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Relative Contribution of LFA-1 and Mac-1 to Neutrophil Adhesion and Migration

Zhi-Ming Ding,* Julia E. Babensee,*$ Scott I. Simon,* Huifang Lu,† Jerry L. Perrard,‡
Daniel C. Bullard,§ Xiao Y. Dai,‡ Shannon K. Bromley,¶ Michael L. Dustin,¶ Mark L. Entman,¶
C. Wayne Smith,* and Christie M. Ballantyne*‡

To differentiate the unique and overlapping functions of LFA-1 and Mac-1, LFA-1-deficient mice were developed by targeted homologous recombination in embryonic stem cells, and neutrophil function was compared in vitro and in vivo with Mac-1-deficient, CD18-deficient, and wild-type mice. LFA-1-deficient mice exhibit leukocytosis but do not develop spontaneous infections, in contrast to CD18-deficient mice. After zymosan-activated serum stimulation, LFA-1-deficient neutrophils demonstrated activation, evidenced by up-regulation of surface Mac-1, but did not show increased adhesion to purified ICAM-1 or endothelial cells, similar to CD18-deficient neutrophils. Adhesion of Mac-1-deficient neutrophils significantly increased with stimulation, although adhesion was lower than for wild-type neutrophils. Evaluation of the strength of adhesion through LFA-1, Mac-1, and CD18 indicated a marked reduction in firm attachment, with increasing shear stress in LFA-1-deficient neutrophils, similar to CD18-deficient neutrophils, and only a modest reduction in Mac-1-deficient neutrophils. Leukocyte influx in a subcutaneous air pouch in response to TNF-α was reduced by 67% and 59% in LFA-1- and CD18-deficient mice but increased by 198% in Mac-1-deficient mice. Genetic deficiencies demonstrate that both LFA-1 and Mac-1 contribute to adhesion of neutrophils to endothelial cells and ICAM-1, but adhesion through LFA-1 overshadows the contribution from Mac-1. Neutrophil extravasation in response to TNF-α in LFA-1-deficient mice dramatically decreased, whereas neutrophil extravasation in Mac-1-deficient mice markedly increased. The Journal of Immunology, 1999, 163: 5029–5038.

Adhesion and transmigration are critical steps required for neutrophils to emigrate from the blood to inflammatory sites (1). Numerous studies have shown that complete inhibition of CD18, the common β2-chain of the leukocyte integrins, or genetic mutations in CD18 profoundly reduce leukocyte emigration at sites of inflammation (2). The genetic disorder leukocyte adhesion deficiency I is caused by mutations in CD18 that lead to a severe or total deficiency of the CD11/CD18 integrins from the leukocyte surface, including CD11a/CD18 (LFA-1, α2β2), CD11b/CD18 (Mac-1, CR3, αMβ2), CD11c/CD18 (p150,95, α2β2), and CD11d/CD18 (α3β2) (2–4). Although leukocyte adhesion deficiency I has given insight into the functional importance of the CD11/CD18 integrins, the relative contributions of each of the CD11 integrins remain unclear.

LFA-1 and Mac-1 are the two most abundant CD11/CD18 integrins on neutrophils. A large number of studies have been performed both in vitro and in vivo to understand the functions of Mac-1 (5), with less evidence for the functional role of LFA-1 in neutrophils (6). Both these molecules have been shown to play roles in the firm adhesion of leukocytes to the endothelium, but LFA-1 appears dominant in transendothelial migration. Although CD11a and CD11b share structural similarities, are both located in a gene cluster on human chromosome 16, and function as ligands for ICAM-1 (CD54), these molecules clearly have different expression and unique functions that are not overlapping. LFA-1 cooperates with the TCR in Ag-stimulated T cell priming, and Mac-1 is an important receptor on phagocytic cells that recognizes ligands such as complement fragment iC3b. Surface levels of Mac-1 and LFA-1 on human neutrophils are approximately equal. However, levels of Mac-1 but not of LFA-1 can be rapidly increased after chemotactic stimulation because of the transport of Mac-1 to the cell surface from secretory granules. Although early in vivo studies using Abs against Mac-1 suggested that Mac-1 plays a dominant role in neutrophil emigration in vivo (7, 8), subsequent studies using Abs have demonstrated the importance of LFA-1 in neutrophil emigration (6, 9, 10).

Recently, mice deficient in Mac-1 have been developed by our group and others and found to have normal migration of neutrophils in the peritoneal cavity after chemical stimulus (6, 11). To define better the functions of LFA-1 that are unique and those that are overlapping with Mac-1, we have now generated mice deficient in LFA-1 by targeted homologous recombination in embryonic stem cells and compared neutrophil function both in vitro and in vivo with mice deficient in Mac-1, mice deficient in CD18, which therefore lack both Mac-1 and LFA-1, and wild-type (WT)3 mice.

*Speros P. Martel Laboratory of Leukocyte Biology, Department of Pediatrics, †Department of Molecular and Human Genetics, and ‡Department of Medicine, Baylor College of Medicine, Houston, TX 77030; ‡Institute of Biosciences and Bioengineering, Rice University, Houston, TX; ³Department of Comparative Medicine, University of Alabama, Birmingham, AL 35294; and ¶Department of Pathology, Washington University School of Medicine, St. Louis, MO 63110

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3 Abbreviations used in this paper: WT, wild-type; KLH, keyhole limpet hemocyanin; ZAS, zymosan-activated serum; MFI, mean fluorescence intensity; CMC, carboxymethylcellulose; MIP, macrophage inflammatory protein; D-PBS, Dulbecco’s PBS.
Materials and Methods

Animals

All animal studies were approved by the Institutional Review Board of Baylor College of Medicine.

Targeting construct and generation of LFA-1-deficient mutant mice

Murine CD11a cDNA was used as a probe to isolate two partially overlapping genomic phage clones from a 129/Sv mouse lambda library (Stratagene, La Jolla, CA). The genomic clones were characterized (Fig. 1A) by restriction mapping, Southern blot analysis, and DNA sequencing. A 7.0-kb genomic restriction fragment containing exons 3–6 and a 3.1-kb genomic restriction fragment upstream of exon 1 were ligated into the polylinker of pBluescript SK+ (Stratagene). A neomycin cassette driven by the mouse RNA polymerase II promoter was inserted between the two fragments (Fig. 1B). The construct was designed so that targeted homologous recombination with the murine CD11a gene will result in a 2.1-kb genomic fragment containing exons 1 and 2 being replaced with the 1.8-kb neomycin cassette (Fig. 1C).

The AB2.1 embryonic stem cell line (provided by Allan Bradley, Baylor College of Medicine) was electroporated with linearized vector (25 μg/ml) as described (12). Following selection with G418, individual colonies were picked and screened for targeted homologous recombination by Southern blotting using a microextraction procedure (13) with a 500-bp probe derived from 5′-flanking genomic DNA that was not included in the construct, as indicated in Fig. 1C. Cells confirmed by Southern blotting to carry the replacement mutation were injected into day 3.5 C57BL/6 blastocysts and transferred into foster mothers. Chimeric males were mated with C57BL/6 females, and germline transmission was confirmed by Southern blotting of tail DNA after digestion with EcoRI as indicated in Fig. 1D.

Mac-1- and CD18-deficient mutant mice

Mac-1-deficient mice were generated as described by Lu et al. (6). CD18-null mice, which do not have CD11a or CD11b on the cell surface (14), were back-crossed for at least 7 generations with C57BL/6J mice. The animals used for these experiments were 8- to 16-wk-old males. WT littermates were used as controls for mixed-background homozygous mutant mice (Mac-1 (−/−) and LFA-1 (−/−)), and WT C57BL/6J mice (Harlan, Indianapolis, IN) were used as controls for CD18-null mice.

Flow cytometric determination of neutrophil receptor expression

Isolated neutrophils were suspended at a concentration of 3 × 10⁶ cells/ml in Hanks HEPES buffer (NaCl, 7.15 g/L; KCl, 0.75 g/L; MgCl₂, 2.03 g/L; glucose, 1.8 g/L; HEPES, 7.15 g/L; pH, 7.35) containing 0.1% (w/v) human serum albumin and 1.5 mM CaCl₂. The cells were treated with and without zymosan-activated serum (ZAS; 10% (v/v)) at 37°C for 10 min. Thereafter, cells were maintained at 4°C and labeled with fluorescence-conjugated mAbs specific for the cell adhesion molecules (final concentration 4 μg/ml, 1 h) and analyzed by FACScan flow cytometry (Becton Dickinson Immunocytometry, San Jose, CA). The following rat mAbs to mouse cell adhesion molecules were used: LFA-1-FITC (KBA (IgG2a), kindly provided by Hideo Yagita, Juntendo University School of Medicine, or M17/4 (IgG2a), PharMingen, San Diego, CA), Mac-1-FITC (M1/70.15 (IgG2b), CalTag Laboratories, Burlingame, CA), CD18-PE (C71/16 (IgG2a), CalTag Laboratories), CD62L-FITC (MEL-14 (IgG2a), CalTag Laboratories).
The chamber was inverted for an additional 500 s. The number of cells (40 objective) under a Nikon phase contrast microscope. The cells were allowed to settle on the coated coverslips for 500 s. The total number of cells was recorded by counting the fluorescence channel 1 (FL1) and 2 (FL2) voltage 650, amplification gain 8; and fluorescence channel 3 (FL3) voltage 600, amplification gain 1.00), and linear mode. The neutrophil population was gated on in the forward-side scatter plot and controlled by characteristic emission of CD45-CY3. The mean fluorescence intensity (MFI) for the labeled receptors was quantified on the green fluorescence channel (FL1) for FITC-labeled Abs, on the red fluorescence channel (FL2) for the PE-labeled Ab, and on the orange fluorescence channel (FL3) for the CY3-labeled Ab, using linear mode. Analysis of cellular events was performed using FACScan analysis software (Becton Dickinson). Quantitation of receptor number on neutrophils under resting and ZAS-stimulated conditions was found by comparing the binding of fluorescently conjugated anti-CD11a and anti-CD11b to the cells with the binding to Quantum Simply Cellular (7) anti-rat microbeads (Flow Cytometry Standards, San Juan, Puerto Rico). These beads were derivatized with a range of rat IgG-binding capacities (bead 1: 3674; bead 2: 11,878; bead 3: 40,487; bead 4: 123,050). Bead suspensions were labeled with the Abs under identical conditions as for the cells. Beads were analyzed by FACScan using the same settings as for cells in the linear mode such that resolution was ~10,000 binding sites. A linear relation of MFI to the number of sites was obtained for each Ab. The relative receptor number under resting and stimulated conditions was computed by subtracting the fluorescence due to nonspecific Ab binding (CD23) from that for anti-CD11a and anti-CD11b and applying the MFI vs site number relation. All data are presented as mean ± SEM, n = 2 separate neutrophil preparations. 

Peripheral white blood cell counts

Blood was drawn from the tail vein. Ten µl of blood was added to Isoton II diluent (Coulter, Miami, FL), and erythrocytes were lysed with Manual Lyse (Stephens Scientific, Riverdale, NJ). The total number of leukocytes was determined by Coulter counter ZM (Coulter Electronics, Hialeah, FL). Blood smears were prepared with Neat stain (Midlantic Biomedical, Paulsboro, NJ) and analyzed under a Nikon phase contrast microscope. 

Static adhesion assay

An adhesion assay was performed as described previously (16) in a static adhesion chamber. One of the coverslips of the chamber was either covered with M1END1 or ICAM-1-liposome-coated coverslips, 5 × 10⁶ cells/ml. The suspensions were mixed and applied to the neutrophils at a concentration of 20 µg/ml. The suspensions were mixed and incubated at 25°C for 20 min. ZAS was added immediately before the cell suspension was injected into the chamber. The detachment assay was performed in a parallel flow chamber connected to a hydrodynamic flow system and a Nikon phase contrast microscope equipped with a video camera. Mouse endothelial cells were cultured in 35-mm tissue culture dishes and stimulated with mouse recombinant TNF-α (5 ng/ml; R&D Systems, Minneapolis, MN) for 4 h. The endothelial monolayer was assembled onto the flow chamber. Isolated bone marrow neutrophils (3 × 10⁶) were injected into the chamber and allowed to settle on the monolayer for 5 min. Afterward, D-PBS was perfused into the system for 1 min at a flow rate that resulted in a shear stress of 2.0 dynes/cm². In the following 4 min, the shear stress was doubled every minute until it reached 32 dynes/cm². The entire experiment was conducted at 37°C. The number of firmly adherent cells was monitored under the microscope and videotaped for analysis. The videotaped images were analyzed with Optimas image analysis software (Bioscan, Edmonds, WA). The percentage of firmly adherent cells that detached was calculated. Firmly adherent cells were defined as cells that moved less than half the cell diameter in a 10-s interval.
Results are presented as mean ± SD. Statistical analysis was performed using one-way ANOVA with Bonferroni correction for multiple comparisons, two-way ANOVA, and $t$ test. A value of $p < 0.05$ was considered significant.

**Results**

**Generation of mice deficient in LFA-1**

Embryonic stem cells with a targeted event had an 8.5-kb EcoRI fragment identified by the 5'-flanking probe on Southern blot compared with the 12.0-kb fragment in WT 129/Sv mice (Fig. 1D). Targeted embryonic stem cell clones were injected into C57BL/6J blastocysts, and male chimeric offspring that were >90% agouti coat color were bred to C57BL/6J mice. Germline transmission was confirmed by Southern blotting studies. The expected targeted allele of 8.5 kb was identified by the 5'-flanking probe on Southern blots of mice heterozygous for the mutation (Fig. 1E). In addition, a probe derived from exon 2 was also used in Southern blots of DNA from mice homozygous for the mutation, which confirmed that exon 2 was deleted in the mutant mice but present in the WT mice (data not shown).

Homozygous and heterozygous mutant mice were born in expected ratio and were fertile. Mutant mice did not demonstrate any gross abnormalities in growth and development and did not develop an increased rate of spontaneous infections when maintained in microisolator cages. Mice that were homozygous for the targeted mutation in CD11a (CD11a/CD11a) had a significantly increased peripheral white blood cell count compared with WT siblings that was primarily due to increased neutrophils (Table I).

**Surface expression of LFA-1, Mac-1, and CD18 in resting and stimulated neutrophils**

Flow cytometric analysis of isolated mouse neutrophils confirmed that mAb recognition of LFA-1 in LFA-1-deficient mutants was identical to the background level bead staining (Fig. 2A) and the nonimmune control. To examine whether targeted mutations in either LFA-1 or Mac-1 would lead to a compensatory increased expression of the other integrin subunit, we quantitated the number of binding sites per neutrophil by using flow cytometric analysis and beads calibrated with known numbers of binding sites. Unstimulated neutrophils from mice deficient in LFA-1 had similar numbers of binding sites for Mac-1 compared with WT littermates, and mice deficient in Mac-1 had similar numbers of binding sites for LFA-1 compared with WT littermates (Fig. 2B). After stimulation with 10% ZAS, the number of binding sites for Mac-1 increased by greater than 3-fold on WT and LFA-1-deficient neutrophils, whereas there was no change in the number of sites for LFA-1 on either WT or Mac-1-deficient neutrophils. LFA-1 was not detected above nonspecific background on neutrophils from LFA-1-deficient mice, and Mac-1 was not detected on neutrophils from Mac-1-deficient mice. CD62L was expressed at similar levels on LFA-1-deficient and Mac-1-deficient neutrophils and was equivalent to WT controls. Activation with ZAS was accompanied by a loss of CD62L surface expression from all the mice, reflecting a similar level of cell stimulation elicited across all the strains (data not shown).

**In vitro studies of neutrophil static adhesion**

To specifically address the interaction of LFA-1 and Mac-1 with ICAM-1, we studied the static adhesion of neutrophils from mice deficient in LFA-1, Mac-1, and CD18 to purified mouse ICAM-1. Neutrophils were isolated from the bone marrow of LFA-1 (−/−), Mac-1 (−/−), CD18 (−/−), and WT mice. The purity of neutrophils was >75% as determined by Neat (Midlantic Biomedical) stained cytospin preps before use in an adhesion assay. With ZAS stimulation, the adhesion of WT neutrophils to ICAM-1 was increased by 166%. The adhesion of CD18-deficient neutrophils was

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**FIGURE 2.** Surface expression of CD11/CD18 integrins on murine neutrophils. A. Representative fluorescence histogram of bone marrow neutrophils labeled with anti-CD11a. Plotted on a linear scale is the MFI distribution of labeled neutrophils from WT (dark gray histogram) and LFA-1 (−/−) mice (CD11a−/−; light gray histogram). The histogram for the Quantum Simply Cellular anti-rat microbeads (black line), labeled with anti-CD11a, is overlaid with the rat IgG-binding capacities indicated. Values of fluorescence intensity are included in parentheses. Neutrophils were incubated with FITC-labeled anti-LFA-1 (M17/4) at 4°C for 1 h and analyzed on a FACSscan flow cytometer. B, Receptor numbers of LFA-1 and Mac-1 expressed on LFA-1 (−/−) and Mac-1 (−/−) mice and the respective WT matched controls with (+) and without (-) stimulation with ZAS. Neutrophils alone or incubated with ZAS (10% [v/v]) at 37°C for 10 min were thereafter incubated with FITC-labeled anti-LFA-1 (KBA) or FITC-labeled Mac-1 (M1/70.15) for 1 h at 4°C. Stained cells were analyzed on the FACSscan. Neutrophils were similarly stained with an FITC-labeled isotype control Ab to CD23 (Ag not expressed on neutrophils). Quantum Simply Cellular anti-rat microbeads with different binding capacities for rat IgG were identically labeled with these Abs, and a linear relation of MFI to the number of sites was obtained for each Ab. The receptor number resulting from nonspecific Ab binding (CD23) was subtracted from the receptor numbers for LFA-1 and Mac-1 to give the relative receptor number. All data are presented as mean ± SEM of two different neutrophil preparations.
not increased with ZAS stimulation (Fig. 3A), as would have been predicted because these neutrophils lack both Mac-1 and LFA-1. Although LFA-1-deficient neutrophils demonstrated activation by ZAS as evidenced by up-regulation of Mac-1 on the cell surface, adhesion to purified ICAM-1 was not increased after ZAS stimulation. In contrast, adhesion of Mac-1-deficient neutrophils to ICAM-1 was significantly increased after ZAS stimulation, although the adhesion after activation was significantly lower than that of WT neutrophils (see Fig. 3B). Thus, stimulated neutrophils lacking LFA-1 did not show increased adhesion to either purified ICAM-1 or endothelial cells despite evidence of ZAS activation with increased surface Mac-1.

To determine the contribution of very late activation Ag 4 (VLA-4) (CD49d/CD29, \(\alpha_4/\beta_1\)) in neutrophil adhesion and to examine whether blocking mAbs gave results similar to the genetic absence of the molecule, we used Abs against LFA-1 (KBA), Mac-1 (M1/70), Mac-1 (M1/70, \(\alpha_4\) (PS2), and IgG2 control (SFDR5) were added to neutrophil suspension to a final concentration of 20 \(\mu\)g/ml. The mixture was incubated at 25°C for 20 min. After incubation, neutrophils were activated by 1% ZAS. Adhesion without Ab blocking was considered as maximum adhesion. Significant differences in adhesion from baseline or between WT and knockouts are indicated by asterisks: \(*, p < 0.05; **, p < 0.01\).
was then incubated at 37°C for 30 min in a CO₂ incubator. A solution (500 μl) containing either fibrinogen or KLH was spread onto a coverslip that was then incubated at 37°C for 30 min in a CO₂ incubator. The coated coverslip was gently rinsed twice with D-PBS, and neutrophils were added for adhesion assay under static conditions. Data were analyzed by two-way ANOVA; *p < 0.01.

The predominant component of infiltrated leukocytes at 4 h after injection of TNF-α was neutrophils, which comprised more than 80% of total leukocytes. Monocytes accounted for about 15%, and eosinophils and fibroblast-like cells made up <5% of the total cells.

To compare directly the contribution of LFA-1, Mac-1, and CD18 to leukocyte extravasation, we injected 40 ng of TNF-α into the air pouches of LFA-1-, Mac-1-, and CD18-deficient mice and analyzed leukocyte influx at 4 h after injection. In LFA-1- and CD18-deficient mice, leukocyte influx was decreased by 67% and 59%, respectively, compared with WT mice. Leukocyte influx in Mac-1-deficient mice was increased by 198% (Fig. 6). The percentage of neutrophils, monocytes, and other cells in the pouch was similar for all groups (data not shown). There was no difference in the number of mast cells attached to or in the wall of the air pouch as assessed by Alcian blue staining.

The total number of leukocytes that accumulate in the subcutaneous pouch may not be the best measure to assess leukocyte extravasation, because the peripheral white blood cell count was significantly increased in both CD18 (-/-) and LFA-1 (-/-) mice compared with Mac-1 (-/-) mice. One way to compensate for the difference in peripheral leukocyte counts is to calculate the emigration ratio (the ratio of total leukocytes or neutrophils in the air pouch to leukocytes or neutrophils in peripheral blood after injection of TNF-α) (27). The results in Table II, which compare the relative change of the emigration ratio, show that, in CD18-deficient mice, emigration ratios of total leukocytes and neutrophils were decreased by 89% and 93%, respectively, whereas in LFA-1-deficient mice, emigration ratios were decreased by 85% and 93%, respectively. Taken together, the data suggest that LFA-1 is responsible for almost all the leukocyte influx contributed by CD11/CD18 integrins in response to TNF-α. In contrast to LFA-1- and CD18-deficient mice, in Mac-1-deficient mice the emigration ratios of total leukocytes and neutrophils were increased by 238% and 270%, respectively. To examine whether the increase in leukocytes noted in Mac-1-deficient mice was due to reduced apoptosis, we examined DNA laddering. As shown in Fig. 7, there was no evidence of apoptosis as assessed by DNA laddering in leukocytes obtained 4 h after TNF-α stimulation in the air pouch, whereas DNA laddering was clearly evident in leukocytes 48 h after TNF-α stimulation.

**Discussion**

The CD18 (β2) integrins have been shown to play a critical role in the complex process of neutrophil extravasation from the peripheral blood to an inflammatory site. Most of our current understanding of the functions of the individual CD18 integrins results from the use of in vitro and in vivo blocking mAbs. While these studies have provided considerable insight into the roles of these integrins, there have been some important limitations due to three factors: 1) Complete blocking is difficult, particularly for those integrins with...
another cell surface receptor, a mAb may cause steric interference with the associated receptor (28). To define better the specific contributions of LFA-1 to the process of extravasation, we have developed mice deficient in LFA-1 and studied neutrophil function both in vivo and in vitro in parallel with functional studies in mice deficient in Mac-1 and CD18. Mice deficient in LFA-1 have a peripheral leukocytosis due to increased neutrophils, which is similar to but not as severe as that noted for mice deficient in CD18, whereas leukocytosis is not observed in Mac-1-deficient mice. Mice with a complete deficiency in LFA-1 have normal growth and development compared with WT littermates, with a very low incidence of spontaneous infection similar to WT mice when housed in microisolator cages. Mice deficient in Mac-1 also appear healthy in these housing conditions; however, under identical housing conditions, mice with a complete deficiency of CD18 have a much higher incidence of poor growth and spontaneous infection (15). Thus, absence of either LFA-1 or Mac-1 does not recapitulate the phenotype of enhanced susceptibility to infection as seen in CD18 deficiency, in which all the CD11/CD18 integrins are absent from the leukocyte surface. One potential therapeutic implication of this observation is that specific pharmacological inhibition of either LFA-1 or Mac-1 may have lower adverse risk for susceptibility to infection than inhibition of CD18.

The number of binding sites for Abs to LFA-1 and Mac-1 was approximately equal in unstimulated murine neutrophils, which is similar to human neutrophils. A deficiency of either LFA-1 or Mac-1 did not cause a compensatory increase in the number of binding sites for the other ligand, and neutrophils deficient in LFA-1 exhibited a similar increase in Mac-1 on the cell surface after ZAS stimulation compared with WT neutrophils. The CD11/CD18 integrins have previously been shown to play a critical role in neutrophil adhesion and emigration, and neutrophils from CD18-deficient mice, which lack both LFA-1 and Mac-1, did not show any increased adhesion to either murine endothelial cells or ICAM-1 with ZAS stimulation. ZAS stimulation of neutrophils from LFA-1-deficient mice also did not show increased adhesion to either murine endothelial cells or ICAM-1, even though the surface expression of Mac-1 increased severalfold. In summary, we found that, after ZAS stimulation of neutrophils, increased expression of Mac-1 in the absence of LFA-1 is not sufficient to increase adhesion to either endothelial cells or ICAM-1, whereas the absence of Mac-1 modestly but significantly reduced adhesion when LFA-1 was present. These results strongly suggest that both LFA-1 and Mac-1 are involved in the adhesion of neutrophils to endothelial cells, but that adhesion through LFA-1 overshadows the contribution from Mac-1. Our observation is consistent with a recently published study by Andrew et al. (29) that also showed that LFA-1 was important for neutrophils to adhere to either resting or TNF-α-activated murine endothelial cells. Cytokine activation of endothelial cells induces a number of other cell adhesion molecules that may potentially influence neutrophil-endothelial adhesion. Utilization of adhesion assay with neutrophils and purified ICAM-1 allowed us to examine specifically whether the absence of either LFA-1 or Mac-1 influences adhesion to purified ICAM-1.

We considered several potential mechanisms that may explain why LFA-1 had a more dominant role than Mac-1. First, we ruled out the possibility that LFA-1 was more abundant than Mac-1 on the surface of murine neutrophils by quantitation of cell surface receptors by Ab-labeled beads. Although immunobead quantitation studies revealed that there are far more binding sites for Mac-1 than for LFA-1 on ZAS-stimulated neutrophils, these studies do not rule out the possibility that more molecules of LFA-1 have undergone a conformational change to a “high-avidity” conformation. However, this would not explain why the absence of LFA-1...
would completely abolish the increase in adhesion, unless initial binding through LFA-1 were required to observe the separate contribution of Mac-1 adhesion.

We also examined whether LFA-1 is required for Mac-1 to function as an adhesion ligand. Neutrophils deficient in LFA-1 exhibited the same adhesion to fibrinogen and KLH as did WT neutrophils, but adhesion to these substrates was absent in neutrophils deficient in Mac-1. Thus, LFA-1 is not absolutely required for Mac-1 on activated neutrophils to function as an adhesion molecule to its ligands other than ICAM-1.

Finally, we examined whether LFA-1 may provide stronger adhesion to ICAM-1 than Mac-1 under shear. ICAM-1 is a common ligand for LFA-1 and Mac-1. It belongs to the Ig superfamily and consists of five Ig-like domains. In humans, LFA-1 binds to the first Ig domain of ICAM-1 (30), whereas Mac-1 binds to the third Ig domain (31). In the mouse, ICAM-1 has been identified as a ligand of LFA-1, resembling the human system (32, 33). Nevertheless, few studies have been conducted to characterize systematically the interaction of murine Mac-1 and ICAM-1. Lub et al. (34) examined the adhesion of murine macrophage cell lines that express varying amounts of LFA-1 and Mac-1 and found that both subunits can function as a ligand for ICAM-1 in the mouse as in the human. However, Mac-1-dependent adhesion to ICAM-1 was observed only when LFA-1 was absent. When LFA-1 and Mac-1 are coexpressed on the macrophage surface at similar levels, the adhesion is predominantly mediated by LFA-1. Therefore, Lub et al. concluded that there is competition between LFA-1 and Mac-1 for ICAM-1 binding. Although our studies also show that LFA-1 can itself mediate the majority of murine neutrophil adhesion to ICAM-1, neutrophils deficient in Mac-1 did have reduced adhesion in both static and shear flow conditions compared with WT neutrophils. Our observations are not consistent with the hypothesis that LFA-1 competes with Mac-1 for binding, because activated neutrophils that lacked LFA-1 and had high levels of Mac-1 did not show increased binding to purified ICAM-1 after ZAS stimulation.

We believe that these observations are consistent with the hypothesis that LFA-1 has greater avidity for ICAM-1 than does Mac-1. Once adhesion is initiated by the interaction of LFA-1 to ICAM-1, the more abundant but lower-avidity binding of Mac-1 to ICAM-1 may strengthen the adhesion. In support of this hypothesis, the absence of Mac-1 led to a significant increase in detachment of neutrophils under shear stress, but the increase in detachment was much more modest and required higher shear forces than that observed for LFA-1-deficient neutrophils. Another possibility is that the expression of LFA-1 and Mac-1 is influenced by receptor topography, though studies of LFA-1 and Mac-1 topography on mouse neutrophils are lacking. Previous studies that examined the topography of Mac-1 on human neutrophils (35) and LFA-1 on

Table II. Leukocyte emigration ratio normalized by peripheral WBC counts

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Emigration Ratio (%)</th>
<th>Decrease of Emigration Ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leukocytes</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>WT</td>
<td>78.3</td>
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<tr>
<td>CD11a (-/-)</td>
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<tr>
<td>WT</td>
<td>67.8</td>
<td>181.1</td>
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<tr>
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<td>226.1</td>
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<td>189.0</td>
</tr>
<tr>
<td>CD18 (-/-)</td>
<td>8.4</td>
<td>12.4</td>
</tr>
</tbody>
</table>

* Emigration ratio = (leukocytes in air pouch/peripheral leukocytes after injection of TNF-α) × 100%. Leukocyte numbers were determined at 4 h after injection of TNF-α.

* Percentage change of emigration ratio = (leukocyte emigration ratio of knock-out mice/leukocyte emigration ratio of WT mice) - 1] × 100%.
human lymphocytes (36) have shown Mac-1 and LFA-1 primarily on the membrane of the cell body and not clustered on microvilli; the topography of LFA-1 on neutrophils is unknown.

Chemical peritonitis induced by thioglycollate has been used in evaluating leukocyte extravasation in LFA-1- (37), Mac-1- (6), and CD18-deficient mice (14). With this model, total extravasated leukocytes in LFA-1-deficient mice were reduced by more than 50% at 4 h (37), whereas leukocyte extravasation in Mac-1-deficient mice was not changed significantly at 4 h (6) but increased substantially at 12 h after administration of thioglycollate, mainly because of an increase in neutrophils (11). However, in CD18-deficient mice, leukocyte accumulation was reported to be increased at 24 h after injection of thioglycollate (14). These results are difficult to compare because each knockout was studied in different experimental protocols and there was no attempt to correct for baseline leukocytosis in the knockout. Furthermore, the molecular signals that lead to leukocyte influx into the peritoneum after thioglycollate injection have not been characterized. Therefore, in this study we examined the effects of a specific cytokine, TNF-α, using a subcutaneous air pouch model, to compare leukocyte extravasation directly in LFA-1-, Mac-1-, and CD18-deficient mice. A pouch created by repeated injection of air results in a connective tissue-lined space resembling the synovial cavity (24). Injection of TNF-α into the pouch triggers the release of a number of chemokines including macrophage inflammatory protein-2 (MIP-2), MIP-1α, and JE. These chemokines, especially MIP-2, induce acute leukocyte influx, which is dominated by neutrophils (18). In LFA-1-deficient mice, leukocyte accumulation was decreased by about 67%, whereas in Mac-1-deficient mice, leukocyte accumulation increased by 198%, compared with WT controls. Furthermore, the reduction in leukocyte accumulation in LFA-1-deficient mice was very similar to that in CD18-deficient mice. The effect of leukocyte migration in vivo that was observed in mice deficient in LFA-1 was consistent with in vitro studies that showed that adhesion of activated neutrophils deficient in LFA-1 to either ICAM-1 or endothelial cells was similar to that of activated neutrophils deficient in CD18. Although the in vitro studies suggested that the absence of Mac-1 would not lead to a marked reduction in leukocyte extravasation, the marked increase in leukocytes in the pouch at an early time (4 h) after TNF-α stimulation was surprising. Coxon et al. (11) observed that, in thioglycollate-induced peritonitis, neutrophil accumulation was increased by 3-fold at 10.5 h. Extravasated neutrophils exhibited a significant decrease in apoptosis at 10.5 h in Mac-1-deficient mice, suggesting that the absence of Mac-1 could facilitate the accumulation of extravasated neutrophils because of a reduction in programmed neutrophil death. Although reduced apoptosis may play a role in the accumulation of neutrophils at later time points, they found no evidence of apoptosis in thioglycollate-induced peritonitis at 4 h. We also found no evidence of apoptosis after 4 h in the TNF-α-stimulated air pouch model by assessing DNA laddering, which was clearly evident at 48 h (see Fig. 7). In addition, mice deficient in Mac-1 have been described to have significantly reduced numbers of mast cells resident in the peritoneal cavity, peritoneal wall, and dorsal skin (38). We found no difference in the number of mast cells attached to or in the wall of the air pouch as assessed by Acanthamoeba blue staining. Thus, the increased number of neutrophils in the pouch is more likely due to an increased rate of influx. One potential explanation for this observation is that Mac-1 may serve as a “brake” after the neutrophil migrates below the endothelium as it moves through the interstitial space and subsequently crosses the mesothelium to enter the pouch. LFA-1 has been shown to support migration of lymphocytes on purified ICAM-1 at a speed of 10 μm/min (17), and WT murine neutrophils also exhibit a similarly rapid migration on purified ICAM-1 (M. L. Dustin, unpublished data). Therefore, it is possible that not only adhesion strength but also the migration rate supported by LFA-1 vs Mac-1 on ICAM-1 or cellular substrata would have to be considered to account for the in vivo results. In contrast to neutrophil-endothelium interactions, the molecular mechanisms that regulate neutrophil migration through the interstitium remain poorly characterized.

In summary, these data both confirm and extend our knowledge of the role that LFA-1 and Mac-1 play in neutrophil adhesion and extravasation. Although both LFA-1 and Mac-1 have structural similarities, function as ligands to ICAM-1, and play a role in the adhesion of neutrophils to endothelial cells, genetic deficiencies in LFA-1 and Mac-1 clearly demonstrate the unique contribution of each molecule to neutrophil adhesion and extravasation. Direct comparison of neutrophil adhesion in vitro demonstrated that both LFA-1 and Mac-1 are involved in the adhesion of neutrophils to endothelial cells and ICAM-1, but that adhesion through LFA-1 overshadows the contribution from Mac-1. Neutrophil extravasation in response to TNF-α in LFA-1-deficient mice was dramatically reduced, whereas extravasation of neutrophils in Mac-1-deficient mice was markedly increased.

Mac-1 has been previously shown to play an important role in the adhesion of neutrophils to parenchymal cells (39, 40), which is unique from LFA-1. The marked difference in neutrophil extravasation observed in mice deficient in LFA-1 and Mac-1 in response to TNF-α further demonstrates the unique role that these molecules play in neutrophil extravasation. LFA-1 appears to be more important in neutrophil adhesion, which is an obligate step preceding extravasation, whereas Mac-1 may be more important in events occurring after transendothelial migration, including regulating adhesive interactions of the neutrophil as it moves through the interstitial space.

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References


