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ICAM-1 and LFA-1 play critical roles in LPS-induced neutrophil recruitment into the alveolar space

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Basit, Abdul, Joerg Reutershan, Margaret A. Morris, Michael Solga, C. Edward Rose Jr., and Klaus Ley. ICAM-1 and LFA-1 play critical roles in LPS-induced neutrophil recruitment into the alveolar space. *Am J Physiol Lung Cell Mol Physiol* 291: L200–L207, 2006. First published February 3, 2006; doi:10.1152/ajplung.00346.2005.—Neutrophil recruitment into lung constitutes a major response to airborne endotoxins. In many tissues endothelial intercellular adhesion molecule-1 (ICAM-1) interacts with lymphocyte function associated antigen-1 (LFA-1) on neutrophils, and this interaction plays a critical role in neutrophil recruitment. There are conflicting reports about the role of ICAM-1 in neutrophil recruitment into lungs. We studied neutrophil recruitment into alveolar space in a murine model of aerosolized LPS-induced lung inflammation. LPS induces at least a 100-fold increase in neutrophil numbers in alveolar space, as determined by flow cytometry of bronchoalveolar lavage fluid. Neutrophil recruitment was reduced by 54% in ICAM-1 null mice and by 45% in LFA-1 null mice. In wild-type mice treated with anti-ICAM-1 and anti-LFA-1 antibodies, there was 51 and 58% reduction in the neutrophil recruitment, respectively. In chimeric mice, generated by the transplantation of mixtures of bone marrows from LFA-1 null and wild-type mice, the normalized recruitment of LFA-1 null neutrophils was reduced by 60% compared with wild-type neutrophils. Neither the treatment of ICAM-1 null mice with a function-blocking antibody to LFA-1 nor the treatment of LFA-1 null mice with anti-ICAM-1 antibody resulted in further reduction in the recruitment compared with untreated ICAM-1 null and LFA-1 null mice. We conclude that ICAM-1 and LFA-1 play critical roles in the recruitment of neutrophils into the alveolar space in aerosolized LPS-induced lung inflammation, and LFA-1 serves as a ligand of ICAM-1 in the lung.

intercellular adhesion molecule; lymphocyte function-associated antigen; lipopolysaccharide; adhesion molecules; inflammation; lung

INHALATION OF ORGANIC DUST causes an inflammatory reaction in lungs (45). Inhalation of air contaminated with gram-negative bacteria results in toxic pneumonitis characterized by fever, chills, and chest tightness, which may be encountered in humidifier disease (38) or during occupational exposures. Lipopolysaccharide (LPS)/endotoxin, a component of the outer membrane of gram-negative bacteria, is one of the primary constituents of organic dust. Inhalation of LPS by healthy volunteers results in the recruitment of neutrophils into alveolar space (26). LPS binds to LPS-binding protein (LBP) in alveolar space, and LPS/LBP complex then binds to CD14 on the surface of alveolar macrophages (27, 48). The ternary complex of CD14-LPS/LBP interacts with Toll-like receptor-4/MD-2 (TLR-4/MD-2) receptor complex on macrophages and results in the assembly of two distinct platforms of adaptor proteins on the cytoplasmic tails of TLR-4. Commercial preparations of LPS may contain contaminants that also stimulate other TLRs. The ensuing signaling events lead to the activation of NF-κB (19, 32, 35, 39). Activated NF-κB induces the secretion of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, and IL-8. These inflammatory mediators are involved in orchestrating neutrophil recruitment.

Neutrophil recruitment from blood into tissues involves multiple sequential steps mediated by the interactions of adhesion molecules (43). Interactions of selectins with their ligands mediate rolling. Rolling allows leukocytes to scan the surface of endothelium for the presence of chemokines. Chemokines on inflamed endothelium interact with their receptors on leukocytes. These interactions result in the activation of leukocyte integrins. Activated integrins interact with members of the immunoglobulin superfamily displayed on the inflamed endothelium, and these interactions cause the rolling cells to arrest on endothelium. Transmigration of arrested leukocytes across the endothelium is not as well understood. For some subsets of leukocytes, homotypic interactions of platelet/endothelial cell adhesion molecule (PECAM)-1 and CD99 are required (30). After leukocytes have transmigrated, they move into the interstitium along a gradient of chemoattractant toward the source of the chemoattractant. In some organs like intestine and lung, leukocytes then migrate across the epithelium to reach the apical side. Molecular requirements of transepithelial migration are not well understood but most likely involve a multistep process that requires sequential interactions of leukocyte adhesion molecules with their ligands on the epithelial cells (25, 49).

Intercellular adhesion molecule-1 (ICAM-1) is a member of the immunoglobulin superfamily of adhesion molecules. Members of this family have a variable number of immunoglobulin-like (Ig-like) domains in their extracellular segment, a single spanning transmembrane segment, and a short cytoplasmic tail. ICAM-1 has five Ig-like domains in its extracellular segment. Its molecular mass ranges between 90 and 110 kDa depending on the degree of glycosylation. It binds to two β_2-integrins on leukocytes, lymphocyte function-associated antigen-1 (LFA-1) and Mac-1. The degree of ICAM-1 glycosylation inversely affects its binding to Mac-1 but has little effect on its binding to LFA-1 (6). ICAM-1 is expressed on the surface of most

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leukocytes, fibroblasts, and endothelial cells, but not on neutrophils. The expression of ICAM-1 is upregulated in many tissues after stimulation with LPS, TNF-α, and IL-1 (11). In lungs, basal levels of ICAM-1 are appreciable and the expression increases further in sepsis (12, 22, 33). There is compelling evidence for the role of endothelial ICAM-1 in the recruitment of leukocytes into many tissues (18, 40, 42).

LFA-1 belongs to the β_2-integrin family of adhesion molecules. β_2-Integrins are heterodimers of α- and β-chains. The β_2-family has four members, which share a common β-chain, CD18, and differ from one other in their α-chains. The four heterodimers are CD11a/CD18 (LFA-1), CD11b/CD18 (Mac 1), CD11c/CD18, and CD11d/CD18. LFA-1 is expressed on all leukocytes, and it binds to ICAM-1 on endothelium and on other cells. Under basal conditions LFA-1 is inactive. On stimulation with chemoattractants like IL-8, LFA-1 is activated and binds to ICAM-1 (5). There are conflicting reports about the role of ICAM-1 in neutrophil recruitment into lungs. Treatment with antibody against ICAM-1 decreases neutrophil recruitment into lungs in wild-type mice, but neutrophil recruitment was reported to be unaffected in ICAM-1 mutant mice (8, 21, 29, 36). Antibody to LFA-1 decreases neutrophil recruitment, but these findings have not been confirmed in LFA-1-deficient mice (36). It has been hypothesized that compensatory mechanisms confound insight into neutrophil recruitment in gene knockout mice (8).

To clarify the role of ICAM-1 and LFA-1 in the recruitment of neutrophils into lungs, we modified a murine model of inflammation induced by aerosolized LPS (31). We counted neutrophils recruited into alveolar space by flow cytometry analysis of bronchoalveolar lavage fluid (BALF). Twenty-four hours after aerosolized LPS treatment, the number of neutrophils in BALF increased at least 100-fold. We used wild-type, ICAM-1 null, LFA-1 null mice, and bone marrow-chimeric mice, as well as function-blocking antibodies to LFA-1 and ICAM-1, to determine the contribution of these adhesion molecules in neutrophil recruitment into the alveolar space.

MATERIALS AND METHODS

Mice. Wild-type mice (C57BL/6 strain) were obtained from Jackson Laboratories (Bar Harbor, ME). ICAM-1 null mice were generated by the deletion of the entire coding region of the ICAM-1 gene (37). LFA-1 null mice have been described previously (7). Knockout mice were backcrossed to wild-type mice (C57BL/6 strain) for at least eight generations. All mice were kept under specific pathogen-free conditions.

Chimeric mice. Chimeric mice were generated by the transplantation of 1:1 mixtures of bone marrows from wild-type mice and LFA-1 null mice as described previously (14). Recipient mice were lethally irradiated by two doses of 600 rads each. The doses were 4 h apart. Donor mice were killed by lethal injections of pentobarbital sodium (Nembutal; Abbott Laboratories, North Chicago, IL). Bone marrow cells from both femurs and tibias were harvested under sterile conditions. Approximately 50 million cells were obtained from each donor mouse. Bones were flushed with RPMI (Life Technologies, Grand Island, NY). Suspended bone marrow cells were washed, and erythrocytes were lysed in lysis buffer (0.15 M NH_4Cl). Unfractionated LFA-1 null bone marrow cells were mixed with unfractionated wild-type bone marrow cells in RPMI. Cell density was adjusted to 10 million cells/500 μl, and 500 μl were injected intravenously into each irradiated wild-type mouse via tail vein. Recipient mice were housed in a barrier facility (individually ventilated cages, HEPA-filtered air, sterile bedding, and autoclaved food) under pathogen-free conditions and were given autoclaved water supplemented with antibiotics [5 mM sulfamethoxazole, 0.86 mM trimethoprim (Sigma, St. Louis, MO)]. These conditions were maintained for 8 wk, after which mice were used for studies. The Animal Care and Use Committee of the University of Virginia approved all studies.

LPS-induced lung inflammation. Up to four mice were placed in a Plexiglas cylindrical chamber (9 × 3 inches). The chamber was connected to a nebulizer (NE-U2VV, Micro*Air*VMT; Omron, Bannockburn, IL) on one side and a vacuum system on the opposite side. Seven milliliters of LPS solution (*Salmonella enteriditis*, Sigma) 0.5 mg/ml were aerosolized into the chamber, and mice were allowed to breathe air containing the aerosolized LPS. No attempt at purification of LPS was made. Control mice were given aerosolized vehicle (PBS). Mice were removed from the chamber after complete aerosolization of LPS or vehicle and returned to room air. Complete aerosolization of the LPS was achieved at 30 min. The average effective dose inhaled by each mouse over 30 min was 216 ng, based on the respiratory parameters determined in resting mice by the Buxco PLY 3211 system (Buxco Electronics, Wilmington, NC). The rate of inhalation of LPS was therefore ~ 7 ng·min^{-1}.

Different techniques of delivering endotoxin to the pulmonary system of animals have been used. The distribution and clearance of the endotoxin in the pulmonary system are specific to the technique used. Foster and colleagues (15) studied the deposition and clearance of insoluble radioisotope particles in mice by three different methods: nose-only inhalation, oropharyngeal aspiration, and intratracheal instillation. Nose-only inhalation was associated with more even distribution of the radioisotope in lung and oropharyngeal regions than the other two methods, which delivered the isotope predominantly to the lung region. Nose-only inhalation was also associated with faster clearance of the radioisotope than the other two techniques. Leong and colleagues (23) compared the deposition and distribution of dye particles in the pulmonary system of rats by three different methods: intratracheal nebulization, intratracheal fast instillation, and nose-only inhalation. Nose-only inhalation was found to be useful for toxicological studies, where uniform deposition of toxin was of prime interest. Our model simulates the nose-only inhalation method except that in our studies mice are not restrained. It is therefore possible that in our studies some LPS was deposited and absorbed from the skin in addition to inhalation.

In some experiments, anti-LFA-1 antibody (TIB 217) or anti-ICAM-1 antibody (YN1/1.7.4) (5 μg/g) or a control rat IgG2a or rat IgG2b, respectively, was injected intraperitoneally before LPS administration. The LFA-1 MAb TIB-217 and the ICAM-1 MAb YN1/1.7.4 were purified from hybridoma supernatant (American Type Culture Collection, Manassas, VA) in the Lymphocyte Culture Center at the University of Virginia. Purified rat IgG2a and rat IgG2b were purchased from BD Pharmingen (San Jose, CA).

Mice were anesthetized 24 h after LPS treatment by intraperitoneal injection of ketamine (125 μg/g, Ketalar; Parke-Davis, Morris Plains, NJ), xylazine (12.5 μg/g; Phoenix Scientific, St. Joseph, MO), and atropine sulfate (0.025 μg/g; Elkins-Sinn, Cherry Hill, NJ), and blood was drawn from the right ventricle. Tracheae were cannulated with a 22-G catheter (BD Insyte I.V., Franklin Lakes, NJ). One milliliter of PBS was infused and then withdrawn from the lungs via a tracheal catheter, and the process was repeated six times. This yielded 7 ml of lavage fluid from each mouse.

Flow cytometry. Lavage fluid was centrifuged, and each cell pellet was resuspended in flow cytometry buffer (1% BSA and 0.1% sodium azide in PBS). Cells in an aliquot were counted in a hemacytometer after staining with Trypan blue (Sigma), and total cell count was calculated for the whole volume of lavage fluid. Lavage fluid cells were then incubated with fluorescent antibodies according to manufacturers' recommendations: CD45 PerCP (clone 30-F11), 7-4 FITC (clone 7/4), LFA-1 phycoerythrin (PE) (clone 2D7), PECAM-1 PE (clone MEC 13.3), ICAM-1 FITC (clone 3E2), and Gr-1 allophyco-

cyanin (APC) (clone RB6-8C5) and corresponding isotypes: rat IgG2b peridinin chlorophyll-a protein (PerCP), rat IgG2a FITC, rat IgG2a PE, and rat IgG2b APC. After a 20-min incubation at 4°C, samples were washed twice in the flow cytometry buffer and fixed in 1% formalin (Sigma). All antibodies were purchased from BD Pharmingen (San Diego, CA) except for 7-4 FITC, Gr-1 APC, Rat IgG2a FITC, and Rat IgG2b APC, which were purchased from Caltag (Burlingame, CA).

Lung samples were cut into small pieces and suspended in a mixture of enzymes: 125 units/ml collagenase XI, 60 units/ml hyaluronidase, and 60 units/ml DNase (Sigma). The suspension was warmed at 37°C for 30 min and filtered through a 70-μm nylon cell strainer to achieve a single cell suspension. Further staining was done as described for the lavage fluid cells.

Staining of blood cells was done as described for lavage fluid cells with a few exceptions. To determine the expression of LFA-1, PE-labeled anti-LFA-1 antibody was used. After staining the samples, we performed red blood cell lysis twice with lysis fluid (0.15 M NH_4Cl) followed by a wash with flow cytometry buffer. After the final wash cells were fixed in 1% formalin. The samples were measured on a FacsCalibur flow cytometer (BD Pharmingen) using Cell Quest Pro software (BD Pharmingen), and data were analyzed by FlowJo software program (Tree Star, Ashland, OR).

Cytospin of lavage fluid. Lavage fluid cells (100,000 cells in 100 μl) were centrifuged onto a slide at 1,000 rpm for 10 min. Slides were air dried, fixed, and stained with Diff Quick (IMEB, San Marcos, CA) according to the manufacturer's recommendations. Images of slides were processed by Image-Pro Plus software (Media Cybernetics, Silver Spring, MD).

Blood counts. Blood samples from tail veins of mice were analyzed for differential counts by Hemavet 850 FS (CDC Technologies, Oxford, CT).

Statistical analysis. Means of two groups were compared by *t*-test, and multiple groups were analyzed by ANOVA followed by Tukey's post hoc test. Data are presented as means ± SE. SPSS12 software (SPSS, Chicago, IL) was used to perform statistical analysis.

RESULTS

Aerosolized LPS induces neutrophil recruitment into BALF. BALF cells were analyzed by gating on CD45-positive cells (Fig. 1*A*). CD45-positive cells were then examined for the expression of 7-4 and Gr-1. 7-4 and Gr-1 double-positive cells are neutrophils (Fig. 1*A*). In mice, neutrophils are 7-4 positive and Gr-1 high, whereas monocytes are Gr-1 low (17). To minimize the inclusion of monocytes, we used two markers of neutrophils: 7-4 and Gr-1. At 24 h after LPS treatment, 62.7% of the leukocytes in the lavage fluid were neutrophils, which was a striking elevation over 0.55% neutrophils in saline-treated mice (Fig. 1*A*). We obtained the total number of neutrophils in BALF by multiplying the percentage of 7-4 and Gr-1 double-positive cells with the total number of cells in BALF. The total number of neutrophils in BALF showed a progressive increase that peaked at 24 h and declined to baseline by 72 h (Fig. 1*B*). There was at least a 100-fold increase in the number of neutrophils 24 h after LPS compared with vehicle (Fig. 1*B*).

LPS induces expression of ICAM-1 on lung endothelium. To determine the expression of ICAM-1 on endothelium, lung cells were investigated for the expression of CD45 and PECAM-1. CD45-negative, PECAM-1-positive cells are endothelial cells (Fig. 2*B*). With regard to the expression of ICAM-1, two populations of pulmonary endothelial cells were seen under basal conditions, one with intermediate intensity of ICAM-1 and the other with high intensity (Fig. 2*D*). After LPS

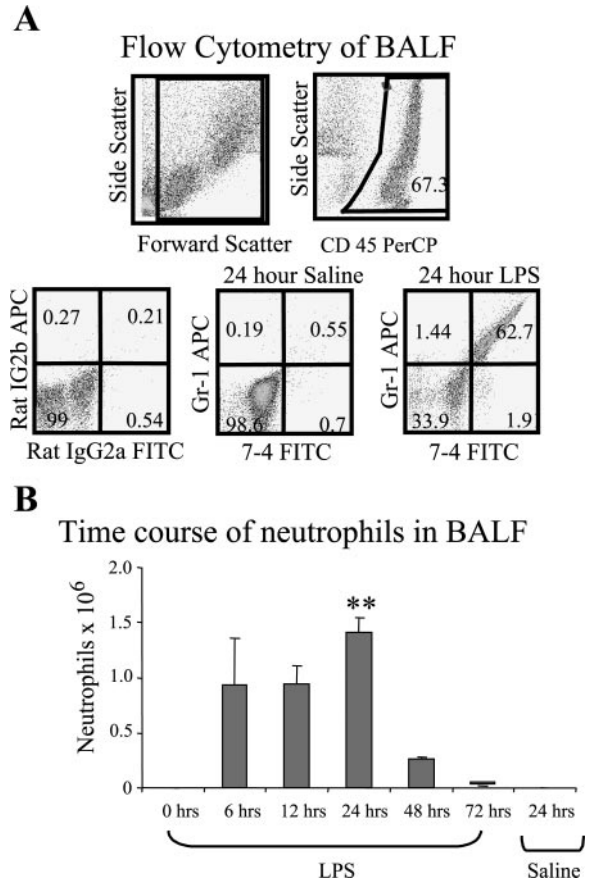

Fig. 1. Aerosolized LPS induces neutrophil recruitment into bronchoalveolar lavage fluid (BALF). *A*: flow cytometry of BALF. BALF cells from wild-type (WT) mice stained with CD45 PerCP, 7-4 FITC, and Gr-1 APC were investigated for the expression of CD45 cells. CD45+ cells were then investigated for the expression of 7-4 FITC and Gr-1 APC double-positive cell to identify neutrophils. Numbers in quadrants reflect the percentage of cells in each respective quadrant. Graphs are representative of 7 experiments. *B*: time course of neutrophils in BALF. BALF cells from WT mice at indicated times after LPS or saline were stained with Trypan blue, and cells were counted in a hemacytometer to obtain total number of cells in the BALF at that time. Samples for fluorescence-activated cell sorting (FACS) were prepared as in *A*. Neutrophil percentage (percentage of 7-4 FITC and Gr-1 APC double-positive cells) at each indicated time point was obtained from FACS. To get the total number of neutrophils at each time, this percentage was multiplied with the total number of cells in the BALF at the corresponding time. $^{**}P < 0.001$ compared with saline-treated group; $n = 2$–7.

treatment, there was increased expression of ICAM-1 on cells with intermediate expression such that by 12 h the percentage of cells with high intensity of ICAM-1 increased significantly (Fig. 2*F*). By 24 h the two populations could be seen again (Fig. 2*G*).

ICAM-1 and LFA-1 play critical roles in neutrophil recruitment. To determine the role of ICAM-1 and LFA-1 in neutrophil recruitment into the alveolar space, we used ICAM-1 null and LFA-1 null mice. We also employed neutralizing antibodies to inhibit these adhesion molecules in wild-type mice. Neutrophil recruitment was decreased by 54% in ICAM-1 null mice and by 45% in LFA-1 null mice compared with wild-type mice (Fig. 3*A*). It has been proposed that the number of neutrophils in circulating blood can affect the recruitment of neutrophils into tissues. To exclude the possibility of neutropenia in null mice that could have resulted in the decreased

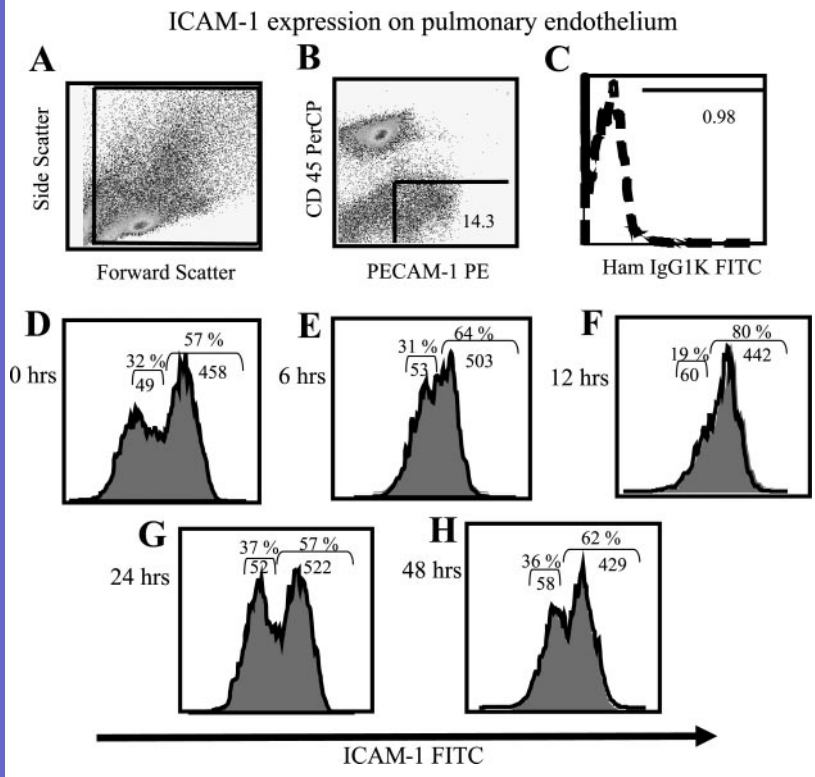

Fig. 2. Intercellular adhesion molecule (ICAM)-1 expression on pulmonary endothelium by flow cytometry. Lung samples obtained from mice at indicated time points after LPS treatment were digested in enzymes as described in MATERIALS AND METHODS, and single cell suspensions were stained with ICAM-1 FITC, platelet/endothelial cell adhesion molecule (PECAM)-1 PE, and CD45 PerCP. *A*: forward scatter and side scatter. *B*: CD45 PerCP-negative and PECAM-1 PE-positive cells are endothelial cells. *C*: isotype control for ICAM-1 on endothelial cells. *D–H*: ICAM-1 expression on endothelial cells at indicated times points after LPS. Numbers on top of gate lines indicate percentage of cells in that particular gate and numbers below a gate line indicate mean fluorescence of endothelial cells for ICAM-1 expression. Graphs represent 2 experiments at each time point.

recruitment of neutrophils in these mice, we performed blood counts before giving aerosolized LPS (Table 1). There was no decrease in circulating neutrophils in knockout mice to account for the reduction in neutrophil recruitment. Neutrophil recruitment was reduced by 51% in wild-type mice treated with anti-ICAM-1 antibody and by 58% in wild-type mice treated with anti-LFA-1 antibody (Fig. 3*B*).

To test whether a compensatory mechanism in LFA-1 null mice may have contributed to the results, we compared the behavior of LFA-1 null neutrophils directly with wild-type neutrophils in reconstituted wild-type mice. To achieve this, we generated chimeric mice as described in methods. Eight weeks after the bone marrow transplant, experiments were performed in these chimeric mice. On average, 63% of the neutrophils in these chimeric mice were of wild-type origin (LFA-1 positive) and 37% were of LFA-1 null origin (LFA-1 negative) (Fig. 4*A*, *right*). To correct for the deviation from a 1:1 ratio of wild-type and LFA-1 null neutrophils in blood, we determined the recruitment of LFA-1 null neutrophils in BALF as a function of LFA-1 null neutrophils in blood and normalized this ratio to the recruitment of wild-type neutrophils in BALF as a function of wild-type neutrophils in blood taken as 100%. The recruitment of LFA-1 null neutrophils was decreased by 63% compared with wild-type neutrophils (Fig. 4*B*, *right*).

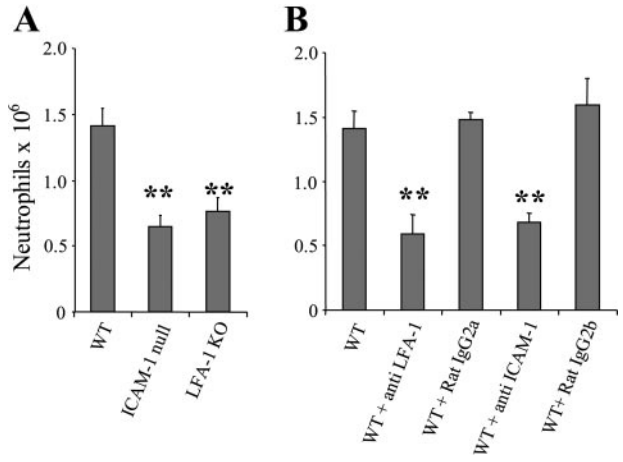

Fig. 3. Role of ICAM-1 and lymphocyte function-associated antigen-1 (LFA-1) in neutrophil recruitment. *A*: neutrophil counts in BALF from WT mice, ICAM-1 null mice, and LFA-1 null (KO) mice were determined as in Fig. 1*C*. $^{**}P < 0.005$ between wild-type and null groups; $n = 6–11$. *B*: neutrophil counts in BALF of WT mice were determined as in Fig. 1*C*. In some experiments, mice were given indicated function-blocking antibodies or isotype control antibodies (5 μg/g ip) 1 h before LPS treatment. $^{**}P < 0.005$ between mice treated with function-blocking antibodies and untreated groups; $n = 4–7$.

LFA-1 and ICAM-1 are in the same adhesion pathway. The recruitment of neutrophils in ICAM-1 null mice treated with a function-blocking antibody to LFA-1 is not different from that seen in untreated ICAM-1 null mice (Fig. 5). Similarly, the recruitment of neutrophils in LFA-1 null mice treated with an anti-ICAM-1 antibody is not different from that in untreated LFA-1 null mice (Fig. 5). These results suggest that LFA-1 is a ligand for ICAM-1 in this model of lung inflammation.

DISCUSSION

We confirm that aerosolized LPS is a potent stimulus of neutrophil recruitment into the bronchoalveolar space. This recruitment is reversible with a return in BALF neutrophil count to baseline by 72 h. We show that ICAM-1 and LFA-1 play critical roles in this process. Because an LFA-1 MAb has no further effect in ICAM-1 null mice nor does an ICAM-1

Table 1. *Blood leukocyte counts ($\times 10^3/\mu l$)*

	Leukocytes	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
WT	10.01±0.95	1.29±0.18*	8.38±1.07	0.29±0.03	0.06±0.01	0.01±0
ICAM-1 KO	12.79±1.85	2.79±0.25	9.54±1.60	0.38±0.04	0.04±0.01	0.01±0
LFA-1 KO	12.53±1.56	3.02±0.38	8.38±1.07	1.05±0.31†	0.06±0.01	0.01±0

Values are means ± SE, $n = 7$ in each group. WT, wild type; ICAM, intercellular adhesion molecule; KO, knockout; LFA, lymphocyte function-associated antigen. *$P < 0.005$ and †$P < 0.05$ compared with other groups.

MAb have further effect in LFA-1 null mice, we conclude that LFA-1 is a ligand for ICAM-1 in the inflamed mouse lung.

The original description of the multistep paradigm of inflammation was developed in the microcirculations of mesentery, skin, and cremaster of animals (1, 24, 46). In lungs some aspects of the process were found to be different from other tissues. First, unlike many tissues, in lungs the major site of neutrophil recruitment is the capillary bed, not postcapillary venules (4). Second, although rolling has been described in pulmonary arterioles and venules, it probably does not contribute to neutrophil recruitment into lungs, as rolling does not occur in the capillaries. The discrepancy between the size of neutrophils and the diameter of pulmonary capillaries requires neutrophils to deform to navigate the capillary bed under basal conditions (16). In inflammation, the neutrophils' ability to deform is impaired significantly due to stiffening induced by inflammatory stimuli. This stiffening slows the neutrophils further. These mechanical changes may make rolling unnecessary in the pulmonary circulation (10). Third, neutrophil CD18 integrins are major contributors to neutrophil recruitment into many tissues, but in the lung their contribution is partial and is limited to some models of lung inflammation (9). Integrins are required to cause the arrest of a rolling leukocyte on inflamed endothelium, and for this to occur, integrins need to be activated. Interactions of chemokines on endothelial surface with their receptors on leukocytes result in the activation of leukocyte integrins. In addition, these interactions of chemokines with their receptors cause directed migration of leukocytes towards the source of chemokines.

ICAM-1 plays a major role in the recruitment of neutrophils and lymphocytes in many tissues (11, 29, 41, 42, 44). Its role in the recruitment of neutrophils in lung inflammation has been studied in different murine models. Bullard and colleagues (2) compared *Streptococcus pneumoniae*-induced recruitment of neutrophils into peritoneum and alveolar space in P-selectin/ICAM-1 double-mutant mice. Neutrophil recruitment into peritoneum was decreased in the ICAM-1 mutant mice by 2.5-fold and in the double mutant mice by >30-fold compared with wild-type mice, but neutrophil recruitment into the alveolar space was not decreased in the double-mutant mice compared with wild-type mice. These findings suggested that in contrast to other tissues, ICAM-1 is not involved in neutrophil recruitment in the pulmonary circulation. The possibility of a compensatory mechanism in the mutant mice that overcame the impairment in neutrophil recruitment could not be excluded. In another report, effects of antisense oligonucleotides directed at ICAM-1 mRNA and function-blocking anti-ICAM-1 antibody were compared with ICAM-1 mutant mice in a model of endotoxin-induced pneumonia (21). Antisense oligonucleotides decreased the mRNA expression. Both antisense oligo-

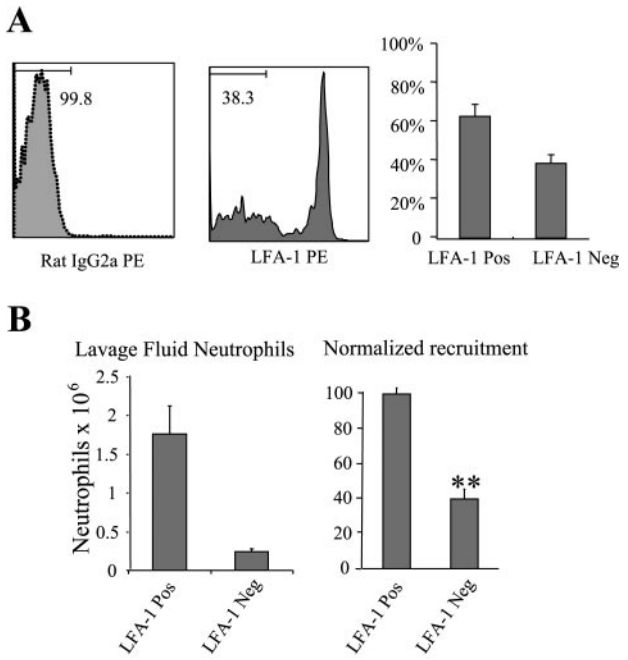

Fig. 4. Chimeric mice. *A*: chimeric mice have a mixture of WT and LFA-1 null neutrophils. Blood from chimeric mice was stained with LFA-1 PE. Neutrophils in blood were gated by forward-scatter and side-scatter criteria. The expression of LFA-1 PE on neutrophils was determined: isotype control (*left*), LFA-1 PE (*middle*), distribution of WT (LFA-1 Pos), and LFA-1 null neutrophils in the chimeric mice (*right*); $n = 4$. *B*: LFA-1 null neutrophil recruitment is impaired in chimeric mice. Cells in the lavage fluid from chimeric mice were stained with CD45 PerCP, 7-4 FITC, LFA-1 PE, and Gr-1 APC. Gr-1 APC and 7-4 FITC double-positive neutrophils were then analyzed for the expression of LFA-1. The number of WT and LFA-1 null neutrophils in BALF from chimeric mice is shown at *left*. The ratio of LFA-1 null neutrophils in BALF to LFA-1 null neutrophils in blood normalized to the ratio of WT neutrophils in BALF to WT neutrophils in blood taken as 100% is shown at *right*. **$P < 0.005$; $n = 4$.

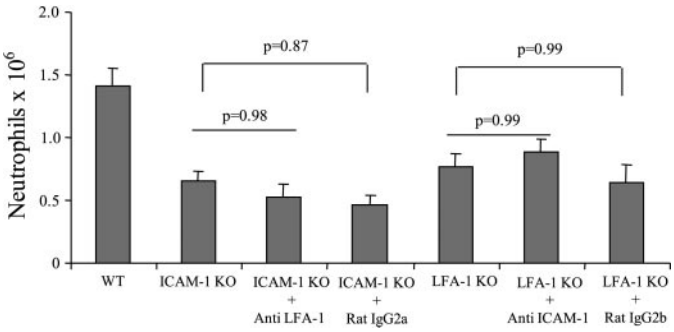

Fig. 5. LFA-1 and ICAM-1 are in the same adhesion pathway. ICAM-1 null mice were treated with anti-LFA-1 antibody or isotype control antibody, and LFA-1 null mice were treated with anti-ICAM-1 antibody or isotype control antibody (5 μg/g ip) 1 h before LPS. Neutrophil recruitment was compared with mice not treated with antibodies; $n = 5$–7.

nucleotides and anti-ICAM-1 antibody reduced the alveolar neutrophil counts in wild-type mice by 58%. As the reduction in neutrophil recruitment was not complete, the possibility of an ICAM-1-independent pathway was raised. In contrast to the reduced neutrophil recruitment in wild-type mice with antisense oligonucleotides and antibody, no reduction was seen in the ICAM-1 mutant mice. Similar findings were obtained in another report that investigated the contribution of ICAM-1 in *Pseudomonas aeruginosa*-induced pneumonia (36). The effect of anti-ICAM-1 antibody on neutrophil recruitment was also investigated by Moreland and colleagues (29), who studied neutrophil recruitment in a model of LPS-induced lung inflammation and reported significant reduction in neutrophil recruitment with anti-ICAM-1 antibody in wild-type mice. These investigators, however, did not investigate ICAM-1 gene-modified mice.

The discrepancy between the effects of acute reduction in ICAM-1 expression obtained by antisense oligonucleotide treatment or reduction in ICAM-1 function by anti-ICAM-1 antibody treatment compared with ICAM-1 mutant mice that have defective ICAM-1 suggested that the duration for which ICAM-1 was defective could explain the discrepancy. ICAM-1 mutant mice have defective ICAM-1 throughout the duration of development and have the opportunity to compensate for this defect by upregulating other mechanisms. This is less likely when ICAM-1 expression or function is blocked acutely with antisense oligonucleotides or with function-blocking antibody, respectively (47). Another explanation offered was that antibody to ICAM-1 could have reduced neutrophil recruitment in wild-type mice by an ICAM-1-independent mechanism. Antibodies can have additional effects by cross-linking the antigen with Fc receptors.

One major difference between the previous reports and our work lies in the ICAM-1-deficient mouse strains. The ICAM-1 mutant mice used in the aforementioned studies were generated by the disruption of exon 5 of the ICAM-1 gene. In these mice, alternatively spliced forms of ICAM-1 have been found in lung endothelium under inflammatory conditions (20). These alternatively spliced variants do retain the first Ig-like domain (D1) of ICAM-1 that binds LFA-1. The antibody to ICAM-1 used in these studies recognizes this domain (36). The finding that this same antibody decreased neutrophil recruitment in two different models of lung inflammation in wild-type mice but failed to reduce neutrophil recruitment in ICAM-1 mutant mice suggests that the role of the alternatively spliced forms of ICAM-1 is not the same as that of full-length ICAM-1. The ICAM-1 exon 5 mutant mice may have other undiscovered abnormalities that might have confounded the results.

The discrepant findings in previous reports fueled a controversy about the role of ICAM-1 in neutrophil recruitment into lungs (2, 28, 47). Given the central role ICAM-1 plays in neutrophil recruitment in systemic circulation, we conducted experiments to resolve the discrepant findings about the role of ICAM-1 in pulmonary circulation. In the ICAM-1 null mice used in our studies, the whole coding region of ICAM-1 gene is absent, and alternatively spliced variants have not been discovered (37). In contrast to previous reports, our findings firmly establish the critical role ICAM-1 plays in neutrophil recruitment into the alveolar space. We found two populations of endothelial cells in mouse lungs that spontaneously expressed ICAM-1 at high and intermediate levels, respectively. Because flow cytometry does not preserve the structural integrity of the lung tissue, we do not know which anatomic locations contribute to these populations, but it is clear from the present data that after LPS inhalation almost all lung endothelial cells express ICAM-1 at high levels.

To test whether LFA-1 is a ligand for ICAM-1, we treated ICAM-1 null and LFA-1 null mice with function-blocking antibodies. We reasoned that if neutrophil LFA-1 were binding an adhesion molecule on endothelium other than ICAM-1, we would see greater reduction in neutrophil recruitment in ICAM-1 null mice and LFA-1 null treated with anti-LFA-1 antibody and anti-ICAM-1 antibody, respectively, than in untreated ICAM-1 null and LFA-1 null mice. We found no further reduction in ICAM-1 null and LFA-1 null mice treated with function-blocking antibodies compared with untreated mice, suggesting that LFA-1 is a ligand for ICAM-1.

To reach the alveolar space from blood, neutrophils must migrate across the pulmonary endothelium, chemotax through interstitial tissue, and finally cross pulmonary epithelium. ICAM-1 has been shown to be expressed on canine lung cultured fibroblasts (3) and on the bronchial epithelium (13, 34). Thus there are three levels during the transit of neutrophils from blood to alveolar space where ICAM-1 in our experiments could have contributed: endothelial, fibroblast, and bronchial epithelium. ICAM-1 on fibroblasts in lung parenchyma has been shown to support neutrophil crawling (4), and the contribution of ICAM-1 to neutrophil recruitment at this level in our model remains a possibility. The orientation of ICAM-1 on the epithelium is such that its ligand-binding domains face the lumen, and this orientation is less likely to allow ICAM-1 to interact with leukocytes migrating from the basal surface of the epithelium (49). However, the possibility remains that bronchial or alveolar epithelial ICAM-1 can contribute to the neutrophil recruitment. With the conclusive evidence for the role of ICAM-1 in neutrophil recruitment into the alveolar space reported here, we think that the stage is set to dissect the level where ICAM-1 is critical. ICAM-1 may contribute at more than one level.

On the basis of previous and present results, we conclude that ~50% of neutrophil recruitment to the alveolar space is mediated by the interaction of LFA-1 with ICAM-1. The nature of the remaining recruitment after LFA-1 and ICAM-1 are blocked or knocked out remains to be determined.

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