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L-selectin is required for fMLP- but not C5a-induced margination of neutrophils in pulmonary circulation

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Olson, Timothy S., Kai Singbartl, and Klaus Ley. L-selectin is required for fMLP- but not C5a-induced margination of neutrophils in pulmonary circulation. *Am J Physiol Regulatory Integrative Comp Physiol* 282: R1245–R1252, 2002. First published December 21, 2001; 10.1152/ajpregu.00540. 2001.—To study the role of L-selectin in neutrophil (PMN) margination and sequestration in the pulmonary microcirculation, maximally active concentrations of C5a (900 pmol/g) and *N*-formylmethionyl-leucyl-phenylalanine (fMLP; 0.34 pmol/g) were injected into the jugular vein of wild-type or L-selectin-deficient C57BL/6 mice. In wild-type mice administered C5a or fMLP, 92 ± 1% and 34 ± 9%, respectively, of peripheral blood PMN were trapped mostly in the pulmonary circulation as determined by immunohistochemistry and myeloperoxidase activity. In wild-type mice treated with $F(ab')_2$ fragments of the L-selectin monoclonal antibody MEL-14 or in L-selectin-deficient mice, C5a-induced neutropenia was not significantly reduced, but the decrease in peripheral PMN in response to fMLP was completely abolished, indicating that L-selectin is necessary for fMLP- but not C5a-induced pulmonary margination. Immunostained lung sections of fMLP- or C5a-treated mice showed sequestered neutrophils in alveolar capillaries with no evidence of neutrophil aggregates. We conclude that chemoattractant-induced PMN margination in the pulmonary circulation can occur by two separate mechanisms, one of which requires L-selectin.

formyl peptides; complement; MEL-14; adhesion molecules

NEUTROPHILS PLAY A PRIMARY role in mediating many types of acute lung injury, including the pathology seen in patients with adult respiratory distress syndrome (ARDS) (12). During this response, circulating neutrophils become marginated within the pulmonary circulation and, along with the large marginated pool of neutrophils present under physiological conditions, become activated to mediate inflammation and subsequent endothelial and alveolar epithelial damage (20). Although the causes of margination are well understood for the peripheral circulation (42), the roles of various adhesion molecules in the margination and subsequent sequestration of neutrophils within the pulmonary circulation are unclear (10).

The size and complex geometry of pulmonary capillary segments are likely responsible for the creation of a large marginated pool of neutrophils within the lungs (21). In animal models, >60% of pulmonary capillary segments are more narrow than the diameters of resting neutrophils (9). While normal flow of erythrocytes is maintained due to the geometry of the capillary beds, neutrophil transit time is increased, and consequently, neutrophils become 60–65-fold more concentrated in the lung vasculature than in the peripheral circulation (8, 16, 22). This marginated pool comprises 40% of total body neutrophils in mice (38), with neutrophils in the pulmonary circulation outnumbering those in the peripheral circulation by two- or threefold (8). The majority of these cells are found within capillaries (36), although rolling neutrophils have occasionally been seen in venules (32).

Further margination of circulating neutrophils followed by a variable period of sequestration, mimicking the pathophysiological reaction seen in ARDS, can be elicited experimentally by a variety of techniques, including intravenous injection of C5a (4, 23), lipopolysaccharide (LPS) (17), leukotriene B_4 (46), or interleukin-6 (IL-6) (45); tracheal or distal airway instillation of bacteria (11), bacterial components (5), or defensins (52); and induction of ischemia followed by reperfusion in lung tissue (41). Although reduced neutrophil deformability on activation can cause sequestration (6, 25, 51), some components of this sequestration depend on adhesion molecules such as β_2 integrins (6, 10), L-selectin (13, 16), and α_4 integrins (5).

C5a binds a heptahelical receptor on neutrophils that signals through pertussis toxin-sensitive G protein pathways to change adhesion molecule expression, increase production of reactive oxygen species, and enhance phagocytosis (14). Via an ill-defined sequence of events, intravenous injection of C5a causes acute neutropenia primarily because of sequestration within pulmonary capillaries (7). Initial margination is not

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dependent on L-selectin, P-selectin, or CD18 integrins, but prolonged sequestration does require CD18 integrins and L-selectin (6, 13, 30). *N*-formylmethionyl-leucyl-phenylalanine (fMLP), a formyl peptide derived from *Escherichia coli*, binds to formyl peptide receptors and also induces signaling events through pertussis toxin-sensitive pathways that activate neutrophils, change adhesion molecule expression, and prime the cell for superoxide production and degranulation (14). In vitro studies (43) have shown that fMLP can induce neutrophil aggregation. fMLP has also been shown (26) to induce pulmonary sequestration of circulating neutrophils, but adhesion molecule involvement in this process has not been studied.

L-selectin is a member of the selectin family of cell surface glycoproteins expressed on the tips of microprocesses on neutrophils (39), where it mediates cell capture under physiological flow conditions and high velocity rolling on microvascular endothelium (27, 35). L-selectin induces homotypic aggregation of neutrophils (43) and neutrophil-neutrophil interactions under flow (2) by binding to P-selectin glycoprotein ligand-1 (47) and other unidentified ligands on neutrophils (40). L-selectin is cleaved and shed from the plasma membrane by a metalloproteinase (15) in a process induced by activating agents, including fMLP and C5a (28), or by cross-linking of CD18 integrins (48).

Here we test the hypothesis that L-selectin is required for fMLP-induced neutrophil margination in the lung. We compare the magnitude of C5a- and fMLP-induced lung-specific sequestration responses in wild-type C57BL/6 mice to that seen in mice with absent or blocked L-selectin by evaluating peripheral blood neutrophil concentrations, marginated neutrophils in lung sections, and the amount of myeloperoxidase activity within harvested lungs. We present evidence that L-selectin is not necessary for the initial margination events in response to C5a, but that it is required for all aspects of fMLP-induced margination and sequestration in the lung.

MATERIALS AND METHODS

Animals and reagent preparation. L-selectin-deficient (L−/−) (1) and wild-type mice (both on C57BL/6 background) were obtained from established colonies at the University of Virginia Health Sciences Center vivarium, Hilltop Lab Animals, and Jackson Labs. All animal experiments were approved by the institutional committee for animal use. This work fully conforms with the "Guiding Principles for Research Involving Animals and Human Beings." All experiments were performed on mice at least 8 wk of age. fMLP (ICN Biomedicals, Aurora, OH) was injected at a dose of 0.34 pmol/g body wt (low dose) because this dose has been shown to produce complete neutropenia in rabbits (34). A higher fMLP dose of 34 pmol/g body wt was used to examine whether the neutrophil margination response was maximal at the low dose. Human recombinant C5a complement fragment (Sigma Chemical, St. Louis, MO) was injected at a dose of 900 pmol/g body wt (high dose), which leads to an initial intravascular C5a concentration of ~12 nM. This concentration has been shown (24, 37) in vitro to produce maximal responses to C5a. A C5a dose of 90 pmol/g body wt (low dose) was used to produce a margination response similar in magnitude to that produced by fMLP. The monoclonal antibody MEL-14 (rat IgG2a, 30 μg/mouse), which blocks all known functions of L-selectin (34, 16), was purified from hybridoma supernatant (American Type Culture Collection, Manassas, VA). $F(ab')_2$ fragments of Mel-14 (30 μg/mouse) were prepared by pepsin digestion (Pierce Chemical, Rockford, IL). Optimal digestion (determined by SDS-PAGE) occurred at a reaction time of 5 h in a 37°C shaking water bath (280 rpm in a Brinkmann Orbimix 1010/incubator 1000). $F(ab')_2$ fragments were purified (confirmed by SDS-PAGE) from undigested Ab and Fc fragments by passage over a protein A AffinityPak column (Pierce Chemical).

Neutropenia time course experiments. Wild-type C57BL/6 and L−/− mice were anesthetized (ip) with ketamine hydrochloride (125 mg/kg; Abbott Laboratories; North Chicago, IL), xylazine (12.5 mg/kg; Vedcom, St. Joseph, MO), and atropine sulfate (0.25 mg/kg; American Pharmaceutical Partners, Los Angeles, CA). The trachea was intubated (PE-90 tubing, Becton Dickinson, Sparks, MD), and the right jugular vein and right carotid artery were cannulated (PE-10 tubing, Becton Dickinson). Anesthesia, hydration, and temperature were maintained through jugular vein injection of anesthetics and saline and the use of a heating pad (37°C) (Physitemp Instruments, Clifton, NJ). Mice were stabilized for 15 min before injection (iv) of 50 μl saline containing high- or low-dose C5a, high- or low-dose fMLP, or 50 μl PBS (control). In some experiments, mice were pretreated with either intact MEL-14 antibody or MEL-14 $F(ab')_2$ fragments (30 μg/mouse) 30 min before injection of fMLP, C5a, or PBS. For wild-type mice with no pretreatment, 28 mice were used for high- (n = 2) or low-dose C5a (n = 5), high- (n = 5) or low-dose fMLP (n = 12), or PBS control (n = 4). For L−/− mice, 24 mice were used for high- (n = 5) or low-dose C5a (n = 5) or high- (n = 6) or low-dose fMLP (n = 8). For wild-type mice pretreated with 30 μg MEL-14 $F(ab')_2$ fragments, 13 mice were used for high- (n = 4) or low-dose C5a (n = 3) or high- (n = 3) or low-dose fMLP (n = 3). Blood samples were collected before and at 1, 2, 3, 4, 5, 10, and 30 min after mediator injection by filling one capillary tube (Drummond Scientific, Broomall, PA) with carotid catheter dead space (10 μl) and then filling a second 10 μl tube with carotid blood. This method produced reliable leukocyte counts and differentials (data not shown). Each sample was stained with 90 μl Kimura [0.05% (wt/vol) toluidine blue; 0.9% NaCl in 22% ethanol (11 ml); 0.03% light-green SF yellowish (0.8 ml); saturated saponin in 50% ethanol (0.5 ml); and 0.07 M phosphate buffer, pH 6.4 (5 ml); all reagents Sigma Chemical], and neutrophil and mononuclear cell concentrations were determined using a hemocytometer (Reichert, Buffalo, NY).

Myeloperoxidase assay. At 1 min postinjection of high- (n = 6) or low-dose C5a (n = 4), high- (n = 4) or low-dose fMLP (n = 6), or 50 μl PBS (n = 5), the thoracic cavity of wild-type C57BL/6 mice was opened, and the great vessels were occluded, stopping blood flow and respiratory effort by 2 min. The lungs and spleens were removed, separated from connective tissue, and kept at −80°C in saline. Neutrophil infiltration into lungs was quantified by measuring myeloperoxidase activity as described previously (44). Organs were homogenized in 1:20 (wt/vol) cold (4°C) 20 mM phosphate buffer (pH 7.4) (Thomas Scientific, Swedesboro, NJ). Samples (1.5 ml) were washed twice (17,000 g at 4°C for 30 min) and resuspended 1:5 (original organ wt/buffer vol) in 50 mM phosphate buffer (pH 6.0) with 0.5% (wt/vol) hexadecyltrimethylammonium bromide and 10 mM EDTA. Samples were sonicated,

placed in liquid nitrogen for 1 min, and then thawed at 37°C. This freeze-thaw procedure was repeated twice and followed by incubation at 4°C for 20 min. After centrifugation (17,000 *g* at 4°C for 15 min), myeloperoxidase activity was measured in triplicate by adding myeloperoxidase assay buffer (50 mM KPO_4, pH 6.0, 0.2 mg/ml *o*-dianisidine, and 0.06% H_2O_2) at a ratio of 4:1 to supernatant. The activity (1 U defined as change in absorbance of 1/min) was calculated from the linear slope of the absorbance vs. time plot (460 nm at 25°C for 5 min; Lab Systems, Needham Heights, MA). The assay was normalized by dividing myeloperoxidase activity by total protein absorbance (A) as determined by bicinchoninic acid assay (Pierce Chemical).

Immunohistochemistry. The lungs from animals treated with high-dose C5a, low-dose fMLP, and PBS were inflated in situ with 10% formalin, at 25 cmH_2O. The lungs were subsequently removed and fixed in 10% formalin for 48 h. Paraffin-embedded sections (10 μm) were stained with rat anti-mouse neutrophil antibody (19) from Serotec (Raleigh, NC), using a protocol similar to that described by Bishop et al. (3). Briefly, sections were incubated [avidin, 10% rabbit serum (NRS; Vector Laboratories, Burlingame, CA), and 0.5% fish skin gelatin oil (FSGO) in PBS] for 1 h in a humidified chamber at 25°C to block nonspecific binding, washed in PBS, and then incubated in a humidified chamber at 4°C overnight with 1 μg/ml rat anti-mouse neutrophil antibody (0.5% FSGO, biotin, and 5% NRS in PBS). Sections were washed and then incubated with 5 μg/ml biotinylated rabbit anti-rat IgG (Vector Laboratories) (0.5% FSGO and 5% NRS in PBS) for 1 h at room temperature in a humidified chamber. After washing, sections were incubated for 30 min with avidin-biotin-peroxidase complexes (Vectastain Elite ABC kit, Vector Laboratories), washed with plain PBS, incubated for 5 min with diaminobenzidine (DAB kit, Vector Laboratories), and counterstained with hematoxylin and blueing solution (Stephens Scientific, Kalamazoo, MI). Slides were viewed using an Axiovert 100 microscope (Carl Zeiss, Thornwood, NY), and pictures were generated using an MDS 100 camera (Kodak, Rochester, NY).

Statistical analysis. Statistical analysis of systemic leukocyte concentrations and myeloperoxidase activity was performed using unpaired Student's *t*-test or for multiple comparisons, one-way pair-wise ANOVA, followed by Mann-Whitney rank sum tests (SigmaStat software). Tests were performed for differences between the treatment group and control, unless otherwise indicated. Statistical significance was set at $P < 0.05$.

RESULTS

General observations. All mice evaluated in this study were healthy and of normal weight. Mouse weights and baseline peripheral blood neutrophil and mononuclear cell concentrations were not significantly different among antibody pretreatment or mediator treatment groups in circulating neutrophil studies, and they also did not vary among treatment groups in myeloperoxidase studies (data not shown). Carotid blood sampling resulted in the removal of 160 μl of blood. This volume was compensated for by addition of saline into the jugular vein catheter, resulting in a slight hemodilution (~6%).

C5a and fMLP decrease circulating neutrophils. Because complement fragments have been shown to produce margination of peripheral blood neutrophils within the microvasculature of many organs, but particularly within pulmonary capillaries (6, 7), the neutropenia produced by intravenous administration of C5a or fMLP was considered to be a measure of neutrophil margination. Peak margination after injection with both mediators and doses occurred at 1 min, followed by a mediator- and dose-specific sequestration period. At 1 min postinjection of 900 pmol C5a/g body wt (high dose), the peripheral blood neutrophil concentration fell to 8 ± 0.7% of baseline (Fig. 1*A*), consistent with the neutropenic effect of complement fragments from zymosan-activated plasma used in other studies (4, 7, 23, 26). High-dose C5a also induced a significant

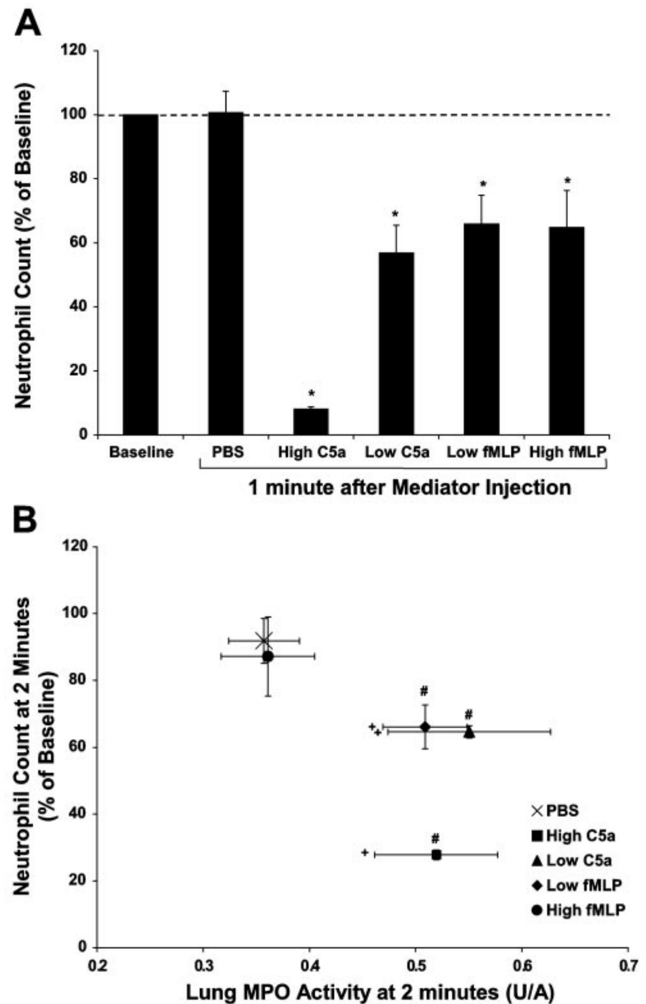

Fig. 1. *A*: peripheral blood neutrophil counts for wild-type C57BL/6 mice at 1 min after intravenous injection of PBS, high- (900 pmol/g body wt) or low-dose C5a (90 pmol/g body wt), or high- (34 pmol/g body wt) or low-dose *N*-formylmethionyl-leucyl-phenylalanine (fMLP; 0.34 pmol/g body wt). Values are %baseline counts ± SE. $*P < 0.05$, neutrophil count significantly decreased vs. baseline or PBS control. *B*: correlation between blood neutrophil counts (%baseline at 2 min) and lung neutrophils as measured by myeloperoxidase (MPO) at 2 min after PBS, high- or low-dose C5a, or high- or low-dose fMLP injection in wild-type C57BL/6 mice. MPO activity is expressed as MPO activity (in U; see *Myeloperoxidase activity*) normalized to total lung protein absorbance (A). $+P < 0.05$, MPO activity significantly increased compared with PBS control; $\#P < 0.05$, peripheral blood neutrophil concentration significantly decreased compared with PBS control.

margination (53 ± 2% of baseline) of mononuclear cells out of the peripheral circulation, whereas neither low-dose C5a nor fMLP induced a drop in mononuclear cells (data not shown). fMLP at 0.34 pmol/g body wt (low dose) induced a smaller but significant percent drop in peripheral blood neutrophils to 66 ± 9% of baseline. A 100-fold increase in the dose of fMLP induced no further decrease in neutrophil counts (65 ± 12% of baseline), indicating that the maximal margination response induced by fMLP is smaller than that induced by C5a. An injection of one-tenth of the original dose of C5a (90 pmol/g body wt) produced neutropenia (57 ± 9% of baseline) that approximated the response induced by fMLP. Peripheral blood neutrophil concentrations after low- and high-dose C5a remained decreased relative to control until 4 and 30 min after injection, respectively (see Fig. 4; other data not shown). In contrast, neutrophil sequestration after fMLP injection was short, with the response to low-dose fMLP terminated by 3 min, and the response to high-dose fMLP terminated by 2 min (see Fig. 4).

Neutropenia correlates with lung neutrophil margination. To verify that the neutropenia produced by C5a and fMLP is primarily due to margination within the pulmonary circulation, we compared the magnitude of the neutropenic effect at 2 min postinjection of mediator with lung neutrophil content as measured by myeloperoxidase activity (in U; see *Myeloperoxidase activity*) normalized to total lung protein absorbance (A) (Fig. 1*B*). Mice treated with high-dose C5a (0.52 ± 0.06 U/A), low-dose C5a (0.55 ± 0.08 U/A), or low-dose fMLP (0.52 ± 0.06 U/A) showed a 50% increase in the number of neutrophils in the lungs compared with PBS-treated control mice (0.36 ± 0.03 U/A). Given that previous morphometric and autoradiographic studies (6, 8) in other animal models have estimated that the normal resting pool of neutrophils in the lungs is at least two times the size of the pool in the peripheral circulation, this observed increase in myeloperoxidase activity is consistent with the lungs as the site of most neutrophil margination in response to C5a or fMLP. In contrast, myeloperoxidase activity in spleens from C5a- or fMLP-treated mice was not significantly increased relative to activity in spleens from PBS-treated controls (data not shown).

Detection of marginated neutrophils in lung sections. Immunohistochemistry was performed on sections of fixed lungs inflated and harvested ~2 min after intravenous injection of high-dose C5a, low-dose fMLP, or PBS (Fig. 2). More neutrophils were found in the pulmonary capillaries and in the surrounding alveolar tissue in the sections taken from C5a- (47 ± 2 neutrophils/high-power field) and fMLP-treated animals (43 ± 1 neutrophils/high-power field) compared with sections from PBS-treated (32 ± 1 neutrophils/high-power field) controls. The increases in neutrophil counts for fMLP- and C5a-treated animals were consistent with the increases in lung myeloperoxidase activity. In addition, C5a-treated sections appeared to have an increased number of neutrophils in pulmonary arterioles and venules compared with fMLP- or PBS-treated animals. Most importantly, virtually all of the marginated neutrophils in the microvasculature were seen as single adherent cells spread along the endothelium. We found no evidence for neutrophil aggregation, one of the potential mechanisms we considered for C5a- or fMLP-induced lung margination.

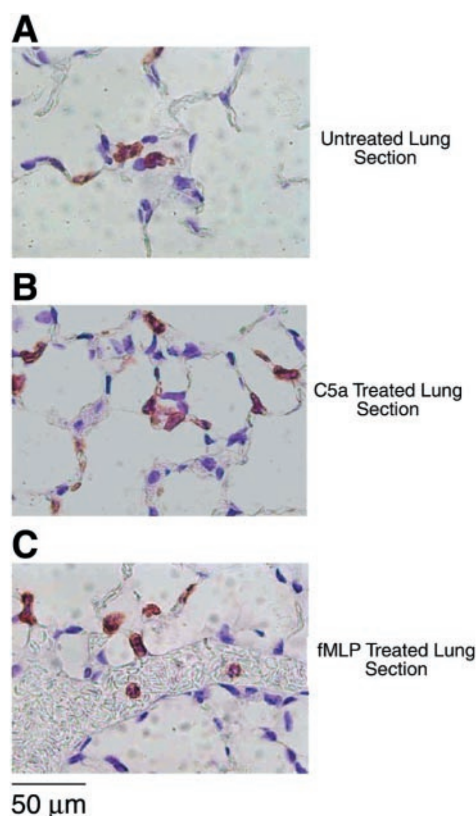

Fig. 2. Neutrophils (red brown) in paraffin-embedded lung sections from wild-type C57BL/6 mice, counterstained with hematoxylin and blueing solution. Lungs were inflated with 10% formalin ~2 min after intravenous injection of PBS (*A*), C5a (900 pmol/g body wt) (*B*), or fMLP (0.34 pmol/g body wt) (*C*). Representative sections are shown. There is no evidence for homotypic (neutrophil-neutrophil) or heterotypic (neutrophil-platelet) aggregation. Objective, ×40; numerical aperture, 1.2.

L-selectin dependence of fMLP-induced margination. We next examined neutropenia 1 min after C5a and fMLP injection in L−/− mice and wild-type mice pretreated with MEL-14, an antibody that blocks all known aspects of L-selectin function (Fig. 3). Peripheral neutrophil counts 30 min after injection of 30 μg intact MEL-14 antibody were reduced to 34 ± 6% (mean ± SE) of baseline, possibly due to an Fc receptor effect. Therefore, we produced $F(ab')_2$ fragments of MEL-14 for blocking studies, which did not cause significant decreases of neutrophils after 30 min (99 ± 7% of baseline). The 30-μg dose of MEL-14 $F(ab')_2$ fragments was demonstrated to saturate L-selectin binding sites on neutrophils by flow cytometry (data not shown). The absence or blockade of L-selectin did not significantly alter the initial margination of neutrophils in response to either high- or low-dose C5a. Peripheral blood neutrophil concentrations for L−/− and MEL-14 $F(ab')_2$ fragment-pretreated wild-type mice

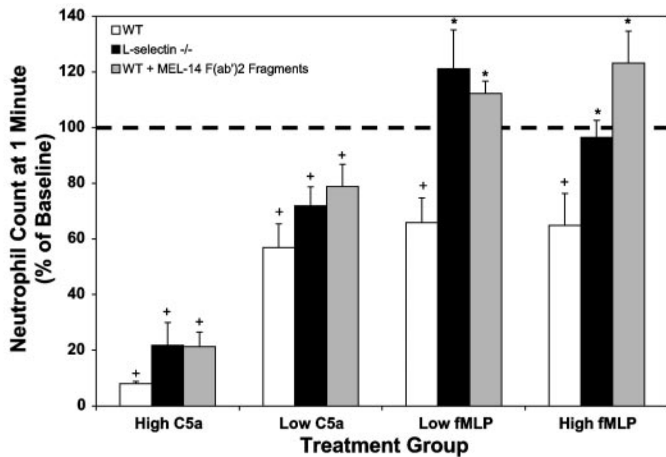

Fig. 3. Peripheral blood neutrophil counts (%baseline) at 1 min after intravenous injection of high- (34 pmol/g body wt) or low-dose fMLP (0.34 pmol/g body wt) or high- (900 pmol/g body wt) or low-dose C5a (90 pmol/g body wt) into wild-type C57BL/6 mice (WT), L-selectin-deficient mice (L-selectin−/−), or wild-type mice pretreated with intravenous injection of 30 μg MEL-14 F(ab′)$_2$ fragments. Absence or blockade of L-selectin has only a minimal effect on C5a-induced neutropenia but blocks fMLP-induced margination completely. *$P < 0.05$, significantly increased over wild type; +$P < 0.05$, significantly decreased compared with baseline.

were 22 ± 8% and 21 ± 5%, respectively, of baseline at 1 min for mice treated with high-dose C5a and 72 ± 7% and 79 ± 8%, respectively, for those treated with low-dose C5a. In contrast, the absence or blockade of L-selectin completely abolished neutrophil margination 1 min after injection of either fMLP dose.

Duration of response with and without L-selectin. The time courses of changes in peripheral blood neutrophil concentrations in L−/− and L-selectin antibody-pretreated wild-type mice were measured out to 5 min after fMLP or C5a injection and compared with similarly treated wild-type mice with no antibody pretreatment. There was no difference in the time course of neutropenia after high- or low-dose C5a injection for mice with absent or blocked L-selectin vs. wild-type (Fig. 4, *A* and *B*). The peripheral blood neutrophil count after low-dose fMLP treatment in L−/− and antibody-pretreated mice was significantly higher than for wild-type mice until 3 min postinjection and did not significantly fall below baseline for at least 30 min after injection (Fig. 4*C*; other data not shown). Counts in high-dose fMLP-treated L−/− or antibody-pretreated mice also did not fall below baseline (Fig. 4*D*).

DISCUSSION

Previous studies looking at the role of adhesion molecules in the pulmonary margination of neutrophils have focused on the complement fragment- and LPS-induced pathways (13, 30, 31). Complement activation leads to margination within the pulmonary capillaries of mice that is initially adhesion molecule independent (30), whereas *E. coli*-derived LPS induces a mechanistically distinct margination event in rabbits that is entirely dependent on L-selectin (31). Interestingly, instillation of *E. coli*, but not *Streptococcus pneumoniae*, into distal airways of mice induces an accumulation of neutrophils within capillaries that is also L-selectin dependent (13). Our finding that margination in response to fMLP is L-selectin dependent suggests that *E. coli*-derived formyl peptides, in addition to LPS, may cause this accumulation of neutrophils in response to *E. coli* instillation.

High- and low-dose fMLP induced approximately the same percentage of neutrophils to disappear from the circulating pool at 1 min, indicating that this shift is the maximal response that can be elicited by a single bolus of fMLP. Issekutz and Ripley (26) induced nearly complete neutropenia with fMLP; however, this study was done in a pig model, and the animals were subject to continuous infusion of fMLP over a 10-min period. Given that at least twice as many neutrophils are

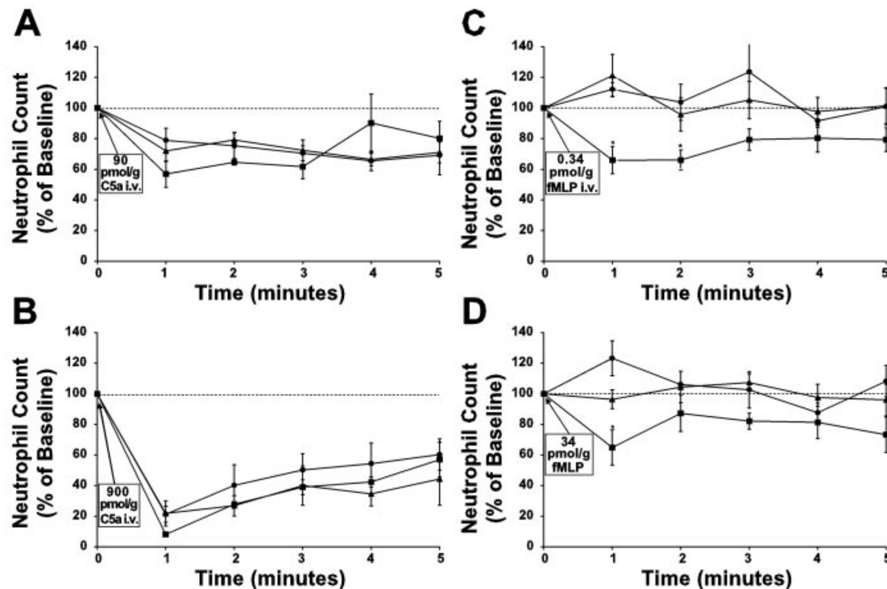

Fig. 4. Peripheral blood neutrophil counts (%baseline) at specified times after low- (90 pmol/g body wt; *A*) or high-dose C5a (900 pmol/g body wt; *B*) or low- (0.34 pmol/g body wt; *C*) or high-dose fMLP (34 pmol/g body wt; *D*) injection in wild-type C57BL/6 mice (■), L-selectin −/− mice (▲), and wild-type mice pretreated with 30 μg Mel-14 Fab(2) fragments (●). Absence or blockade of L-selectin completely prevents neutropenia after fMLP injection. *$P < 0.05$, significantly decreased compared with baseline and mice with absent or blocked L-selectin.

marginated in the lungs than in the peripheral circulation under normal conditions (8), our observed increase in lung myeloperoxidase activity of 50% compared with PBS control for C5a or low-dose fMLP accounts for virtually all of the neutrophils that disappeared from the peripheral circulation. High-dose C5a seems to induce margination into organs other than the lung as well, because myeloperoxidase activity in the lung is equal in C5a- and fMLP-treated animals, despite the much larger neutropenia induced by C5a.

Neutropenia produced by fMLP in pigs is accompanied by thrombocytopenia (26). Neutrophil-platelet aggregation occurs in vitro (29), and fMLP can produce L-selectin-dependent homotypic human neutrophil aggregates in vitro (43). Therefore, we looked for neutrophil-platelet or neutrophil-neutrophil aggregates. However, the vast majority of neutrophils within capillaries were single cells, adherent and spread out along the endothelium, and we saw no evidence of aggregation. These findings do not preclude leukocyte-leukocyte interactions, because they may not be readily detectable by immunohistochemistry. Secondary tethering has previously been reported (2) to play a role in fMLP-induced margination. Although this type of interaction contributes little to leukocyte accumulation in the peripheral circulation (33), vascular parameters in the pulmonary circulation are vastly different (9, 21), and the L-selectin dependence of both secondary tethering and fMLP-induced sequestration within the pulmonary circulation makes this potential mechanism worth investigating.

The lung sections also showed that most of the neutrophils from C5a- or fMLP-treated animals were marginated within capillaries, similar to the findings by Doyle et al. (13) for C5a-induced margination. Therefore, both L-selectin-dependent and L-selectin-independent mechanisms of neutrophil margination exist in lung capillaries and are differentially used depending on the chemoattractant activator present. Both C5a and fMLP activate neutrophils through G protein-coupled receptors and $G\alpha_i$-mediated pathways (14). Given the different physiological responses demonstrated here, it should be interesting to identify differences in signaling pathways and downstream effector systems that might account for the differential requirements for L-selectin. The discovery that fMLP-induced margination is entirely L-selectin dependent represents the first evidence of a chemoattractant response of this type. Previous reports on L-selectin-dependent margination in response to LPS are different, because LPS acts through another set of receptors, specifically Toll-like receptor-2 (TLR-2) and TLR-4 (49).

C5a receptors from different species bind C5a with similar [dissociation constant (K_d), ~1 nM] affinity (50). fMLP is a low-affinity agonist for murine formyl peptide receptors (K_d, ~100 nM), whereas human and rabbit formyl peptide receptors bind fMLP with much higher (K_d, ~1 nM) affinity (18). The low dose of fMLP used in this study induces complete neutropenia in rabbits (34), as opposed to the 35% decrease in peripheral neutrophils in mice that we report here. Although affinity differences may play a role in this species-dependent neutrophil responsiveness, increasing the dose of fMLP 100-fold did not increase the total amount of neutrophil margination in the pulmonary circulation. If affinity differences were solely responsible for the species-specific margination responses, this higher dose would produce an amount of neutropenia similar to that seen in rabbits. Therefore, our results suggest that the biological response to fMLP receptor stimulation may be limited in mice and may cause the observed species differences in neutrophil margination in the lungs.

In conclusion, we have shown that injection of both C5a and fMLP into the jugular vein of wild-type C57Bl6 mice induced significant decreases in peripheral neutrophil concentrations. Through myeloperoxidase activity assay and immunohistochemistry, we show that most neutrophils were trapped within capillaries in the pulmonary circulation. Blocking or eliminating L-selectin had no significant effect on C5a-induced decreases in peripheral neutrophils, but fMLP-induced margination was completely blocked, indicating that L-selectin is necessary for fMLP-induced, but not C5a-induced, neutrophil sequestration in the lung microcirculation. We conclude that to become marginated in the lungs neutrophils use at least two separate mediator-dependent mechanisms, one of which requires L-selectin.

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