L-Selectin–deficient Mice Have Impaired Leukocyte Recruitment into Inflammatory Sites

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Summary

L-selectin, a cell surface adhesion molecule that is expressed by most leukocytes, mediates leukocyte rolling along vascular endothelium at sites of inflammation. The contribution of L-selectin to leukocyte migration in models of chronic inflammation was assessed by using mice that lack cell surface L-selectin expression. Significant inhibition of neutrophil (56–62%), lymphocyte (70–75%), and monocyte (72–78%) migration into an inflamed peritoneum was observed 24 and 48 h after administration of thioglycollate, an inflammatory stimulus. L-selectin–deficient mice were also significantly impaired in delayed-type hypersensitivity reactions. Footpad swelling in response to sheep red blood cell challenge was reduced 75% in L-selectin–deficient mice compared with wild-type mice. Ear swelling in a model of contact hypersensitivity induced by oxazolone challenge was also reduced by 69% compared to wild-type mice. Consistent with L-selectin–mediating leukocyte migration into diverse vascular beds during inflammation, L-selectin–deficient mice were significantly resistant to death resulting from lipopolysaccharide (LPS)-induced toxic shock. LPS administration resulted in a 90% mortality rate in control mice after 24 h, while there was a 90% survival rate in L-selectin–deficient mice. These results demonstrate that L-selectin plays a prominent role in leukocyte homing to nonlymphoid tissues during inflammation and that blocking this process can be beneficial during pathological inflammatory responses.

The recruitment of leukocytes from the vasculature and their extravasation into tissues is critical for a successful host response to tissue injury, but it also contributes to the pathophysiology of many inflammatory diseases. This complex process is regulated in part by the selectin family of adhesion molecules that mediate the initial interactions of leukocytes with endothelium that results in leukocyte rolling along the venular wall (1). The selectin family consists of three closely related cell surface molecules with differential expression by leukocytes (L-selectin, CD62L), platelets (P-selectin, CD62P), and vascular endothelium (E-selectin, CD62E; and P-selectin). Although structurally similar, the selectins perform distinct adhesive functions. L-selectin mediates the binding of lymphocytes to the specialized high endothelial cells that are present in postcapillary venules of lymph nodes and leukocyte rolling on vascular endothelium at sites of tissue injury or inflammation. In contrast, P-selectin is rapidly mobilized to the surface of activated endothelium or platelets, and it binds myeloid cells and a subset of T cells. E-selectin is expressed primarily on the endothelium after activation with inflammatory cytokines, and it mediates the attachment of myeloid cells and a subset of memory T cells.

The generation of mice lacking cell surface P-selectin (2), L-selectin (3), or E-selectin (4) has provided further insight into the function of these receptors. P- and L-selectin–deficient mice have dramatic defects in leukocyte rolling (2, 3, 5). Con- sistent with this, both P- and L-selectin–deficient mice have a pronounced defect in neutrophil accumulation in the peritoneum 2 h after thioglycollate injection, but they appear to have more normal numbers of neutrophils in the peritoneal cavity by 4 h (2, 3). Similar results are obtained in normal mice that have been injected with L-selectin neutralizing antibodies or a soluble L-selectin–IgG chimera (6). Recruitment of neutrophils into an inflamed rat peritoneum at 4 h is partially blocked by E-selectin–neutralizing mAb (7). However, E-selectin–deficient mice have no obvious changes in neutrophil trafficking during inflammatory responses unless they are first treated with a P-selectin–blocking mAb (4). Neutrophil accumulation in the peritoneum of E-selectin–deficient mice 6 h after thioglycollate injection is significantly inhibited (85%) by P-selectin mAb, while this treatment has no effect on neutrophil accumulation in normal mice. These results demonstrate that neutrophil extravasation into the peritoneum during acute inflammation is dependent on the expression of either endothelial P- or E-selectin in combination with L-selectin on leukocytes. However, the consequences of selectin deficiency on neutrophil migration at later time points have not been determined, and their effects on monocyte and lymphocyte migration have not been assessed.

Inflammation is also a central feature of delayed-type hypersensitivity reactions. Allergic contact dermatitis is a hyper-

sensitivity response of the skin that involves the recruitment of antigen-specific T cells to the site of challenge which release cytokines that attract other leukocytes. As opposed to classical delayed-type hypersensitivity reactions, the induction of effector T cells in SRBC-induced delayed-type hypersensitivity is dependent on the presence of neutrophils (8). Edema precedes lymphocyte, neutrophil, and eosinophil immigration, which occurs during the first 24 h, and there is a predominance of lymphocytes within the tissues by 48–72 h. A partial requirement for L-selectin function in leukocyte short-term homing to sites of allergic contact hypersensitivity (4 h) or acute inflammation (1 h) has been demonstrated in mice by injecting labeled leukocytes that were treated with a L-selectin–blocking mAb (9, 10). However, the role of L-selectin in the development and duration of delayed-type hypersensitivity responses is unknown. Therefore, inflammatory responses were experimentally induced in L-selectin–deficient mice to determine the role of the L-selectin adhesion pathway in the recruitment of leukocytes during inflammation.

Materials and Methods

Mice. L-selectin–deficient mice were generated as described (3), and were back-crossed with C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) at least four times. A lack of L-selectin cell surface expression was verified by indirect immunofluorescence staining of blood leukocytes with an mAb (LAM1-116) that reacts with human and mouse L-selectin (Engel, P., and T. F. Tedder, unpublished data). Mice were 8–14 wk old at the time of use. Control mice were either age-matched C57BL/6 mice (The Jackson Laboratory) or mice with a mixed 129/Sv × C57BL/6 background. All studies and procedures were approved by the Animal Care and Use Committee at Duke University Medical Center. Mice were anesthetized using methoxyflurane (Pitman-Moore, Inc., Mundelein, IL) and were monitored at least twice daily for signs of morbidity or distress.

Leukocyte Accumulation into the Inflamed Peritoneum. 1 ml of thioglycollate solution (3% wt/vol; Sigma Immunochemicals, St. Louis, MO) was injected intraperitoneally into mice. Mice were killed, and 5 ml of warm (37°C) medium (RPMI 1640, 2% FCS, and 2 mM EDTA) was injected into the peritoneum followed by gentle massage of the abdomen. The lavage solution was recovered and viable cells were counted using a hemocytometer. The relative percentages of macrophages, lymphocytes, and neutrophils were determined from differential counts of cytospin preparations after Wright-Giemsa staining.

Allergic Contact Dermatitis Model. Mice were sensitized with 25 μl of a solution consisting of 100 mg/ml of oxazolone (4-ethyoxymethylene-2-phenyloxazolone; Sigma) in acetone/sesame seed oil (4:1) applied evenly for two consecutive days on a shaved hind flank as described (11). On day 5, sensitized mice were challenged by applying 10 μl of a 10 mg/ml solution of oxazolone in acetone/sesame seed oil (4:1) to the right ear (5 μl on the dorsal side and 5 μl on the ventral side). An identical amount of acetone/sesame seed oil (4:1) was administered to the left ear. The thickness of the central portion of each ear lobe was measured at timed intervals after challenge using a constant force, calibrated digital thickness gage (Mitutoyo Corp., Tokyo, Japan). Each ear lobe was measured three times at each time interval, and the mean of these values was used for analysis.

SRBC-induced Delayed Hypersensitivity Reaction. SRBC, stored in Alsever's solution at 4°C, were washed three times in PBS before use. Mice were immunized s.c. in the shaved abdomen or back with 2 × 10⁵ SRBC in a 50-μl vol of PBS. The mice were challenged on day 5 after the initial sensitization by injecting 1 × 10⁸ SRBC in a volume of 50 μl PBS s.c. into the right hind footpad. PBS (50 μl) was also injected into the left hind footpad as a control. Footpad thickness was measured in both feet at the same relative location using a constant force, calibrated digital thickness gage (Mitutoyo Corp.). Mice were monitored twice per day for discomfort or tissue necrosis, but none was observed. Each footpad was measured two to three times at each time interval, and the mean value was used for analysis.

Septic Shock Model. Mice were injected i.p. with LPS (40 μg of LPS/g of body wt, *Escherichia coli* serotype 0111:B4; Sigma) and were subsequently monitored for morbidity and mortality as described (12). Some L-selectin–deficient mice appeared dehydrated and were given water orally by dropper at least four times per day.

Statistics. All data are shown as mean values ± SEM. Analysis of variance was used to analyze the data, and the Student's *t* test was used to determine the level of significance of population differences. The survival rate for LPS-treated mice was tested by χ^2 analysis.

Results

Experimentally Induced Peritonitis. The role of L-selectin in inflammatory responses was assessed by injecting thioglycollate into the peritoneal cavity of control and L-selectin–deficient mice. The number of infiltrating neutrophils in the inflamed peritoneum of L-selectin–deficient mice was significantly less (24 h, 62% less, $P = 0.05$; and 48 h, 56%, $P = 0.03$) than the number of immigrant neutrophils observed in wild-type mice (Fig. 1 *B*). The number of infiltrating macrophages in the inflamed peritoneum of L-selectin–deficient mice was 72 and 78% ($P = 0.006$) lower than in wild-type mice at 24 and 48 h, respectively. Although the average number of resident peritoneal lymphocytes in L-selectin–deficient mice was 43% higher ($P = 0.006$) than in wild-type mice, the number of infiltrating lymphocytes found 24 and 48 h after thioglycollate injection was 70 and 75% ($P = 0.007$) lower, respectively, compared to wild-type mice (Fig. 1 *D*). These results indicate that neutrophils, monocytes, and lymphocytes lacking L-selectin are unable to exit the bloodstream effectively during the late stages of an inflammatory response.

Delayed-type Hypersensitivity Reactions in L-selectin–deficient Mice. L-selectin–deficient and wild-type mice were sensitized with oxazolone on the flank and were challenged with oxazolone on the ear pinnae 5 d later (Fig. 2). Swelling in the oxazolone-challenged ears of wild-type mice was detected at 12 h (36 ± 13% increase in thickness compared to the carrier-challenged ear), peaked by 24 h (65 ± 13% increase, $P = 0.003$), and began to subside by 48 (43 ± 7% increase, $P = 0.0002$) to 72 h (25 ± 6% increase, $P = 0.005$). In contrast, significant swelling was not observed in L-selectin–deficient mice, with only an average 20 ± 10% increase in ear thickness at 24 h (swelling was <5% in five out of the nine

A ALL LEUKOCYTES **B NEUTROPHILS** **C MACROPHAGES** **D LYMPHOCYTES**
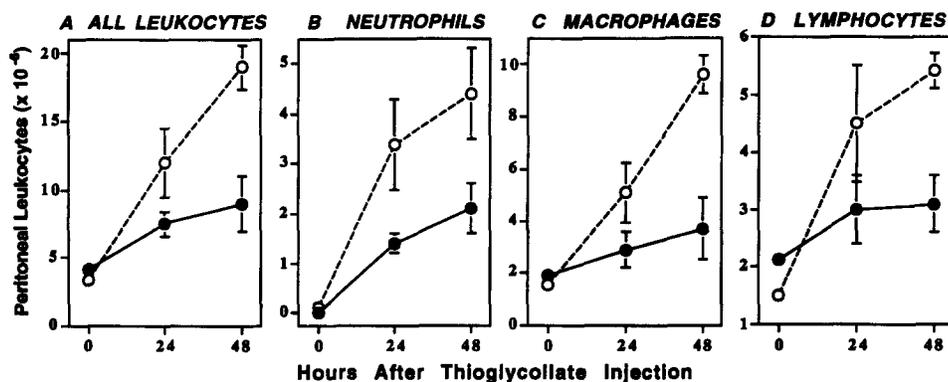
Hours After Thioglycollate Injection

Figure 1. Peritoneal leukocyte influx after thioglycollate-induced peritonitis. Thioglycollate was injected in the peritoneal cavity of L-selectin-deficient (*closed circles*) or control (B6 × 129)F1 (*open circles*) mice. Values are the mean number of leukocytes (± SEM) in peritoneal lavage samples isolated at the indicated times after thioglycollate administration. A minimum of four mice per data point were examined in two independent experiments.
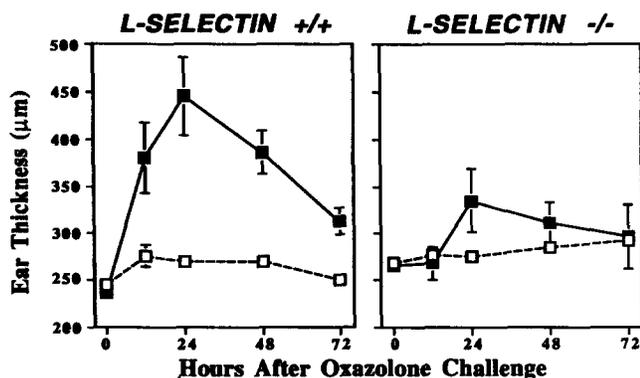

L-SELECTIN +/+   **L-SELECTIN -/-**

Hours After Oxazolone Challenge

Figure 2. The kinetics of oxazolone-induced allergic contact dermatitis in L-selectin-deficient mice. Values are expressed as the mean thickness (± SEM) of the ear pinnae at a similar position in nine wild-type and nine L-selectin-deficient mice. Data were combined from two independent experiments. Filled squares represent oxazolone-challenged ears, while open squares represent ears treated with carrier alone.

L-selectin-deficient animals). Therefore, lack of L-selectin expression resulted in an overall 69% (*P* = 0.06) reduction in swelling at 24 h.

Inflammation was also examined in an SRBC-induced model of delayed-type hypersensitivity. Mice were sensitized by s.c. injection of SRBC and were subsequently challenged with SRBC in the footpad 5 d later. The inflammatory response was quantitated by measuring changes in footpad thickness (Fig. 3). Significant swelling in the challenged footpads versus the unchallenged footpads of wild-type mice was detected by 12 h (9 ± 1% increase in thickness), peaked by 24 h (25 ± 3% increase, *P* <0.01), and remained by day 3 (27 ± 5% increase, *P* <0.004). In contrast, significant swelling was only observed in L-selectin-deficient mice at 12 h (2.0 ± 0.1%, *P* = 0.03) with only a mean 6 ± 3% increase in footpad thickness at 24 h. Therefore, lack of L-selectin translated into a 75% (*P* = 0.002) reduction in swelling at 24 h, an 89% (*P* = 0.002) reduction at 48 h, and a 69% (*P* <0.03) reduction at 72 h (Fig. 3).

The ability of lymphocytes to migrate into draining lymph nodes after SRBC challenge was assessed in four L-selectin-deficient and four wild-type mice 7 d after challenge, the time

of maximal increase in node cellularity. Popliteal nodes isolated from wild-type mice were significantly increased in weight (~3.5-fold, *P* = 0.001) in the challenged foot (3.78 ± 0.55 mg) compared with the foot injected with PBS (1.10 ± 0.06 mg). In contrast, there was no significant change in the weight of popliteal nodes from L-selectin-deficient mice; 1.23 ± 0.46 mg for SRBC-challenged footpads; and 1.55 ± 0.42 mg for unchallenged footpads. Therefore, there was decreased leukocyte infiltration and/or proliferation in the lymph nodes draining the site of challenge of L-selectin-deficient mice.

L-selectin-deficient Mice Are Resistant to Lethal Doses of Endotoxin. Lethal endotoxin shock was induced by the i.p. injection of a high dose of LPS. Wild-type mice demonstrated symptoms of shock that included ruffled fur, shivering, lethargy, watery eyes, and most of them died within 12 h of LPS injection (Fig. 4). Symptoms of endotoxin shock were also observed in L-selectin-deficient mice. Although there were signs of dehydration and wastage by day three, most mice recovered and appeared fairly normal by day four. Most importantly, the majority (60%) of the L-selectin-deficient mice survived LPS-induced shock.

Discussion

These studies demonstrate that L-selectin mediates leukocyte recruitment into sites of chronic inflammation. Leukocyte migration into the inflamed peritoneum was significantly impaired in L-selectin-deficient mice during a 48-h time span (Fig. 1). Monocyte and lymphocyte recruitment was most dramatically inhibited, suggesting a prominent role for L-selectin in mononuclear leukocyte adhesion to endothelium. These findings are consistent with earlier in vitro results where L-selectin-blocking mAbs significantly reduced lymphocyte, neutrophil, monocyte, and eosinophil adhesion to cytokine-activated endothelial cells under nonstatic conditions for time periods of up to 72 h (13–16). These findings are also consistent with results obtained in normal mice treated with a function-blocking L-selectin mAb for up to 72 h (17). The development of contact hypersensitivity and SRBC-induced delayed-type hypersensitivity reactions were also significantly dependent on L-selectin function (Figs. 2 and 3). Since inflammation in L-selectin-deficient mice was inhibited at both early
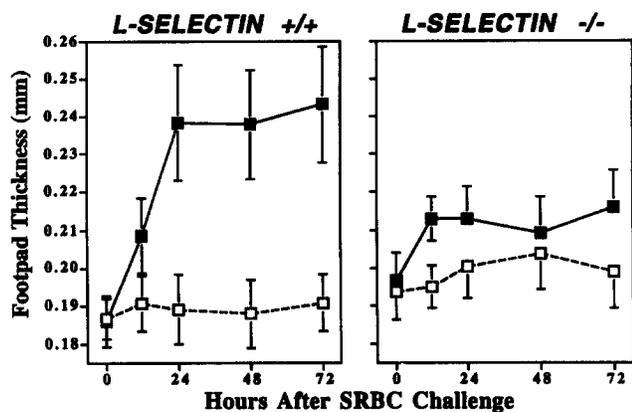
L-SELECTIN +/+ **L-SELECTIN -/-**

Hours After SRBC Challenge

Figure 3. The kinetics of SRBC-induced delayed-type hypersensitivity in L-selectin–deficient mice. Values are expressed as the mean thickness of the footpad (± SEM) at a similar position in nine wild-type and nine L-selectin–deficient mice. Data were combined from two independent experiments. Filled squares represent SRBC-challenged footpads, while open squares represent footpads injected with PBS.

Days After LPS Injection

Figure 4. Survival of mice treated with a lethal dose of LPS. Mice were given 40 μg of LPS i.p. per g of body weight on day 0. Survival data are combined from two independent experiments, with 10 age- and body weight–matched mice of each genotype.

and late time points, L-selectin appears necessary for both the initiation and amplification of delayed-type hypersensitivity responses. Therefore, the reduced inflammatory response is likely to result from inhibiting leukocyte interactions with endothelium, as was observed in thioglycollate-induced peritonitis (Fig. 1).

The magnitude of the decrease in inflammation observed in the current studies suggests a predominant requirement for L-selectin function during inflammation. Although E- and P-selectin are involved in lymphocyte homing to the skin (18–21), delayed hypersensitivity responses are not inhibited in normal mice that have been treated with a P-selectin mAb or in E-selectin–deficient mice (4). However, treatment of E-selectin–deficient mice with a P-selectin mAb significantly reduces inflammation and neutrophil accumulation during contact hypersensitivity responses (4). Therefore, maximal leukocyte accumulation in a given vascular bed or disease site is likely to be dependent on the function of leukocyte L-selectin in conjunction with the endothelial selectins, rather than function of a single selectin. Reductions in physiologic shear rates during vasodilation may also lead to integrin-dependent, selectin-independent leukocyte rolling in vivo (22). Consistent with this, blocking both very late antigen (VLA)-4 and leukocyte function–associated molecule 1 function is required to maximally inhibit delayed-type hypersensitivity reactions (12, 23–26). VLA-4 and L-selectin are each expressed by overlapping subpopulations of circulating lymphocytes in humans, while all blood monocytes express both VLA-4 and L-selectin (27, 28). Therefore, L-selectin may mediate endothelial attachment by the majority of T cells and monocytes, while other adhesive mechanisms may direct the migration of L-selec-
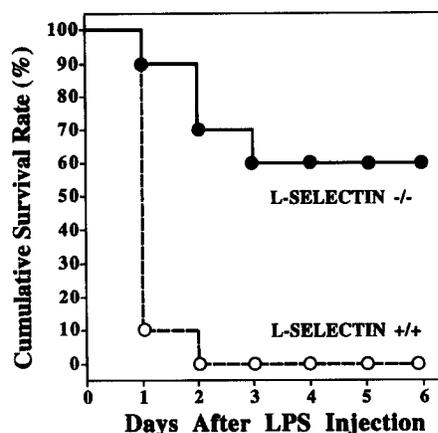
tin–negative cells. Nonetheless, the current studies demonstrate a central role for L-selectin in inflammatory processes and are consistent with leukocyte rolling, arrest, and spreading on vascular endothelium being mediated via the sequential action of selectins, β_1-integrins, and β_2-integrins (21, 29).

Excessive accumulation of leukocytes can be harmful and can lead to a variety of pathologic inflammatory disorders. This is well demonstrated in septic shock, a systemic response to infection with a very high mortality rate. A major finding of the current studies was that L-selectin–deficient mice are dramatically resistant to the lethal effects of high dose endotoxin shock. The mechanism underlying protection appeared to be distal from endotoxin-initiated cytokine production since the L-selectin–deficient mice developed many of the symptoms characteristic of systemic release of inflammatory mediators. It is therefore likely that the genetic loss of L-selectin inhibits leukocyte accumulation in tissues, an event that precedes the pathophysiological response that leads to lethality during endotoxin shock. So far, the only other adhesion molecule shown to have a definitive role in the development of septic shock is intracellular adhesion molecule-1 (12). Since L-selectin function is only associated with leukocyte interactions with the vasculature and the genetic loss of L-selectin does not confer susceptibility to multifocal infections, it is possible that L-selectin–directed therapies may also positively affect the outcome of septic shock. Therefore, efficacious inhibitors of L-selectin function will have a considerable impact on multiple acute inflammatory conditions and are also likely to ameliorate long-term disorders associated with chronic inflammation.

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