Impairment of neutrophil emigration in CD18-null mice

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Walzog, Barbara, Karin Scharffetter-Kochanek, and Peter Gaehtgens. Impairment of neutrophil emigration in CD18-null mice. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G1125–G1130, 1999.—This study was undertaken to investigate the requirement of β2-integrins (CD11/CD18) for extravasation of neutrophils in mice. After intraperitoneal thioglycollate injection, an in vivo model of inflammation, leukocyte extravasation into the peritoneal cavity was studied in CD18-deficient and wild-type control mice. Before the induction of peritonitis, total and differential leukocyte counts in the circulation were analyzed and found to be 10 fold elevated in CD18-deficient animals compared with wild-type animals. This was largely due to neutrophilia, with a 30 fold increase in neutrophil counts. In CD18-deficient animals, extravasated white blood cells in the peritoneal cavity were diminished by 46%. The neutrophil number in the peritoneal fluid was severely reduced to 13% compared with control animals. In contrast, the number of emigrated monocytes was enhanced and lymphocyte emigration was not altered in the absence of CD18. The emigration efficiency of the neutrophils, calculated as ratio of the cell number in the lavage fluid and the circulating blood, was reduced to 0.4% in CD18-deficient mice compared with wild-type animals. Thus efficient neutrophil emigration into the peritoneal cavity strongly required CD11/CD18.

METHODS

Induction of peritonitis. In all experiments, CD18-null mice (13) or wild-type control animals of the same genetic background were used (mixed 129/Sv and C57BL/6J). Mice were injected intraperitoneally with 2 ml of 3% thioglycollate. After 4 h, animals were killed by CO2 inhalation and injected intraperitoneally with 5 ml of PBS. All peritoneal fluid was collected, and total leukocyte numbers were analyzed using a Coulter Electronics counter. Differential leukocyte counts were determined under the microscope using smears of Hematoxylin-stained cells as well as by flow cytometry (see below). Peritoneal lavage was carefully confirmed to be negative for erythrocytes by microscopy to exclude the possibility that leukocyte accumulation in the peritoneal cavity was due to microbleeding, which may occur during animal

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preparation. Animal experiments were subject to institutional approval.

Flow cytometry. Peripheral blood was collected by resection of the tip of the tail, and aliquots of heparinized whole blood (20 µl) were diluted 1:4 with PBS. Samples of blood and peritoneal washout fluid were stained using a phycoerythrin-labeled rat anti-mouse CD18 antibody (clone C71/16) as well as a FITC-labeled rat anti-mouse Gr-1 antibody (clone RB6–8C5) from PharMingen (San Diego, CA). After antibody incubation for 1 h at 4°C in the dark, cells were washed twice. Blood samples were treated with a fluorescence-activated cell sorter (FACS) lysis solution according to supplier's instructions (Becton Dickinson). In each sample, 10^6 cells were counted (FACScan, Becton Dickinson) and gated off-line for granulocytes, monocytes, and lymphocytes, using CellQuest software. Differential leukocyte numbers were calculated from absolute leukocyte numbers and values obtained by flow cytometry. In all experiments, >90% of cells gated for granulocytes were neutrophils as determined by staining for Gr-1, a marker of mature neutrophils. Statistical significance was determined using Student's t-test where applicable; P < 0.05 was considered statistically significant.

RESULTS

Neutrophilia in CD18-deficient mice. Leukocyte extravasation into the peritoneal cavity was studied in an in vivo model of inflammation using wild-type (n = 5) and CD18-deficient (n = 5) mice. Before the induction of peritonitis, total and differential blood leukocyte counts were analyzed. Total leukocyte counts were ~10-fold elevated in CD18-deficient mice (92 × 10^6/µl) compared with wild-type controls (8.9 × 10^6/µl). This was largely due to granulocytosis as shown in Fig. 1. The CD18-deficient mice revealed an ~30-fold increase of granulocyte counts in the circulation compared with wild-type animals. Analysis of Hematocolor-stained leukocyte populations under the microscope revealed that elevated granulocyte counts were due to neutrophilia. Neutrophilia was the most pronounced effect, but absolute monocyte counts (~10-fold) as well as lymphocyte counts (~3-fold) were also elevated in CD18-deficient animals compared with the wild-type control.

Leukocytes of the peripheral blood were analyzed for expression of CD18 as well as Gr-1 on the cell surface, which represents a marker for mature neutrophils (Fig. 2). Analysis of Gr-1 expression demonstrated that both wild-type as well as CD18-deficient neutrophils expressed high amounts of Gr-1 on their cell surface, revealing the presence of mature neutrophils in the circulation. For control, monocytes as well as lymphocytes were shown to be negative for Gr-1 expression. As expected, CD18 expression was high on wild-type leukocytes but completely absent on the surface of cells derived from CD18-deficient animals.

Reduced emigration of CD18-deficient neutrophils. Next, peritonitis was induced by injection of 3% thioglycollate. After 4 h, extravasated leukocytes were harvested from the peritoneal fluid, counted, and subjected to flow cytometric analysis. As shown in Fig. 3, the majority of emigrated cells in wild-type animals were granulocytes. In contrast, monocytes were the dominant population of emigrated cells in CD18-deficient animals. To confirm that neutrophil emigration was reduced in the CD18-deficient animals, extravasated leukocytes were stained for Gr-1 as shown in Fig. 4. In the wild-type animal, ~65% of emigrated cells showed high Gr-1 expression on the cell surface. In contrast, only ~16% of extravasated cells were positive for high Gr-1 expression in CD18-deficient animals, demonstrating a defect in neutrophil extravasation.

The total number of emigrated leukocytes in the peritoneal fluid revealed a marked reduction of emigration by ~46%, from 5.9 × 10^6 cells in wild-type animals to 3.2 × 10^6 cells in CD18-deficient mice. The differential leukocyte counts are shown in Fig. 5. In wild-type animals, the majority (60.4%) of emigrated cells represented neutrophils (5.00 × 10^6), whereas monocyte and lymphocyte emigration was poor. In contrast, only 0.66 × 10^6 neutrophils were detected in the peritoneal fluid of CD18-deficient animals (20.6% of emigrated leukocytes). On the basis of the total number of neutrophils in the lavage, this corresponded to a reduction of neutrophil emigration to ~13% compared with the wild-type animals. In contrast, the number of emigrated monocytes was substantially enhanced (536%) in CD18-deficient mice (1.93 × 10^6) compared with the wild-type animals (0.36 × 10^6). Lymphocyte emigration was similar in both CD18-deficient (113%) and wild-type animals.

As mentioned above, emigration in the CD18-deficient animals occurred from a substantially larger leukocyte pool in the circulating blood compared with wild-type animals. Therefore, a quantitative parameter of emigration efficiency can be estimated by calculating the ratio of cell number in the lavage fluid and the circulating blood. This revealed that emigration efficiency of neutrophils in the CD18-deficient mice was severely diminished to 0.4% of that in wild-type mice (100%) but only mildly reduced for monocytes (45%) and lymphocytes (35%).

DISCUSSION

In this study, evidence was obtained that β2-integrins play a pivotal role in the extravasation of neutrophils.
With the use of CD18-null mice, a severe reduction of neutrophil emigration was observed in response to intraperitoneal thioglycollate injection compared with control animals. This finding is in agreement with previous data obtained from LFA-1-deficient mice in which the lack of CD11a caused a marked decrease in neutrophil emigration under similar experimental conditions (14). This is also consistent with recent data showing a severe impairment of neutrophil emigration in response to toxic dermatitis in CD18-deficient mice.
Thus the β2-integrins seem to be required for neutrophil emigration, which is probably a consequence of their pivotal role for firm adhesion to the microvascular endothelium. This is supported by the finding that the bacterial-derived tripeptide N-formyl-Met-Leu-Phe was ineffective in inducing leukocyte adhesion to endothelial cells in CD18-null mice, as measured by intravital microscopy in the cremaster muscle (13). The observed absence of substantial neutrophil recruitment is also consistent with observations in patients suffering from leukocyte adhesion deficiency type I. In these patients, the absence of neutrophil emigration was observed despite the presence of proinflammatory agents, e.g., in the gastrointestinal tract (3, 9).

The present data are strictly the opposite of the results presented by Mizgerd et al. (11) who found elevated neutrophil emigration in response to thioglycollate-induced peritonitis in the same strain of CD18-null mice. We have no direct evidence to demonstrate the reason for this difference; however, varying housing or breeding conditions between laboratories may cause some variability in animal responsiveness. Besides this, the only explanation for this obvious discrepancy that we have is that microbleeding in the peritoneal cavity, which can occur during animal preparation for peritoneal lavage, may be responsible for the observed neutrophil accumulation in the above-mentioned study. Because leukocyte numbers in the peripheral blood are profoundly elevated in CD18-deficient animals, contamination of lavage fluid with peripheral blood would have a rather large impact on the estimated leukocyte number in the lavage fluid, particularly in these animals. About 10 µl of peripheral blood of CD18-deficient mice contain the number of neutrophils that were found within 4 h after thioglycollate injection in the peritoneum. A 30-fold blood volume would be required for accumulation of the same neutrophil number in the wild-type animal via bleeding. Thus bleeding can substantially affect the measurements of neutrophil accumulation in the peritoneal cavity, especially in the CD18-deficient animals.

In the present study, care was taken to rule out an erroneous interpretation of the cellular content of lavage fluid. First, the peritoneal lavage was proved to be negative for erythrocytes by microscopy to exclude an accumulation of neutrophils via injured vascular beds. Moreover, neutrophils in the peritoneal fluid were not only identified by morphology but also by Gr-1 staining, a marker for mature neutrophils. The small number of neutrophils observed in the lavage of the CD18-null mice may indicate that CD11/CD18-independent mechanisms exist that allow neutrophil emigration. Although it is not clear to what degree neutrophil emigration depends on the circulating neutrophil count, the calculated emigration efficiency in these animals, which recognizes the profoundly elevated neutrophil counts in the circulation, reveals that the dimension of a CD11/CD18-independent recruitment is extremely small. A possible candidate for CD18-independent adhesion is the α4β1-integrin very late antigen-4 (VLA-4), which has previously been shown to mediate firm adhesion of neutrophils to tumor necrosis factor-α-stimulated endothelium under flow conditions (12). This molecule also mediates monocyte
emigration by binding to vascular cell adhesion molecules (4) and may be responsible for the profound monocyte emigration observed in CD18-deficient animals. In CD18 hypomorphic mice, an enhanced P-selectin expression was observed in endothelial cells subsequent to tumor necrosis factor-α administration (7), suggesting that this molecule may have a compensatory function in leukocyte recruitment in the absence of CD18. Although we have no evidence for altered adhesion molecule expression in the CD18-null mice, it has to be taken into account that the constitutive absence of CD18 may putatively cause compensatory upregulation of other adhesion molecules that may promote CD18-independent emigration.

Altogether, this study demonstrates that neutrophil recruitment in mice depends on CD11/CD18 because emigration as well as emigration efficiency of neutrophils was severely compromised in CD18-deficient animals. In contrast, substantial monocyte and lymphocyte emigration was detectable in the absence of CD18, demonstrating that these cells can efficiently emigrate by employment of CD11/CD18-independent mechanisms. These mechanisms seem to be substantially less important for neutrophils, since the residual neutrophil emigration in the absence of CD18 was extremely small. Thus neutrophils, monocytes, and lymphocytes showed different requirements for extravasation in the peritoneal cavity.

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**Fig. 4.** Expression of Gr-1 on the surface of CD18-deficient and wild-type leukocytes emigrated in response to intraperitoneal thioglycollate injection. Fluorescence histograms are shown of leukocytes obtained from peritoneal lavage 4 h after induction of peritonitis in a wild-type and a CD18-deficient animal. Samples were stained with FITC-labeled rat anti-mouse Gr-1 antibody or were left untreated (control). In each sample, 10⁴ cells were counted and left ungated. Regions define cells with high (M1) and low (M2) surface expression of Gr-1. Numbers at top of each panel indicate cells with high Gr-1 surface expression in percentage of total cell number. Results are representative of 5 wild-type and 5 CD18-deficient animals.

**Fig. 5.** Differential leukocyte numbers in the peritoneal fluid of wild-type and CD18-deficient mice in response to intraperitoneal thioglycollate injection. Data represent means ± SD, n = 5. ***P < 0.05 vs. wild-type control. ns, Not significant.
REFERENCES


