Effects of CD18 Deficiency on the Emigration of Murine Neutrophils During Pneumonia[1]

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We hypothesized that CD18 deficiency would impair the ability of neutrophils to emigrate from pulmonary blood vessels during certain pneumonias. To directly compare the abilities of wild-type (WT) and CD18-deficient neutrophils to emigrate, mice with both types of leukocytes in their blood were generated by reconstituting the hemopoietic systems of lethally irradiated C57BL/6 mice with mixtures of fetal liver cells from WT and CD18-deficient mice. Percentages of CD18-deficient neutrophils in the circulating and emigrated pools were compared during experimental pneumonias. Similar percentages were observed in the blood and bronchoalveolar lavage fluid 6 or 24 h after intratracheal instillation of *Streptococcus pneumoniae*, demonstrating that no site on the CD18 molecule was required for either its adhesive or its signaling functions during neutrophil emigration. However, 6 h after instillation of *Escherichia coli* LPS or *Pseudomonas aeruginosa*, the percentage of CD18-deficient neutrophils in the bronchoalveolar lavage fluid was only about one-fourth of that observed in the blood. This difference persisted for at least 24 h after instillation of *E. coli* LPS. Thus, neutrophil emigration elicited by the Gram-negative stimuli *E. coli* LPS or *P. aeruginosa* was compromised by deficiency of CD18. These data, based on comparing WT and gene-targeted CD18-deficient neutrophils within the same animals, provide evidence for molecular pathways regulating neutrophil emigration, which could not be appreciated in previous studies with pharmacological blockade or genetic deficiency of CD18. *The Journal of Immunology,* 1999, 163: 995–999.

The β_2 integrins are expressed by all leukocytes and, among other functions, mediate adhesion to endothelial cells. They are heterodimeric transmembrane glycoproteins composed of one invariant CD18 chain and one of four possible CD11 chains (CD11a, -b, -c, or -d). The spontaneous genetic deficiency of CD18 results in the loss of all immunologically recognizable CD11 and CD18 and the absence of all CD11/CD18 function (see Refs. 1 and 2 for review). Infected tissues from human or veterinary CD18-deficient patients are typically devoid of extravascular neutrophils, suggesting that CD11/CD18 complexes are essential to emigration from the blood vessels (1–7). Confirming this, blocking Abs against CD11/CD18 significantly inhibit neutrophil emigration from the systemic circulation during diverse inflammatory processes in various tissues (8–13).

In contrast, pneumonic lungs from CD18-deficient human or veterinary patients display abundant emigrated neutrophils (6, 7, 14), demonstrating that CD18-independent pathways can be used for neutrophil emigration from the pulmonary circulation. Blocking Ab studies suggest that neutrophils use CD18-dependent or CD18-independent pathways in the lungs, depending on the stimulus inducing pneumonia. Blocking Abs against CD11/CD18 prevent neutrophil emigration by ~70% during 4–6 h of pneumonia induced by *Escherichia coli* LPS, *E. coli*, *Pseudomonas aeruginosa*, IgG immune complexes, IL-1α, and phorbol esters (12, 15–18), but blocking Abs against CD11/CD18 do not affect neutrophil emigration during 4–6 h of pneumonia induced by *S. pneumoniae*, group B *Streptococcus*, *Staphylococcus aureus*, complement fragment C5a, hyperoxia, or hydrochloric acid (12, 16, 18–20).

Consistent with clinical observations of neutrophils in the lungs of CD18-deficient patients, but contrasting with predictions based on blocking Ab studies, mice rendered CD18-deficient by gene targeting show no defect in neutrophil emigration compared with wild-type (WT)[5] mice during experimental pneumonias induced by either *E. coli* or *S. pneumoniae* (21). Furthermore, experimental pneumonias induced by *Pasteurella haemolytica* in cows with spontaneous deficiencies of CD18 show no defect in neutrophil emigration compared with normal (CD18-expressing) cows (22). Thus, the results from all studies to date suggest that CD18 deficiency does not compromise the emigration of neutrophils from the pulmonary circulation. However, the genetic deficiency of CD18 results in systemic phenotypic alterations (1, 7, 23), including soft tissue infections, neutrophilia, splenomegaly, and lymphadenopathy, which may affect neutrophil emigration and confound comparisons of CD18-deficient and control animals. To directly examine the roles of CD11/CD18 in neutrophil emigration in the lungs, we endeavored to compare the emigration of WT and CD18-deficient neutrophils within the same physiological environment. To accomplish this, mice with both types of neutrophils in their blood were generated after reconstitution of the hemopoietic

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[5] Abbreviations used in this paper: WT, wild type; BAL, bronchoalveolar lavage; i.t., intratracheal.

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systems of lethally irradiated mice with mixtures of fetal liver cells from WT and CD18-deficient mice.

Materials and Methods

Hemopoietic reconstitution

The hemopoietic systems of lethally irradiated C57BL/6 host mice were reconstituted after injection of fetal liver cells as described (24). In short, CD18-deficient mice (23), provided by Dr. Arthur L. Beaudet, and WT mice of similar randomly mixed C57BL/6 × 129/Sv background were mated with the like genotype, and fetuses were collected after 14 days of gestation. Single-cell suspensions were prepared from WT and CD18-deficient fetal livers, and mixtures of 1–2 × 10^6 total cells were injected i.v. into host mice that had received radiation doses of 800 and 400 rad, separated by 3 h, from a ^{137}Cs source. To minimize possible systemic physiological effects caused by CD18 deficiency, these studies were performed using mice reconstituted with mixtures of fetal liver cells in which a minority of the inoculum was from CD18-deficient animals (≤10% in the *E. coli* LPS and *S. pneumoniae* experiments and 25% in the *P. aeruginosa* experiments). After transplantation, mice received trimethoprim-sulfamethoxazole in their drinking water and were maintained under barrier conditions. Animals were analyzed a minimum of 4 wk after irradiation to allow for reconstitution. Several mice reconstituted with mixed WT and CD18-deficient fetal liver cells had their lungs lavaged (see below) and then fixed by instillation of 10% formalin at 22 cm H_2O. Lavage fluids were analyzed for neutrophil content, and histological sections from the fixed lungs were examined to determine whether irradiation and reconstitution resulted in pulmonary inflammation.

Pneumonia

Pneumonias were induced by intratracheal (i.t.) instillations (21). Mice were anesthetized by i.m. injection of ketamine hydrochloride (100 mg/kg) and acepromazine maleate (5 mg/kg). The tracheas were surgically exposed, and 50 μl of *E. coli* LPS serotype O55:B5 (Sigma, St. Louis, MO) at 2 mg/ml, of *P. aeruginosa* at 1 × 10^8 CFU/ml, or of *S. pneumoniae* at 5 × 10^9 CFU/ml were instilled i.t. At the indicated times, mice were killed by inhalation of a lethal overdose of halothane. Blood was collected from the inferior vena cava, and erythrocytes were hypotonically lysed. Bronchoalveolar lavage (BAL) was performed after cannulating the trachea. A syringe containing 1 ml of ice-cold PBS containing 0.6 mM EDTA was inserted into the cannula, 0.5 ml were injected while the chest was massaged, and then as much volume as possible was recovered. The lavage steps were twice repeated without changing the syringe, after which the syringe was removed from the cannula and the contents were evacuated into a test tube and kept on ice. The entire procedure was repeated twice more, so that the lungs received a total of 9 lavages of 0.5 ml pooled in ≤3 ml final volume. Blood and BAL leukocytes were washed with fresh PBS/EDTA, and WT and CD18-deficient cells were differentiated using mAb against CD11a and CD11b.

For flow cytometric analyses, blood and BAL cells were stained with saturating concentrations of a FITC-conjugated mAb against murine CD11a (M17/4, PharMingen, San Diego, CA) and a PE-conjugated mAb against the murine granulocyte marker Gr-1 (RB6-8C5, PharMingen). Overlapping spectra of FITC and PE were accounted for by adjusting compensation using cells stained with single Abs. Gr-1 bright cells had forward and right angle scatter characteristics consistent with granulocytes. The percentage of neutrophils that were CD18 deficient was assessed for each sample by scoring 5000 Gr-1 bright cells as either positive or negative for CD11a/CD18.

For immunohistochemical analyses, blood and BAL cells were cytocentrifuged onto glass slides, allowed to air dry, and then fixed with acetone-methanol (1:1). Slides were treated with a rat mAb against murine CD11b (M1/70, PharMingen), and M1/70 was visualized using biotinylated goat anti-rat IgG and a streptavidin-alkaline phosphatase detection system (Kirkegaard and Perry, Gaithersburg, MD). Control slides demonstrated no staining of WT or CD18-deficient cells when nonspecific rat IgG replaced M1/70 and no staining of cells from CD18-deficient mice by M1/70. Slides were counterstained with hematoxylin, and the percentage of neutrophils which were CD18-deficient was assessed for each sample by scoring 300 polymorphonuclear cells as either positive or negative for CD11b/CD18.

Statistics

In each group, the percentages of CD18-deficient neutrophils in the blood and in the BAL fluid were compared by paired *t* tests, and differences were considered significant when $p < 0.05$. Results were expressed as mean and SEM. Each group consisted of five to eight mice.

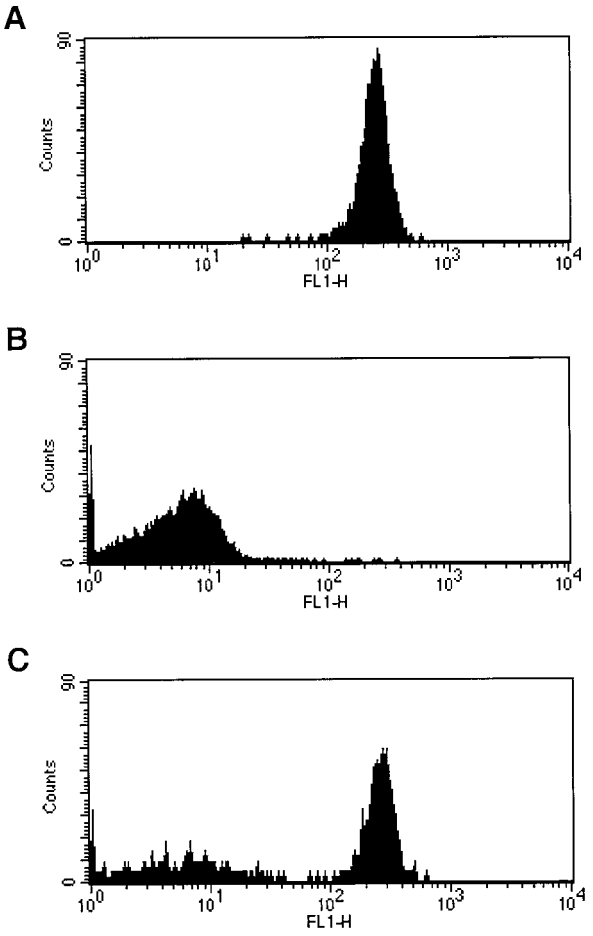

FIGURE 1. Circulating WT and CD18-deficient neutrophils after hemopoietic reconstitution. Neutrophils were identified by bright staining with a PE-conjugated mAb against murine Gr-1 (RB6-8C5). WT and CD18-deficient cells were differentiated using a FITC-conjugated mAb against CD11a/CD18 (M17/4). The *y*-axes depict relative numbers of peripheral blood neutrophils that express the level of CD11a/CD18 designated on the *x*-axes. *A,* Neutrophils from peripheral blood of mice reconstituted with WT fetal liver cells; *B,* neutrophils from peripheral blood of mice reconstituted with CD18-deficient fetal liver cells; *C,* neutrophils from peripheral blood of mice reconstituted with mixtures of WT and CD18-deficient fetal liver cells.

Results

Hemopoietic reconstitution

Whereas peripheral blood neutrophils from WT mice are uniformly positive for CD11a, CD11b, and CD18, neutrophils from CD18-deficient mice are uniformly negative for CD11a and CD11b as well as CD18 (21, 23), similar to patients with spontaneous deficiencies of CD18 (1, 2). Abs against CD11a and CD11b were used to differentiate WT and CD18-deficient neutrophils in the present studies. When lethally irradiated C57BL/6 mice were reconstituted with WT fetal liver cells, their peripheral blood neutrophils expressed CD11a/CD18 (Fig. 1*A*). When lethally irradiated C57BL/6 mice were reconstituted with CD18-deficient fetal liver cells, their peripheral blood neutrophils did not express CD11a/CD18 (Fig. 1*B*). When lethally irradiated C57BL/6 mice were reconstituted with mixtures of WT and CD18-deficient fetal liver cells, anti-CD11a/CD18 staining revealed both WT and CD18-deficient neutrophils in the circulating blood (Fig. 1*C*). Similar results were observed when WT and CD18-deficient neutrophils were differentiated using immunohistochemistry for CD11b/CD18 (data not shown).

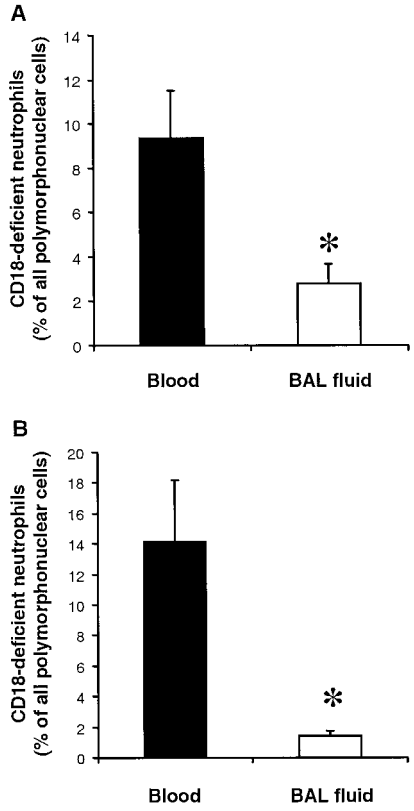

FIGURE 2. Percentages of blood and BAL neutrophils which were CD18-deficient during *E. coli* LPS pneumonia. Mice had hemopoietic systems reconstituted from mixtures of WT and CD18-deficient fetal liver cells, and WT and CD18-deficient neutrophils from blood and BAL fluid were differentiated using immunohistochemical staining for CD11b/CD18 in cytospin preparations. *A*, Percentages of CD18-deficient neutrophils in blood and BAL fluid collected 6 h after instillation of *E. coli* LPS. *B*, Percentages of CD18-deficient neutrophils in blood and BAL fluid collected 24 h after instillation of *E. coli* LPS. ∗, Significant differences between blood and BAL fluid.

The lungs of mice which had been irradiated and reconstituted with mixed WT and CD18-deficient fetal liver cells were examined for signs of inflammation. In the absence of experimentally induced pneumonia, neutrophils were not recovered by BAL. Fixed sections from the lungs of these mice did not reveal evidence of infection, radiation-induced pneumonitis, or neutrophilic infiltration of the interstitium or air spaces.

E. coli *LPS pneumonia*

The intratracheal instillation of *E. coli* LPS to mice with hemopoietic systems reconstituted with mixed WT and CD18-deficient fetal liver cells resulted in neutrophil emigration by 6 h, as measured by the recovery of neutrophils by BAL ($1.8 \pm 0.6 \times 10^5$ neutrophils/ml). To determine whether the WT and CD18-deficient neutrophils were equally capable of emigrating 6 h after *E. coli* LPS instillation, the percentage of CD18-deficient neutrophils in the circulating pool (peripheral blood) and in the emigrated pool (BAL fluid) were compared. In these animals, 9.3% of the circulating neutrophils were CD18-deficient, but only 2.8% of the emigrated neutrophils were CD18-deficient (Fig. 2*A*), indicating that CD18-deficient neutrophils had a defect in emigration compared with neutrophils expressing CD11/CD18. The BAL fluid contained a smaller fraction of CD18-deficient neutrophils than the circulating blood in every mouse, whether the cells were differentiated by anti-CD11b staining of polymorphonuclear cells examined by immunohistochemistry (Fig. 2*A*) or by anti-CD11a staining of Gr-1-positive cells examined by flow cytometry (data not shown).

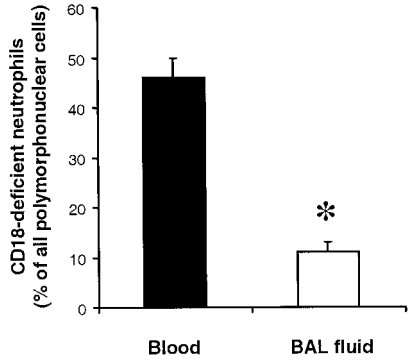

FIGURE 3. Percentages of blood and BAL neutrophils that were CD18-deficient during *P. aeruginosa* pneumonia. Mice had hemopoietic systems reconstituted from mixtures of WT and CD18-deficient fetal liver cells, and WT and CD18-deficient neutrophils from blood and BAL fluid were differentiated using immunohistochemical staining for CD11b/CD18 in cytospin preparations. ∗, Significant difference between blood and BAL fluid.

After 24 h of *E. coli* LPS pneumonia in mice with hemopoietic systems reconstituted from mixtures of WT and CD18-deficient stem cells, $2.7 \pm 0.5 \times 10^6$ neutrophils/ml were recovered in the BAL fluid. Cytospins immunohistochemically stained for CD11b/CD18 revealed that only 1.4% of these emigrated neutrophils were CD18-deficient, whereas 14.1% of the circulating neutrophils were CD18-deficient (Fig. 2*B*). Similar results were observed when WT and CD18-deficient cells were differentiated by anti-CD11a staining of Gr-1-positive cells examined by flow cytometry (data not shown).

P. aeruginosa *pneumonia*

To determine whether CD18-deficient neutrophils were compromised in emigration during pneumonia induced by living Gram-negative organisms, *P. aeruginosa* were instilled i.t. into mice with CD18-negative and -positive neutrophils in their blood. After 6 h, $3.4 \pm 0.6 \times 10^5$ neutrophils/ml were recovered in the BAL fluid. Immunohistochemical staining for CD11b/CD18 revealed that 46% of the neutrophils were CD18-deficient in the peripheral blood, whereas only 11% of the neutrophils were CD18-deficient in the BAL fluid (Fig. 3).

S. pneumoniae *pneumonia*

To determine whether CD18-deficient neutrophils were compromised in emigration during streptococcal pneumonia, *S. pneumoniae* were instilled i.t. into mice with hemopoietic systems reconstituted from mixtures of WT and CD18-deficient stem cells. After 6 h, $1.4 \pm 0.6 \times 10^6$ neutrophils/ml were recovered in the BAL fluid. At this time, 12.7% of the circulating neutrophils were CD18-deficient and 12.8% of the emigrated neutrophils were CD18-deficient (Fig. 4A), indicating that CD18-deficient neutrophils did not have a defect in emigration compared with CD18-positive neutrophils.

After 24 h of *S. pneumoniae* pneumonia in mice reconstituted with mixtures of WT and CD18-deficient stem cells, $1.0 \pm 0.2 \times 10^6$ neutrophils/ml were recovered by BAL. Similar percentages of neutrophils were CD18-deficient in the blood and the BAL fluid (Fig. 4*B*), suggesting that neutrophil emigration over this time frame remained free of requirements for CD11/CD18 adhesion complexes.

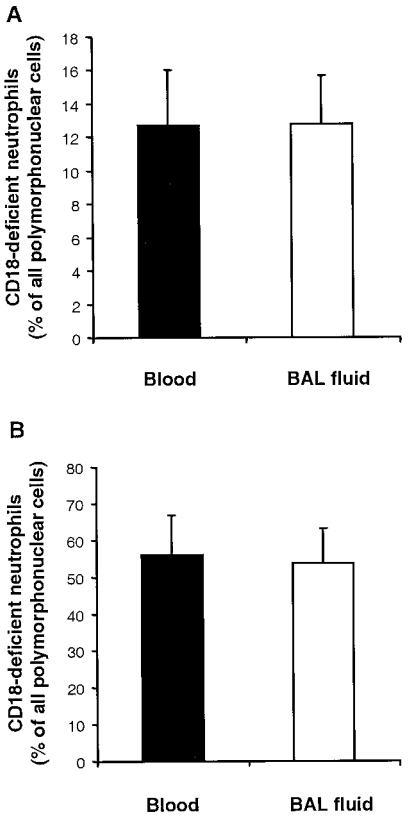

FIGURE 4. Percentages of blood and BAL neutrophils that were CD18-deficient during *S. pneumoniae* pneumonia. Mice had hemopoietic systems reconstituted from mixtures of WT and CD18-deficient fetal liver cells, and WT and CD18-deficient neutrophils from blood and BAL fluid were differentiated using immunohistochemical staining for CD11b/CD18 in cytospin preparations. *A*, Percentages of CD18-deficient neutrophils in blood and BAL fluid collected 6 h after instillation of *S. pneumoniae*. *B*, Percentages of CD18-deficient neutrophils in blood and BAL fluid collected 24 h after instillation of *S. pneumoniae*. Blood and BAL fluid did not differ significantly.

Discussion

Blocking Abs against CD11/CD18 prevent neutrophil emigration by ~70% during 4–6 h of pneumonia induced by *E. coli* LPS, *E. coli*, or *P. aeruginosa* (12, 17, 18). However, no defect in emigration is observed in neutrophil emigration in CD18-deficient mice during 6 h of *E. coli* pneumonia (21). The reason for these discrepant results is unclear but may reflect confounding effects of either Ab treatments or targeted gene deletions. Blocking Abs against CD11/CD18 may have effects other than simply the prevention of ligand binding. For example, they may cross-link CD11/CD18 molecules or interact with Fc receptors. The targeted deletion of CD18 induces systemic phenotypic changes (23), including peripheral blood neutrophilia and chronic inflammatory lesions, which also may confound comparisons of neutrophil emigration in CD18-deficient and WT mice.

The present results compared the emigration of WT and CD18-deficient neutrophils within the same animal and are free from these confounding factors. Pneumonias were induced in hemopoietically reconstituted mice with both WT and CD18-deficient neutrophils circulating in their peripheral blood, to directly compare the emigrating abilities of these neutrophils. The percentage of neutrophils that were CD18-deficient was significantly smaller in the BAL fluid than in the blood 6 h after i.t. instillation of *E. coli* LPS to mice with ~10% of their circulating cells CD18-deficient. Similar results were observed 6 h after the i.t. instillation of living *P. aeruginosa* to mice with ~50% of their circulating cells CD18-deficient. These data suggest that CD18 deficiency compromises neutrophil emigration during acute pneumonia elicited by either of these two Gram-negative stimuli.

Blocking Ab studies and comparisons of WT and CD18-deficient animals investigate the effects of inhibiting CD11/CD18 function on all hemopoietic cells. Accordingly, any observed differences might be the result of CD11/CD18 expressed by either neutrophils or other cells. For example, macrophages express CD11/CD18 molecules. Because CD11/CD18 can mediate uptake or signaling in response to complement-opsonized particles, LPS, and other stimuli (23, 25–30), the expression of inflammatory mediators produced by macrophages could be affected by blocking CD11/CD18 function, leading to decreased emigration through mechanisms other than blockade of neutrophil CD18. In the present studies, CD11/CD18-positive and CD11/CD18-negative neutrophils were directly compared within the same environment, while exposed to the same chemoattractants, cytokines, and lipid mediators. Thus, the compromised emigration of CD18-deficient neutrophils under these conditions demonstrates a critical role for the CD11/CD18 molecules expressed by neutrophils per se.

Although the present studies were free from several limitations discussed above inherent to comparisons of Ab-treated or gene-targeted mice with controls, limitations inherent to the present techniques also bear consideration. First, neutrophil emigration was studied by analysis of lavaged cells. It is conceivable that CD18 deficiency affects the adhesion of emigrated neutrophils to the alveolar epithelium, and such differential adhesion could affect the relative recovery by lavage of WT and mutant cells. Second, the mice in these studies were lethally irradiated and then hemopoietically reconstituted before the studies of pneumonia. These experimental manipulations could potentially affect the regulation of acute inflammatory responses in the lungs in as yet unrecognized ways. Blocking Ab studies (12, 17) and the studies reported in this article are subject to different experimental limitations, but the results collected with either approach suggest that CD11/CD18 is critical to neutrophil emigration during 4–6 h of pneumonia elicited by LPS or *P. aeruginosa*.

To determine whether the requirements for CD11/CD18 changed as the pneumonia progressed, the percentages of CD18-deficient neutrophils were compared in the blood and BAL fluid 24 h after the i.t. instillation of *E. coli* LPS. Similar to observations at the earlier time points, a significantly smaller percentage of neutrophils were CD18-deficient in the BAL fluid compared with the blood 24 h after instillation of *E. coli* LPS. In previous studies of peritonitis induced in rabbits by the injection of *E. coli* or protease peptone, blocking Abs against CD18 compromised neutrophil emigration during the first several hours, but emigration during 24 h of peritonitis was no longer affected by blocking Abs (31). These results indicate that CD18-independent pathways become available over this time frame of peritonitis. The present data suggest that neutrophil emigration during *E. coli* LPS pneumonia remains dependent on CD18 for at least 24 h. Although these studies used different animal species and inflammatory stimuli, the present results suggest that the temporal regulation of CD18 dependence in mediating neutrophil emigration may differ in the vascular beds of the lungs and of the peritoneum.

In contrast to the results with *E. coli* LPS or *P. aeruginosa* pneumonias, similar percentages of CD18-deficient neutrophils were present in the blood and BAL fluid 6 h after the instillation of *S. pneumoniae*. Previous studies demonstrate that blocking Abs against CD11/CD18 do not affect neutrophil emigration during 4–6 h of pulmonary inflammation induced by *S. pneumoniae*,

group B *Streptococcus*, *Staphylococcus aureus*, complement fragment C5a, hyperoxia, or hydrochloric acid (12, 16, 18–20). Several factors have been suggested as responsible for the inability of blocking Abs to inhibit neutrophil emigration during such pneumonias, including: 1) failure to obtain sufficient Ab concentrations to the required anatomic or cellular locations to completely prevent ligand binding; 2) alternative epitopes on CD11/CD18 that mediate neutrophil emigration and are not blocked by the Abs; or 3) a distinct pathway for neutrophil emigration that does not require CD11/CD18. The present data, indicating that the emigration of CD18-deficient neutrophils is not compromised compared with WT during streptococcal pneumonia, suggest that *S. pneumoniae* elicits a distinct pathway for neutrophil emigration in the lungs that is truly CD18 independent, not utilizing any region of CD11/CD18 complexes for either adhesion or signaling events essential for emigration. Furthermore, the present results demonstrate that neutrophil emigration remains CD18 independent throughout 24 h of pneumonia induced by *S. pneumoniae*.

These roles for CD11/CD18 in mediating neutrophil emigration during pneumonias could not be appreciated when comparing neutrophil emigration in CD18-deficient and WT mice (21), likely due to the diverse physiological effects resulting from the genetic deficiency of CD18 by all leukocytes. However, these roles for CD11/CD18 became clear in mice with reconstituted hemopoietic systems in which the behaviors of cells of different genotypes were studied within the same physiological environment. To our knowledge, these data are the first to demonstrate that the deficiency of an adhesion molecule compromises neutrophil emigration during pneumonia. Furthermore, these studies identified CD11/CD18 expressed by neutrophils per se as critical to mediating emigration during *E. coli* LPS and *P. aeruginosa* pneumonias. These requirements for CD11/CD18 persisted for at least 24 h of *E. coli* LPS pneumonia. Finally, the data conclusively demonstrate that in contrast to *E. coli* LPS or to *P. aeruginosa*, *S. pneumoniae* induces neutrophil emigration in the lungs that does not require CD11/CD18.

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