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Leukocyte Entry into Sites of Inflammation Requires Overlapping Interactions Between the L-Selectin and ICAM-1 Pathways

Douglas A. Steeber, Mimi L. K. Tang, Nathan E. Green, Xiu-Qin Zhang, Jennifer E. Sloane, and Thomas F. Tedder

Leukocyte interactions with vascular endothelium during inflammation depend on cascades of adhesion molecule engagement, particularly during selectin-mediated leukocyte rolling. Leukocyte rolling is also facilitated by members of the integrin and Ig families. Specifically, leukocyte rolling velocities during inflammation are significantly increased in ICAM-1-deficient mice, with ICAM-1 expression required for optimal P- and L-selectin-mediated rolling. Elimination of ICAM-1 expression in L-selectin-deficient mice significantly reduces leukocyte rolling. Whether disrupted leukocyte rolling in L-selectin and ICAM-1 double-deficient (L-selectin/ICAM-1−/−) mice affects leukocyte entry into sites of inflammation in vivo was assessed in the current study by using experimental models of inflammation; thioglycollate-induced peritonitis, chemokine-induced neutrophil migration to the skin, delayed-type hypersensitivity responses, rejection of allogeneic skin grafts, and septic shock. In many cases, the loss of both L-selectin and ICAM-1 expression dramatically reduced leukocyte migration into sites of inflammation beyond what was observed with loss of either receptor alone. In fact, the effects from loss of both L-selectin and ICAM-1 effectively eliminated multiple chronic inflammatory responses in L-selectin/ICAM-1−/− mice. By contrast, the combined loss of L-selectin and ICAM-1 expression had minimal effects on the generation of Ag-specific T cell responses or humoral immunity. Thus, members of the selectin and Ig families function synergistically to mediate optimal leukocyte rolling and entry into tissues, which is essential for the generation of effective inflammatory responses in vivo. 

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decrease in trauma-induced leukocyte rolling that persists much longer than in mice deficient in P-selectin alone (4), which results in an almost complete lack of neutrophil emigration into an inflamed peritoneum at early time points (5). By contrast, recent studies in L-selectin/ICAM-1−/− mice reveal complex interactions between P-selectin- and ICAM-1-mediated adhesion during leukocyte rolling in the absence of L-selectin (3). Elimination of ICAM-1 expression in L-selectin−/− mice results in a further 50% reduction in the flux of rolling leukocytes during TNF-α-induced inflammation compared with what is observed with L-selectin deficiency alone. Consistent with this, circulating neutrophil, monocyte, and lymphocyte numbers are markedly increased in L-selectin/ICAM-1−/− mice. Collectively, the differences in leukocyte rolling behavior observed in these adhesion molecule-deficient mice demonstrate that ICAM-1 expression is required for optimal P- and L-selectin-mediated rolling in vivo.

The significant reduction in P-selectin-mediated leukocyte rolling observed in L-selectin/ICAM-1−/− mice has therefore prompted us to determine whether altered leukocyte rolling (i.e., increased velocity and/or decreased rolling flux fractions) would affect inflammatory responses in L-selectin/ICAM-1−/− mice. Moreover, would the reduction in P-selectin-mediated leukocyte rolling due to loss of ICAM-1 and L-selectin expression affect leukocyte entry into sites of inflammation following the effects resulting from the loss of ICAM-1 or L-selectin alone, or would this affect leukocyte entry under conditions where ICAM-1 expression does not appear to be required. To address these issues, the development of inflammation was assessed in L-selectin/ICAM-1−/− mice during thioglycollate-induced peritonitis, IL-8-induced neutrophil migration to the skin, contact hypersensitivity reactions, mucus secretion, and trauma-induced leukocyte rolling. In vivo sites of inflammation were injected with thioglycollate to determine the relative percentages of neutrophils, monocytes, and eosinophils.

Materials and Methods

Animals

L-selectin−/− mice were produced as described (10). ICAM-1−/− mice (17) were from The Jackson Laboratory (Bar Harbor, ME). These ICAM-1−/− mice express residual amounts of ICAM-1 splice variants in the thymus and spleen but not in other organs such as brain, heart, liver, kidney, skin, and gut (19). Mice lacking both L-selectin and ICAM-1 were generated by crossing F1 offspring from crosses of homozygous L-selectin−/− mice with homozygous ICAM-1−/− mice. Lack of L-selectin surface expression was confirmed by direct immunofluorescence staining of blood leukocytes with FITC-conjugated LAM1–116 Ab (20). The presence of the mutated ICAM-1 gene was verified by PCR analysis of DNA from tail biopsies. The L-selectin/ICAM-1−/− mice were healthy, fertile, and did not display any evidence of infection or disease. All mice were backcrossed between 5 to 10 generations onto the C57BL/6 background (H-2b).

Chemokine injections and immunohistochemistry

Recombinant endothelial cell-derived human IL-8 (Genzyme Diagnostics, Cambridge, MA) was reconstituted in endotoxin-free water (Sigma) and diluted to a final concentration of 100 µg/ml in PBS containing 0.1% low-endotoxin BSA (Sigma). One microgram of IL-8 (10 µl) was injected intradermally (i.d.) into a shaved flank region of anesthetized mice. An equal volume of 0.1% BSA in PBS was injected i.d. into a separate site at least 3 cm away from the IL-8 injection site to serve as a control. The injection sites were marked to facilitate subsequent accurate identification of the area. Four hours after injection, 4 mm full thickness skin biopsies were harvested and snap frozen in OCT compound (Miles, Elkhart, IN) and stored at −70°C until processed.

Serial 5-µm tissue sections of skin biopsies were acetone-fixed and then incubated with 10% normal goat serum in PBS (10 min, 37°C) to block nonspecific staining. Sections were then incubated with primary antibodies to neutrophils, monocytes, and eosinophils (clone RR1, clone RB6-8C5, kindly provided by Dr. R. Coffman, DNAX, Palo Alto, CA) and monocytes (clone F4/80, American Type Culture Collection, Manassas, VA). Rat IgG (Southern Biotechnology Associates, Birmingham, AL) was used as a control for nonspecific staining. Sections were then incubated sequentially (20 min, 37°C) with a biotinylated goat anti-rat IgG secondary Ab (Southern Biotechnology Associates), then HRP-conjugated avidin-biotin complexes (Vectastain ABC method, Vector Laboratories, Burlingame, CA). Sections were washed three times with PBS between incubations, developed with 3,3′-diaminobenzidine tetrahydrochloride (Sigma) and hydrogen peroxide, and then counterstained with hematoxylin. In all cases, multiple serial sections from each biopsy were compared with ensure that representative planes were assessed.

Contact hypersensitivity responses

Mice were sensitized by application of 25 µl of oxazolone (100 mg/ml, 4-ethoxysxymethylene-2-phenylazoxone; Sigma) in acetone/seasme seed oil (4:1) for 2 consecutive days on a shaved hind flank as described (13). On day 5 after sensitization, mice were challenged with 10 µl of a 10 mg/ml solution of oxazolone in acetone/seasme seed oil (4:1) applied to the right ear (5 µl applied dorsally, 5 µl applied ventrally). An equal volume of acetone/seasme seed oil (4:1) was applied to the left ear in a similar manner. A calibrated digital thickness gauge (Mitutoyo, Tokyo, Japan) was used to measure the thickness of the central portion of the ear lobes at 0, 12, 24, and 48 h after challenge. Each lobe was measured three times at
vested from three recipient mice from each genotype 14 days after place-
as assays were performed after preactivation in vitro (16). Spleens were har-
erally yielded low cpm with poor signal-to-background differentials, all
methods. Because direct CTL activity was inconsistently detected and gen-
activation in individual mice demonstrated good correlation between these
studies comparing direct CTL activity and CTL activity after in vitro pre-

FIGURE 1. Leukocyte influx after thioglycollate-induced peritonitis in adhesion molecule-deficient mice. Values represent the mean number of leu-
kocytes (±SEM) obtained by peritoneal lavage following i.p. injection of thioglycollate medium into 4–10 mice of each genotype. Statistical analysis
results are presented in the text of the Results section.

each time point, and the mean of these values was used for analysis. The
increase in ear thickness was determined by subtracting the ear thickness
before challenge from the ear thickness after challenge. No significant in-
creases in ear thickness were observed at any time point in the ear chal-
lenged with the acetone/sesame seed oil vehicle alone. Pinnae were har-
vested from some of the mice 24 h after challenge, cut longitudinally into
equal halves, and fixed in 10% neutral-buffered formalin. Tissue sections
were stained with hematoxylin and eosin.

Allogeneic skin grafts
Wild-type littermates generated from heterozygous breedings were used as
controls to avoid any possible contributions by minor histocompatibility
differences between test mice and controls as described (16). Briefly, dorsal
skin was dissected from anesthetized donor mice after removal of hair with
electric clippers and cleansing with 70% ethanol. Subcutaneous fascia was
gently removed from the undersurface of donor skin with a no. 15 scalpel.
Recipient mice were anesthetized with pentobarbital administered i.p. at a
dose of 0.7 mg/g body weight. Following depilation, the dorsolateral skin of
recipient mice was cleansed with 70% ethanol and painted with flexible
collodian. Graft beds (8 mm diameter) were prepared by removing the
epidermis and superficial dermis, leaving the fascia layer intact. Fitted skin
grafts were then placed by trimming donor skin to fit graft beds. Grafts
were dressed with petroleum-coated Telfa gauze (Johnson & Johnson Med-
ical, Arlington, TX) held in place with circumferential bandages. Dressings
were removed on day 3 after placement of grafts. Mice were monitored
daily following surgery.

Duplicate BALB/c grafts were placed on each mouse. Duplicate syn-
geneic skin grafts placed on each mouse served as controls for nonspecific
inflammation related to surgery. Grafts were followed daily for graft pro-
gression and time to rejection. Grafts were considered to be rejected at the
time of sloughing or upon complete conversion to a hard avascular eschar.
Time to rejection was monitored independently by two observers based
upon the above criteria.

In vitro CTL assays
Specific in vitro CTL activity was measured by $^{51}$Cr release (23). Previous
studies comparing direct CTL activity and CTL activity after in vitro pre-
activation in individual mice demonstrated good correlation between these
methods. Because direct CTL activity was inconsistently detected and gen-
erally yielded low cpm with poor signal-to-background differentials, all
assays were performed after preactivation in vitro (16). Spleens were har-
vested from three recipient mice from each genotype 14 days after place-

ment of skin grafts. Splenocytes were isolated by density gradient centrif-
guration over Ficoll (Fico/Lite, Atlanta Biologicals, Norcross, GA) and
then incubated with irradiated (2500 Rad) BALB/c splenocytes at an effector:
stimulator ratio of 4:1 for 5 days at 37°C in 5% CO$_2$. Primed effector cells
were then harvested and incubated with $^{51}$Cr- (Amersham, Arlington
Heights, IL) labeled BALB/c or syngeneic splenocyte targets at 100:1, 33:1, 10:1, 3:1 E:T ratios. Specific killing ability of effector cells was
determined by measurement of $^{51}$Cr release over 4 h at 37°C. Spontaneous
$^{51}$Cr release in culture medium and maximal $^{51}$Cr release after addition of
2% Triton X-100 were determined for each target population in all assays.
Specific $^{51}$Cr release was determined by the following formula: specific
$^{51}$Cr release = [(experimental release − spontaneous release)/(maximum
release − spontaneous release)] × 100.

All experiments were carried out in triplicate wells. Lytic units/10$^6$ effector
cells were calculated from E:T lysis curves by standard methods whereby
1 lytic unit was defined as 50% of maximal lysis. This unit of measurement
was selected as the majority of E:T lysis curves were linear at this value.
Total lytic units/spleen were calculated based on lytic units/10$^6$ spleen
cells.

Immunization of mice and T cell proliferation assays
Two-month-old mice were immunized in both hind limbs and at the base of
the tail s.c. with DNP-conjugated keyhole limpet hemocyanin (DNP-
KLH, 100 µg, Calbiochem-Novabiochem, La Jolla, CA) in CFA. Mice
were bled from the retroorbital venous plexus before and 5 days after
immunization and hapten-specific serum Ab levels were measured by
ELISA as described (24). Relative levels of Ag-specific IgM and IgG were
determined for each group of mice using individual and pooled serum
samples.

Single-cell suspensions of lymphocytes from the spleens, MLN, and
pooled PLN pairs draining the sites of immunization (inguinal, popliteal,
and para-aortic) were isolated from mice that had been immunized with
KLH as described above. The lymphocytes were cultured in triplicate in
96-well plates in RPMI 1640 medium (2 × 10$^5$ cells/well in 200 µl) con-
taining 10% FCS, 2 mM L-glutamine, 55 µM 2-ME, antibiotics, and vary-
ing concentrations of KLH. Cellular proliferation was quantified by the
addition of 1 µCi of $[^{3}$H]thymidine (Amersham) during the last 18 h of a
5-day culture, and incorporation of radioactivity was assayed by liquid
scintillation counting.
FIGURE 2. Migration of adhesion molecule-deficient lymphocytes into the peritoneum of wild-type mice during thioglycollate-induced peritonitis. Splenocytes from wild-type and L-selectin−/− mice were isolated, calcine-labeled, mixed with an internal standard population of PKK26-labeled wild-type splenocytes, and then injected i.v. into 2-mo-old wild-type or ICAM-1−/− mice that had been injected i.p. with thioglycollate medium 24 h earlier. After 24 h of migration, single-cell suspensions were obtained by peritoneal lavage and from different lymphoid organs (PL, peritoneal lavage; popliteal and inguinal PLN) and the numbers of fluorescent cells determined by flow cytometry. The ratio of calcine-/PKH26-labeled cells resident in each organ (Ro) was determined and normalized by dividing it with the ratio determined for the injected cell mixture (Ri). The values represent the mean (±SEM) ratios obtained in 4–5 independent experiments. Results significantly different from wild-type mice, *, p < 0.05.

**Results**

**Thioglycollate-induced peritonitis**

The numbers of resident leukocytes within the peritoneum of L-selectin/ICAM-1−/− mice are similar to those of wild-type mice (3). However, 2 h after i.p. injection of L-selectin/ICAM-1−/− mice with thioglycollate, the concurrent loss of both adhesion molecules reduced neutrophil transmigration far greater than observed with loss of either molecule alone (3). To further assess the extent that disrupted leukocyte rolling in L-selectin/ICAM-1−/− mice affects leukocyte entry, leukocyte influx in an experimental model of peritonitis was further analyzed at early (4 h) and late (24 and 48 h) time points. After 4 h of peritonitis, the loss of both L-selectin and ICAM-1 resulted in a 69% decrease in infiltrating neutrophils relative to wild-type mice (p < 0.01; Fig. 1B). L-selectin−/− and ICAM-1−/− mice also had significant reductions (55% and 75%, respectively) in the number of infiltrating neutrophils (p < 0.01, Fig. 1B). By contrast, normal numbers of neutrophils entered the peritoneum of ICAM-1−/− mice by 24 h following the injection of thioglycollate (Fig. 1B), but neutrophil emigration was significantly inhibited in both L-selectin−/− and L-selectin/ICAM-1−/− mice (Fig. 1B, p < 0.002). At 48 h, neutrophil emigration in L-selectin/ICAM-1−/− mice was 66% lower (p < 0.01) than in L-selectin−/− mice. Eosinophil emigration was significantly inhibited in both the L-selectin−/− (by 52%) and L-selectin/ICAM-1−/− (43%) mice compared with ICAM-1−/− and wild-type mice at 48 h (p < 0.002; Fig. 1E). These results imply that the repertoire of functional adhesion molecules expressed at different stages of inflammation varies and that the requirements for overlapping L-selectin and ICAM-1 functions are dependent on the relative expression densities of other adhesion molecules.

Lymphocyte entry into the peritoneum of L-selectin/ICAM-1−/− mice was significantly more impaired relative to L-selectin−/− mice at both 24 (p < 0.02) and 48 h (p < 0.002, Fig. 1C). Similarly, emigration of monocytes into the peritoneum of L-selectin/ICAM-1−/− mice was reduced to a greater extent than in L-selectin−/− mice (48 h, p < 0.01; Fig. 1D). In fact, lymphocyte and monocyte numbers at 24 and 48 h remained the same as at 4 h following thioglycollate administration. ICAM-1 loss alone did not significantly impair leukocyte entry into the inflamed peritoneum at these time points. These findings suggest that the emigration of lymphocytes and monocytes during the later stages of thioglycollate-induced peritonitis that occurs in the absence of L-selectin expression is completely dependent on the expression of ICAM-1.

Rather than reflecting adhesion molecule function, decreased lymphocyte entry into the inflamed peritoneum of adhesion molecule-deficient mice could result from decreased neutrophil entry and blunting of the inflammatory response. This issue was addressed directly by quantifying the migration of labeled lymphocytes injected into the circulation of wild-type or ICAM-1−/− mice that had been injected 24 h earlier with thioglycollate. Wild-type or L-selectin−/− lymphocytes were calcine labeled and mixed with equivalent numbers of PKK26-labeled wild-type splenocytes. After 24 h of recirculation, the numbers and ratio of calcine-/PKH26-labeled cells migrating into the peritoneum were determined. L-selectin−/− lymphocyte migration into the inflamed peritoneum of wild-type mice was half that of wild-type splenocytes (see Table I; Table 1: Roles for L-selectin and ICAM-1 during lymphocyte migration

<table>
<thead>
<tr>
<th>Tissue</th>
<th>PKH+ wild type</th>
<th>Cal⁺ wild type</th>
<th>PKH⁺ L-selectin−/−</th>
<th>PKH⁺ ICAM-1−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peritoneum</td>
<td>61 ± 19</td>
<td>59 ± 11</td>
<td>35 ± 8</td>
<td>17 ± 3*</td>
</tr>
<tr>
<td>PLN</td>
<td>115 ± 7</td>
<td>115 ± 10</td>
<td>131 ± 34</td>
<td>2 ± 1*</td>
</tr>
<tr>
<td>MLN</td>
<td>243 ± 41</td>
<td>247 ± 34</td>
<td>219 ± 41</td>
<td>21 ± 5†</td>
</tr>
<tr>
<td>Blood</td>
<td>83 ± 20</td>
<td>78 ± 17</td>
<td>62 ± 9</td>
<td>99 ± 8†</td>
</tr>
<tr>
<td>Spleen</td>
<td>761 ± 134</td>
<td>685 ± 94</td>
<td>561 ± 101</td>
<td>1210 ± 166†</td>
</tr>
</tbody>
</table>

*Calcine (Cal⁺)-labeled splenocytes from wild-type or L-selectin−/− mice were mixed with PKH-labeled wild-type splenocytes and injected into individual host mice (wild-type or ICAM-1−/−) that had been injected i.p. 24 h earlier with thioglycollate as described in Fig. 2. After 24 h, single-cell suspensions of tissues were isolated and the percentages of calcine- or PKH-labeled lymphocytes was determined by two-color fluorescence cytometry. Absolute numbers of cells were then calculated from individual tissue cell counts and are expressed as mean values (±SEM) from four to five separate experiments. Differences in numbers of calcine- and PKH-labeled lymphocytes were significant: *, p < 0.05; †, p < 0.01.

Mean values for blood are given as cells/ml.
Fig. 2). L-selectin−/− lymphocyte migration to PLNs and MLNs was mostly eliminated, whereas these cells were found at higher levels in blood and spleen. Injection of L-selectin−/− lymphocytes into ICAM-1−/− mice with peritonitis generated similar results (Table I, Fig. 2). Thus, L-selectin was required for optimal lymphocyte migration into an inflamed peritoneum, whereas ICAM-1 did not appear to contribute substantially to migration during the later stages of peritonitis (Figs. 1 and 2).

Chemokine-induced neutrophil migration into the skin

The overlapping roles of L-selectin and ICAM-1 in leukocyte migration into other sites of inflammation was assessed by injecting the four groups of mice i.d. with IL-8, a neutrophil chemoattractant. This assay eliminates differences during the induction stage of inflammatory responses in the different genotypes of mice. Therefore, the effect of adhesion molecule loss on effector cell migration can be assessed directly. Full-thickness skin biopsies were taken from injection sites after 4 h and examined by immunohistochemistry. Injection of IL-8 induced an intense influx of GR1+ neutrophils into the dermis of wild-type mice, with dense collections of neutrophils throughout the dermis (Fig. 3B). No influx of F4/80+ monocytes was observed. In L-selectin/ICAM-1−/− mice, the level of neutrophil recruitment was reduced by >97% (p < 0.001, Fig. 3E) with minimal evidence of dermal thickening (Fig. 3E). Indeed, the IL-8 injection site in L-selectin/ICAM-1−/− mice resembled BSA control injection sites, which consistently contained
few or no neutrophils. A reduction in neutrophil recruitment into the IL-8 injection site was also noted in L-selectin $^{-/-}$ (by 56%, $p < 0.001$) and the ICAM-1 $^{-/-}$ mice (60%, $p < 0.001$), although considerable numbers of neutrophils were still scattered throughout the dermis, which was noticeably thickened as a result of inflammation (Fig. 3, C and D). The $>97\%$ reduction observed in the L-selectin/ICAM-1 $^{-/-}$ mice suggests that these two molecules function synergistically to mediate neutrophil migration rather than reflecting the additive effects from blocking a L-selectin-dependent first step and a subsequent independent, nonoverlapping ICAM-1-dependent step.

Contact hypersensitivity responses
The ability of leukocytes to migrate into the skin following contact hypersensitivity reactions was assessed by oxazolone challenge on the ear 5 days following sensitization. Histologic examination of ear biopsies revealed that the ears of wild-type mice were edematous and contained large numbers of leukocytes in both the dermis and epidermis with frequent microabscesses (Fig. 4B). Edema was significantly reduced in ear sections from L-selectin $^{-/-}$ or ICAM-1 $^{-/-}$ mice, although infiltrating neutrophils and occasional microabscesses remained apparent (Fig. 4, C and D). By contrast, microabscesses were completely absent in L-selectin/ICAM-1 $^{-/-}$ mice, and the ears of these mice were identical in appearance to ears challenged with vehicle only (Fig. 4E). Reductions in ear swelling were observed in L-selectin/ICAM-1 $^{-/-}$ mice at all time points examined compared with wild-type mice (by 76–98%, $p < 0.001$), ICAM-1 $^{-/-}$ mice (62–81%, $p < 0.001$) and L-selectin $^{-/-}$ mice (70–96%, $p < 0.02$, Fig. 4). ICAM-1 deficiency significantly reduced swelling by 31% at 12 h.

FIGURE 4. Contact hypersensitivity in adhesion molecule-deficient mice. Representative hematoxylin and eosin-stained tissue sections of ear biopsies from oxazolone-sensitized mice undergoing contact hypersensitivity responses 24 h after challenge on the ear pinnae. Shown are biopsies from wild-type mice following challenge with the carrier alone (A) or wild-type (B), L-selectin $^{-/-}$ (C), ICAM-1 $^{-/-}$ (D), and L-selectin/ICAM-1 $^{-/-}$ (E) mice following challenge with oxazolone. The accumulation of neutrophils within microabscesses is indicated by arrows (magnification, $\times 130$). The graph shows the mean increases in ear pinnae thickness ($\pm$ SEM) following challenge with oxazolone (wild-type, ■; L-selectin $^{-/-}$, ●; ICAM-1 $^{-/-}$, △; L-selectin/ICAM-1 $^{-/-}$, ○). Data points were collected from three independent experiments using a total of 13 wild-type, 12 L-selectin $^{-/-}$, 11 ICAM-1 $^{-/-}$, and 10 L-selectin/ICAM-1 $^{-/-}$ mice. *, $p < 0.05$, significantly different from wild-type mice. #, $p < 0.05$, significantly different from all other genotypes. Additional statistical analysis results are presented in the Results section.
but not at later time points, as compared with wild-type mice (Fig. 4). L-selectin$^{-/-}$ mice had significant reductions (20–39%, $p < 0.05$) in ear swelling at all time points following oxazolone challenge relative to wild-type littermates. Again, the combined L-selectin/ICAM-1 loss resulted in a greater reduction in leukocyte migration and edema than would be predicted if L-selectin and ICAM-1 functioned in an independent manner.

**Rejection of allogeneic skin grafts**

The roles of L-selectin and ICAM-1 in the migration of mononuclear cells into allogeneic grafts were assessed by transplanting MHC-disparate BALB/c (H-2$b$) skin onto adhesion molecule-deficient mice. Rejection of BALB/c skin grafts by L-selectin/ICAM-1$^{-/-}$ mice (mean ± SD, 16.25 ± 0.5 days) was significantly delayed when compared with wild-type littermates (9.25 ± 0.5, $p < 0.001$) and L-selectin$^{-/-}$ littermates ($p < 0.003$, Fig. 5). Rejection of BALB/c skin grafts by L-selectin$^{-/-}$ mice (mean ± SD, 14 ± 0 days) was significantly delayed when compared with wild-type littermates (9.75 ± 0.5, $p < 0.001$, Fig. 5). However, ICAM-1$^{-/-}$ mice (10.0 ± 0.8) and their wild-type littermates (9.0 ± 2.2) rejected BALB/c skin grafts similarly. Control syngeneic grafts remained healthy for up to 3 mo. Therefore, although skin graft rejection was normal in ICAM-1$^{-/-}$ mice, skin graft rejection by L-selectin/ICAM-1$^{-/-}$ mice was significantly delayed beyond the delay associated with L-selectin deficiency alone.

The ability of adhesion molecule-deficient mice to generate specific CTL responses to BALB/c splenocytes in vivo was assessed. The CTL activity of spleen cells from mice was assessed 14 days following placement of BALB/c skin grafts and was similar among all groups of mice (Table II). These findings suggest that delayed rejection of allogeneic grafts in adhesion molecule-deficient mice resulted from decreased migration of CTL effector cells into the skin rather than from reduced or impaired priming of lymphocytes during the sensitization phase of allograft rejection.

**T cell responses to Ag challenge**

Whether adhesion molecule deficiencies reduced or impaired priming of lymphocytes during the sensitization phase of contact hypersensitivity or allograft rejection responses was assessed further by examining T cell responses to KLH immunization. Five days after mice were immunized with KLH, their MLN, spleen, and draining PLN T cells were isolated and cultured in vitro with KLH for 5 days. Significant T cell proliferation was observed for T cells from all tissues, although the highest levels of proliferation were observed for PLN T cells and the least for MLN T cells (Fig. 6). In three experiments, similar levels of proliferation were observed for T cells from wild-type, L-selectin$^{-/-}$, ICAM-1$^{-/-}$, and L-selectin/ICAM-1$^{-/-}$ mice. The only exception was that T cell proliferation by splenocytes from L-selectin/ICAM-1$^{-/-}$ mice was consistently higher than for T cells from mice of other genotypes.

These results differ markedly from previous studies of others using lymphocytes from a different line of L-selectin$^{-/-}$ mice (14). T cell proliferative responses to Ag were reported to be markedly impaired in that study, although the mice were immunized with identical amounts of KLH and T cell proliferative responses were also assessed 5 days after immunization. The only apparent difference between experimental conditions was that $5 \times 10^5$ lymphocytes were cultured per well in 96-well culture plates whereas the current study assessed proliferation of $2 \times 10^5$ cells/well. Because large numbers of PLN cells would be required to carry out the studies as described (14), it would have been necessary to pool lymphocytes from large numbers of L-selectin$^{-/-}$ mice for each assay. In the current experiments, lymphocytes from individual mice were assessed. These experimental differences may have affected the magnitude or kinetics of the proliferative response observed on day 5. This may have been the case because in that study T cells isolated from L-selectin$^{-/-}$ mice 9 days after immunization generated wild-type proliferative responses (14).

Despite the inability of L-selectin$^{-/-}$ lymphocytes to enter PLNs across high endothelial venules (HEV) during Ag challenge (24), the high frequency of KLH-reactive T cells within the draining PLNs of adhesion molecule-deficient mice is explained by their entry through the afferent lymphatics. Although PLN cellularity was greatly reduced in adhesion molecule-deficient mice, node cellularity increased ~400–1300% following Ag challenge (Table III). By contrast, MLN and spleen cellularity did not change significantly following Ag challenge.

**Humoral immune responses**

To further assess immune responses in adhesion molecule-deficient mice, serum Ab responses were assessed 5 days following KLH immunization. All mice generated significant Ab responses (Fig. 7). IgM and IgG Ab responses were reduced slightly in both L-selectin$^{-/-}$ and L-selectin/ICAM-1$^{-/-}$ mice as previously reported for L-selectin$^{-/-}$ mice (24), but ICAM-1 deficiency had no apparent effect on humoral responses.

**Resistance to septic shock**

Susceptibility to lethal endotoxin shock was assessed in adhesion molecule-deficient mice by the i.p. injection of LPS. Wild-type mice and adhesion molecule-deficient mice demonstrated similar symptoms of shock including ruffled fur, shivering, lethargy, diarrhea, and watery eyes within 12 h of LPS injection as described...
previously for L-selectin<sup>−/−</sup> and ICAM-1<sup>−/−</sup> mice (13, 18). Most mice succumbed to shock within 3 days. For wild-type mice, the LD<sub>50</sub> for LPS treatment was ~2 × 10<sup>5</sup> endotoxin units (EU)/g of body weight (Fig. 8). By contrast, the LD<sub>50</sub> for ICAM-1<sup>−/−</sup> and L-selectin<sup>−/−</sup> mice was about 10-fold higher, 1.5–2.0 × 10<sup>6</sup> EU/g of body weight. The double adhesion molecule deficiency was slightly more protective with an LD<sub>50</sub> of ~2.7 × 10<sup>6</sup> EU/g of body weight. Thus, the protective effects of L-selectin and ICAM-1 deficiencies appear to operate through overlapping pathways or mechanisms in this in vivo model of systemic inflammation.

**Discussion**

Intravital microscopic analysis of leukocyte interactions with endothelial cells at sites of inflammation has demonstrated complex overlapping functions for the selectins and members of the Ig superfamily (3, 4). The current studies also suggest a role for ICAM-1 during leukocyte rolling in inflamed vessels in several in vivo models of inflammation, in addition to its well-established roles in firm adhesion and transmigration of leukocytes at sites of inflammation (25). The loss of both ICAM-1 and L-selectin led to reductions in leukocyte entry into tissues beyond those caused by loss of L-selectin alone and under conditions where the loss of ICAM-1 alone had no demonstrable effect. In acute inflammation such as peritonitis, the majority of leukocyte entry into the peritoneum was eliminated in L-selectin/ICAM-1<sup>−/−</sup> mice (Fig. 1A). Although ICAM-1 deficiency alone did not significantly affect neutrophil migration into an inflamed peritoneum at 24 and 48 h, the majority of neutrophil entry into the peritoneum was eliminated in L-selectin/ICAM-1<sup>−/−</sup> mice (Fig. 1B). Similarly, lymphocyte and monocyte migration into the peritoneum during peritonitis was essentially eliminated in L-selectin/ICAM-1<sup>−/−</sup> mice, whereas ICAM-1 deficiency alone had no significant effect (Fig. 1, C and D). These results demonstrate a high degree of cooperation between the adhesion molecules that mediate leukocyte rolling and those that were previously presumed to only mediate firm adhesion. This also suggests a significant role for ICAM-1 in regulating leukocyte rolling mediated by P-selectin during inflammation in vivo.

The effects from loss of both L-selectin and ICAM-1 were more than would be expected if these molecules functioned independently. L-selectin/ICAM-1<sup>−/−</sup> loss resulted in dramatically reduced inflammatory responses in the skin following IL-8 treatment (Fig. 3), contact hypersensitivity responses (Fig. 4), and allogeneic skin transplantation (Fig. 5). The ICAM-1<sup>−/−</sup> mice used in this study do not express alternate isoforms of ICAM-1 in the resting skin, although systemic LPS treatment induces significant expression of hypomorphic ICAM-1 molecules (19). However, the in vivo physiological significance of hypomorphic ICAM-1 expression is unknown because IgG fusion proteins generated with hypomorphic ICAM-1 isoforms have only been shown to support LFA-1-dependent binding in vitro. In fact, the finding that the ICAM-1<sup>−/−</sup> mice are extremely resistant to LPS-induced septic shock (Fig. 8 and Ref. 18) argues against these ICAM-1 isoforms playing a dominant role in vivo. Nonetheless, that the combined loss of L-selectin and ICAM-1 dramatically reduced inflammation in four different experimental models demonstrates that L-selectin and ICAM-1 mediate optimal leukocyte accumulation during inflammation through...
In situ cellular responses in L-selectin/ICAM-1−/− mice following KLH immunization

<table>
<thead>
<tr>
<th>Mouse Genotype</th>
<th>Day 0</th>
<th>Day 5</th>
<th>% Increase</th>
</tr>
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<tbody>
<tr>
<td>Wild type</td>
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<td>0.6 ± 0.1</td>
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<td>455</td>
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<td>ICAM-1−/−</td>
<td>3.1 ± 0.5</td>
<td>39.1 ± 6.3*</td>
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<tr>
<td>L-selectin/ICAM-1−/−</td>
<td>0.3 ± 0.1</td>
<td>1.2 ± 0.1*</td>
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<th>% Increase</th>
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<tr>
<td>ICAM-1−/−</td>
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</tr>
<tr>
<td>L-selectin/ICAM-1−/−</td>
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<td>5.4 ± 1.6</td>
<td>NS</td>
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<th>Day 5</th>
<th>% Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
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<td>66.1 ± 6.5</td>
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<tr>
<td>L-selectin−/−</td>
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<td>ICAM-1−/−</td>
<td>56.3 ± 3.3</td>
<td>61.3 ± 7.5</td>
<td>NS</td>
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<tr>
<td>L-selectin/ICAM-1−/−</td>
<td>154.5 ± 29.6</td>
<td>122.8 ± 16.6</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values represent the mean number of cells (×10^6) ± SEM from three age-matched mice of each genotype immunized with KLH as described in Fig. 6. *Values represent results from pooled inguinal, popliteal and para-aortic lymph node pairs. Not significantly different from day 0. NS, < 0.01; results significantly different from the day 0 value.

An overlapping as well as synergistic functions, despite these receptors having distinct mechanical capacities. A deficiency in ICAM-1 alone did not significantly affect neutrophil or lymphocyte migration into an inflamed peritoneum at 24 and 48 h (Figs. 1 and 2, Table I) or affect allogeneic skin graft rejection (Fig. 5). Therefore, in the absence of ICAM-1 expression, P-selectin, E-selectin, LFA-1/ICAM-2, or α4/β1 integrin interactions may be sufficient to facilitate optimal rolling when L-selectin function is intact. The differential requirements for simultaneous L-selectin and ICAM-1 expression at different time points also emphasizes that the collective array of adhesion molecules expressed at each time point dictates the requirement for function of individual adhesion receptors during these dynamic processes.

That excessive leukocyte accumulation can lead to a variety of pathologic inflammatory disorders is well demonstrated in septic shock, a systemic response to infection with a very high mortality rate. Previous studies have demonstrated that L-selectin−/− and ICAM-1−/− mice are dramatically resistant to the lethal effects of high-dose endotoxin shock (13, 18). In both cases, the protective mechanism appears to be distal from endotoxin-initiated cytokine production, but is related to the inhibition of leukocyte accumulation in tissues, an event that precedes the lethal pathophysiological response. Surprisingly, the combined loss of L-selectin and ICAM-1 did not result in remarkably extended survival. This finding suggests that blockade of adhesion receptor function may be protective to a major extent, but that high-dose LPS-induced shock may be refractory to this treatment due to the complexity of this multiorgan inflammatory disorder. Nonetheless, efficacious inhibitors of selectin and integrin function may have a considerable impact on multiple acute and chronic inflammatory conditions when used in combination during therapy.

A central role for ICAM-1 in the initiation and generation of immune responses raises the issue of whether the current findings with ICAM-1−/− mice result from a lack of sensitization or the appropriate generation of effector cells during inflammatory responses (17). Similarly, the requirement for lymphocytes to express L-selectin to enter PLNs across HEVs (10, 24) has lead to the suggestion that L-selectin−/− mice are immunocompromised (14). However, there is ample experimental evidence in both cases to suggest that the decreases in inflammation observed in the current study results from a lack of leukocyte entry into sites of inflammation rather than simply a deficiency in effector cells. In the current study, ICAM-1 deficiency did not inhibit the in vivo generation of T cells able to mount in vitro proliferative responses to KLH (Fig. 6) or inhibit humoral immune responses following KLH immunization (Fig. 7). Similarly, wild-type numbers of lymphocytes were retained within draining PLNs following KLH immunization (Table III). CTL responses were also generated at wild-type levels in ICAM-1−/− skin allograft recipients (Table II). Furthermore, the absence of ICAM-1 expression on donor tissue or in the recipients of allografts had no effect on skin transplant rejection (Fig. 5 and Ref. 16), and does not significantly prolong cardiac allograft survival (26). Ab (IgG) and cellular (DTH) immune responses to type II collagen are also similar between ICAM-1−/− and wild-type mice (27). Although APC from ICAM-1−/− mice are poor stimulator cells in vitro, ICAM-1−/− T cells respond well in mixed lymphocyte reactions (17). These findings suggest that the reductions in inflammation observed in these ICAM-1−/− mice are due to leukocyte migration defects rather than immunodeficiency.

Although defective neutrophil migration during inflammation in L-selectin−/− mice is well accepted, concern has been raised that decreased lymphocyte migration into sites of inflammation is
Similarly, L-selectin pending on the immunization regime (24). In addition, germinal either augment or delay humoral responses to haptenated Ags de-
munization with KLH (Fig. 7), and has previously been shown to
graft recipients (Table II, and ref. 16). L-selectin deficiency did not
mice. L-selectin deficiency did not inhibit the in vivo
L-selectin-deficient lymphocytes to enter the inflamed
entry. However, naive T cells migrate into both
the afferent lymphatics while restricting naive lymphocytes to
naive phenotype (31). Therefore, there are L-selectin-dependent
substantial portion of L-selectin-bearing lymphocytes require L-
selectin function for effective migration.
Defective effector lymphocyte generation does not explain de-
creased lymphocyte migration into sites of inflammation in L-se-
selectin−/− mice. L-selectin deficiency did not inhibit the in vivo function
of T cells able to mount in vivo proliferative responses to KLH (Fig. 6) or the generation of CTL responses in skin allo-
genous skin transplantation (16), and do enter PLNs through the
afferent lymphatics that drain sites of Ag challenge (Table III, and
Therefore, although there are L-selectin–independent
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