Essential roles for granulocyte-macrophage colony-stimulating factor (GM-CSF) and G-CSF in the sustained hematopoietic response of *Listeria monocytogenes*-infected mice. *Blood* 1998;91:863-9.
  15. Condliffe AM, Kitchen E, Chilvers ER. Neutrophil priming: pathophysiological consequences and underlying mechanisms. *Clin Sci (Lond)* 1998;94:461-71.
  16. Fleischmann J, Golde DW, Weisbart RH, Gasson JC. Granulocyte-macrophage colony-stimulating factor enhances phagocytosis of bacteria by human neutrophils. *Blood* 1986;68:708-11.
  17. Graves V, Gabig T, McCarthy L, Strour EF, Leemhuis T, English D. Simultaneous mobilization of Mac-1 (CD11b/CD18) and formyl peptide chemoattractant receptors in human neutrophils. *Blood* 1992;80:776-87. [Erratum, *Blood* 1993;81:1668.]
  18. Clark RA, Nauseef WM. Isolation and functional analysis of neutrophils. In: Bierer B, Coligan JE, Margulies DH, Shevach EM, Strober W, eds. *Current protocols in immunology*. New York: John Wiley, 2005:2-6.
  19. Berclaz PY, Zsengeller Z, Shibata Y, et al. Endocytic internalization of adenovirus, nonspecific phagocytosis, and cytoskeletal organization are coordinately regulated in alveolar macrophages by GM-CSF and PU.1. *J Immunol* 2002;169:6332-42.
  20. Bainton DE, Ullyot JL, Farquhar MG. The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. *J Exp Med* 1971;134:907-34.
  21. Berclaz PY, Shibata Y, Whitsett JA, Trapnell BC. GM-CSF, via PU.1, regulates alveolar macrophage Fc $\gamma$  R-mediated phagocytosis and the IL-18/IFN- $\gamma$  mediated molecular connection between innate and adaptive immunity in the lung. *Blood* 2002;100:4193-200.
  22. Hassan NF, Campbell DE, Douglas SD. Phorbol myristate acetate induced oxidation of 2',7'-dichlorofluorescein by neutrophils from patients with chronic granulomatous disease. *J Leukoc Biol* 1988;43:317-22.
  23. DeForge LE, Billeci KL, Kramer SM. Effect of IFN- $\gamma$  on the killing of *S. aureus* in human whole blood: assessment of bacterial viability by CFU determination and by a new method using alamarBlue. *J Immunol Methods* 2000;245:79-89.
  24. Boxio R, Bossenmeyer-Pouric C, Steinckewich N, Dournon C, Nusse O. Mouse bone marrow contains large numbers of functionally competent neutrophils. *J Leukoc Biol* 2004;75:604-11.
  25. Rosenzweig SD, Holland SM. Phagocyte immunodeficiencies and their infections. *J Allergy Clin Immunol* 2004;113:620-6.
  26. Meager A, Wadhwa M, Bird C, et al. Spontaneously occurring neutralizing antibodies against granulocyte-macrophage colony-stimulating factor in patients with autoimmune disease. *Immunology* 1999;97:526-32.
  27. Roberts AW. G-CSF: a key regulator of neutrophil production, but that's not all! *Growth Factors* 2005;23:33-41.
  28. Treweeke AT, Aziz KA, Zuzel M. The role of G-CSF in mature neutrophil function is not related to GM-CSF-type cell priming. *J Leukoc Biol* 1994;55:612-6.
  29. Hellmich B, Csernok E, Schatz H, Gross WL, Schnabel A. Autoantibodies against granulocyte colony-stimulating factor in Felty's syndrome and neutropenic systemic lupus erythematosus. *Arthritis Rheum* 2002;46:2384-91.
  30. Iwasaki H, Somoza C, Shigematsu H, et al. Distinctive and indispensable roles of PU.1 in maintenance of hematopoietic stem cells and their differentiation. *Blood* 2005;106:1590-600.
  31. Guthridge MA, Powell JA, Barry EF, et al. Growth factor pleiotropy is controlled by a receptor Tyr/Ser motif that acts as a binary switch. *EMBO J* 2006;25:479-89.
  32. Rosenbloom AJ, Linden PK, Dorrance A, Penkosky N, Cohen-Melamed MH, Pinsky MR. Effect of granulocyte-macrophage colony-stimulating factor therapy on leukocyte function and clearance of serious infection in nonneutropenic patients. *Chest* 2005;127:2139-50.
  33. Krinner EM, Raum T, Petsch S, et al. A human monoclonal IgG1 potently neutralizing the pro-inflammatory cytokine GM-CSF. *Mol Immunol* 2007;44:916-25.
  34. Mann BS, Chung KF. Blood neutrophil activation markers in severe asthma: lack of inhibition by prednisolone therapy. *Respir Res* 2006;7:59.
  35. Koller DY, Urbanek R, Gotz M. Increased degranulation of eosinophil and neutrophil granulocytes in cystic fibrosis. *Am J Respir Crit Care Med* 1995;152:629-33.
  36. Rivkind AI, Siegel JH, Littleton M, et al. Neutrophil oxidative burst activation and the pattern of respiratory physiologic abnormalities in the fulminant post-traumatic adult respiratory distress syndrome. *Circ Shock* 1991;33:48-62.
  37. Bozinovski S, Jones J, Beavitt SJ, Cook AD, Hamilton JA, Anderson GP. Innate immune responses to LPS in mouse lung are suppressed and reversed by neutralization of GM-CSF via repression of TLR-4. *Am J Physiol Lung Cell Mol Physiol* 2004;286:L877-L885.

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