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

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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. ***The Journal of Immunology*****, 1999, 163: 2176–2186.**

The recruitment of leukocytes into sites of acute and chronic inflammation involves leukocyte interactions with vascular endothelium under conditions of shear flow. L-selectin (CD62L) constitutively expressed by most leukocytes and P-selectin expressed by activated endothelial cells predominantly mediate leukocyte capture and rolling along the vessel wall (1, 2). Recent studies have shown that ICAM-1 and CD18 expression are also required for optimal selectin-mediated rolling under in vivo conditions of shear flow (3–6). Leukocyte rolling through P-selectin does not absolutely require ICAM-1 expression, but ICAM-1 expression is required for P-selectin to mediate rolling at characteristic velocities in vivo (3, 4). By contrast, ICAM-1 expression is essential for leukocyte rolling through L-selectin in the absence of P-selectin expression (4). Thus, L-selectin can only mediate leukocyte rolling in vivo when either ICAM-1, P-selectin, or both, are expressed, whereas P-selectin can mediate leukocyte rolling in the absence of L-selectin and ICAM-1 expression, albeit at significantly faster velocities. ICAM-1 is constitutively expressed at low levels by endothelial cells but is rapidly up-regulated during inflammation, resulting in increased leukocyte-endothelial cell adhesion (7). Subsequently, interactions between integrins and Ig superfamily members, along with E-selectin, arrest rolling cells and mediate firm adhesion that leads to migration into sites of inflammation (8, 9). These studies reveal the complex and overlapping functions of adhesion molecules during inflammation in vivo.

L-selectin-deficient (L-selectin$^{-/-}$)[4] mice have decreased trauma- and TNF-α-induced rolling of leukocytes in venules (10–12). L-selectin$^{-/-}$ mice also demonstrate decreased leukocyte recruitment into an inflamed peritoneum at early and late time points, decreased delayed-type hypersensitivity (DTH) responses, delayed rejection of allogeneic skin transplants, and resistance to LPS-induced septic shock (10, 12–15). Similarly, ICAM-1$^{-/-}$ mice have significantly reduced numbers of infiltrating neutrophils during the early stages of peritonitis, reduced susceptibility to LPS-induced septic shock, and impaired DTH reactions, although allogeneic skin graft rejection is normal (16–18). Moreover, leukocyte-rolling velocities are significantly increased in ICAM-1$^{-/-}$ mice during inflammation (3). Recent studies in P-selectin/ICAM-1$^{-/-}$ mice demonstrate a requirement for ICAM-1 expression when L-selectin mediates leukocyte rolling in the absence of P-selectin expression (4, 5). P-selectin/ICAM-1$^{-/-}$ mice display a profound

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[4] Abbreviations used in this paper: L-selectin$^{-/-}$, L-selectin-deficient; DTH, delayed-type hypersensitivity response; HEV, high endothelial venule; ICAM-1$^{-/-}$, ICAM-1-deficient; KLH, keyhole limpet hemocyanin; L-selectin/ICAM-1$^{-/-}$, L-selectin/ICAM-1-deficient; MLN, mesenteric lymph node; PLN, peripheral lymph node; P-selectin/ICAM-1$^{-/-}$, P-selectin/ICAM-1-deficient; Ri, ratio of calcein-labeled test cells to PKH26-labeled internal control cells injected into mice for migration assays; Ro, ratio of calcein-labeled test cells to PKH26-labeled internal control cells within each tissue after migration assays; i.d., intradermally; EU, endotoxin units.

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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 beyond 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, skin graft rejection, and LPS-induced septic shock. The results further confirm that the selectins and ICAM-1 have overlapping functions essential for optimal leukocyte rolling and entry into in vivo sites of inflammation.

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 F_1 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-2^b). BALB/c mice (H-2^d) were purchased from The Jackson Laboratory. Mice were 7–12 wk old for all experiments, and age-matched wild-type littermates or C57BL/6 mice (The Jackson Laboratory) were used as controls. All mice were housed in a specific pathogen-free barrier facility and screened regularly for pathogens. All studies and procedures were approved by the Animal Care and Use Committee of Duke University Medical Center.

Thioglycollate-induced peritonitis

One milliliter of thioglycollate solution (3% w/v; Sigma, St. Louis, MO) was injected i.p. into mice as described (13). Mice were euthanized, and the leukocyte infiltrate was recovered by peritoneal lavage using 6 ml of warm (37°C) RPMI 1640 medium supplemented with 2% FCS, 5 mM EDTA, 10 mM HEPES, and 10 U/ml heparin. Viable cells were counted with a hemocytometer, and differential counts of peritoneal lavage smears were performed following staining to determine the relative percentages of neutrophils, macrophages, lymphocytes, and eosinophils.

In vivo lymphocyte migration assays

Single-cell suspensions of mouse splenocytes were prepared in RPMI 1640 medium (Sigma). Erythrocytes were lysed with a 0.83% ammonium chloride solution. Cells were then washed, counted, and labeled with either calcein-AM (Molecular Probes, Eugene, OR) or PKH26 (Sigma). Calcein labeling was conducted as previously described (21). Briefly, cells ($5–10 \times 10^7$) were incubated on ice for 30 min in 2 ml RPMI 1640 medium containing 1 μM of calcein-AM. PKH26 labeling was conducted following the vendor's recommendations. Cells (5×10^7) were resuspended in 1 ml of PKH26 diluent, immediately added to an equal volume of a 3 μM PKH26 dye solution, and allowed to incubate for 2 min at room temperature. Labeling was stopped by the addition of FCS. After labeling, the cells were washed three times with PBS, counted, and resuspended in PBS. Calcein-labeled lymphocytes (20×10^6, control or test populations) were mixed with an equal number of an internal standard population of PKH26-labeled control lymphocytes and injected in a volume of 400 μl into the lateral tail vein of recipient mice that had been injected i.p. with thioglycollate 24 h earlier. An aliquot of the injected cell mixture was also analyzed by flow cytometry to calculate the injected ratio of calcein-/PKH26-labeled cells (Ri). After 24 h, single-cell suspensions from spleen, peripheral lymph nodes (PLN, inguinal and axillary), mesenteric lymph nodes (MLN), blood, and peritoneal lavage were prepared and the percentage of calcein- and PKH26-labeled cells was determined by flow cytometry. Cells presenting light scattering properties of dead cells were excluded from the analysis. A minimum of 1000 viable fluorescence bright cells was counted for each sample, and the ratio of calcein-/PKH26-labeled cells was determined (Ro). Results were expressed as previously described (22) or as the total number of labeled cells in each sample.

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 hybridoma culture supernatant fluid containing primary Ab used neat or diluted in PBS containing 1% BSA and 0.02% thiomerosal (30 min, 37°C). Abs used included rat mAbs specific for neutrophils (GR1, 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-ethyoxymethylene-2-phenyloxazonone; Sigma) in acetone/sesame 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/sesame seed oil (4:1) applied to the right ear (5 μl applied dorsally, 5 μl applied ventrally). An equal volume of acetone/sesame seed oil (4:1) was applied to the left ear in a similar manner. A calibrated digital thickness gauge (Mitsutoyo, 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

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 increases in ear thickness were observed at any time point in the ear challenged with the acetone/sesame seed oil vehicle alone. Pinnae were harvested 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 Medical, 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 syngeneic skin grafts placed on each mouse served as controls for nonspecific inflammation related to surgery. Grafts were followed daily for graft progression 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 preactivation in individual mice demonstrated good correlation between these methods. Because direct CTL activity was inconsistently detected and generally yielded low cpm with poor signal-to-background differentials, all assays were performed after preactivation in vitro (16). Spleens were harvested from three recipient mice from each genotype 14 days after placement of skin grafts. Splenocytes were isolated by density gradient centrifugation 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) containing 10% FCS, 2 mM L-glutamine, 55 μM 2-ME, antibiotics, and varying 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.

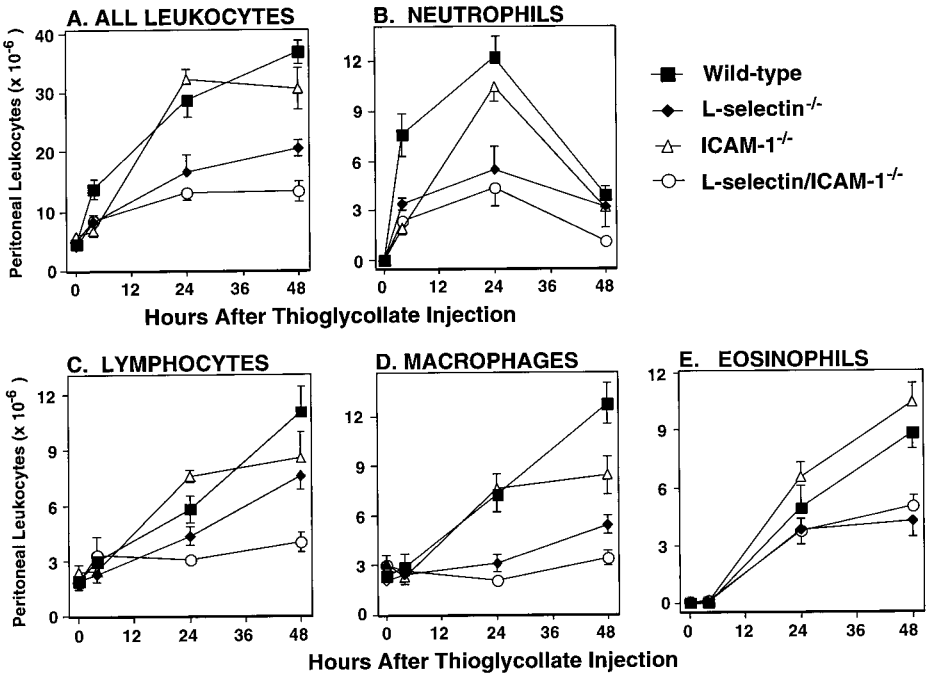

FIGURE 1. Leukocyte influx after thioglycollate-induced peritonitis in adhesion molecule-deficient mice. Values represent the mean number of leukocytes (±SEM) obtained by peritoneal lavage following i.p. injection of thioglycollate medium into 4–10 mice of each genotype. Statistical analysis results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

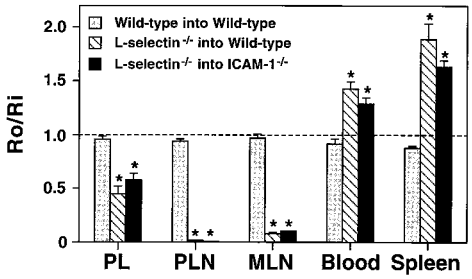

FIGURE 2. Migration of adhesion molecule-deficient lymphocytes into the peritoneum of wild-type mice during thioglycollate-induced peritonitis. Splenocytes from wild-type and L-selectin$^{-/-}$ mice were isolated, calcein-labeled, mixed with an internal standard population of PKH26-labeled wild-type 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 calcein-/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, *, $p < 0.05$.

Septic shock

Mice were injected i.p. with varying doses of LPS (*Escherichia coli* serotype 0111:B4; Sigma) and monitored for morbidity and mortality as described (18). Mice that appeared dehydrated were given water orally by dropper at least four times a day.

Statistical analysis

Data are shown as mean values ± SEM unless otherwise stated. ANOVA was used to analyze the data, and the Student's *t* test was used to determine the level of significance of differences in population means.

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 into 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. 1*B*). L-selectin$^{-/-}$ and ICAM-1$^{-/-}$ mice also had significant reductions (55% and 75%, respectively) in the number of infiltrating neutrophils ($p < 0.01$, Fig. 1*B*). By contrast, normal numbers of neutrophils entered the peritoneum of ICAM-1$^{-/-}$ mice by 24 h following the injection of thioglycollate (Fig. 1*B*), but neutrophil emigration was significantly inhibited in both L-selectin$^{-/-}$ and L-selectin/ICAM-1$^{-/-}$ mice (Fig. 1*B*, $p < 0.002$). 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. 1*E*). 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. 1*C*). 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. 1*D*). 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 calcein labeled and mixed with equivalent numbers of PKH26-labeled wild-type splenocytes. After 24 h of recirculation, the numbers and ratio of calcein-/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 I. *Roles for L-selectin and ICAM-1 during lymphocyte migration*[a]

	Number (±SEM) of Labeled Lymphocytes Entering Each Tissue ($\times 10^{-3}$)					
	Wild-type mice		Wild-type mice		ICAM-1$^{-/-}$ mice	
	PKH$^+$ wild type	Cal$^+$ wild type	PKH$^+$ wild type	Cal$^+$ L-selectin$^{-/-}$	PKH$^+$ wild type	Cal$^+$ L-selectin$^{-/-}$
Peritoneum	61 ± 19	59 ± 11	35 ± 8	17 ± 3*	25 ± 3	18 ± 3*
PLN	115 ± 7	115 ± 10	131 ± 34	2 ± 1†	88 ± 21	0.7 ± 0.2†
MLN	243 ± 41	247 ± 34	219 ± 41	21 ± 5†	157 ± 10	20 ± 3†
Blood[b]	83 ± 20	78 ± 17	62 ± 9	99 ± 8†	115 ± 24	170 ± 23†
Spleen	761 ± 134	685 ± 94	561 ± 101	1210 ± 166†	699 ± 75	1405 ± 200†

[a] Calcein (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 calcein- 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 calcein- and PKH-labeled lymphocytes were significant: *, $p < 0.05$; †, $p < 0.01$.

[b] Mean values for blood are given as cells/ml.

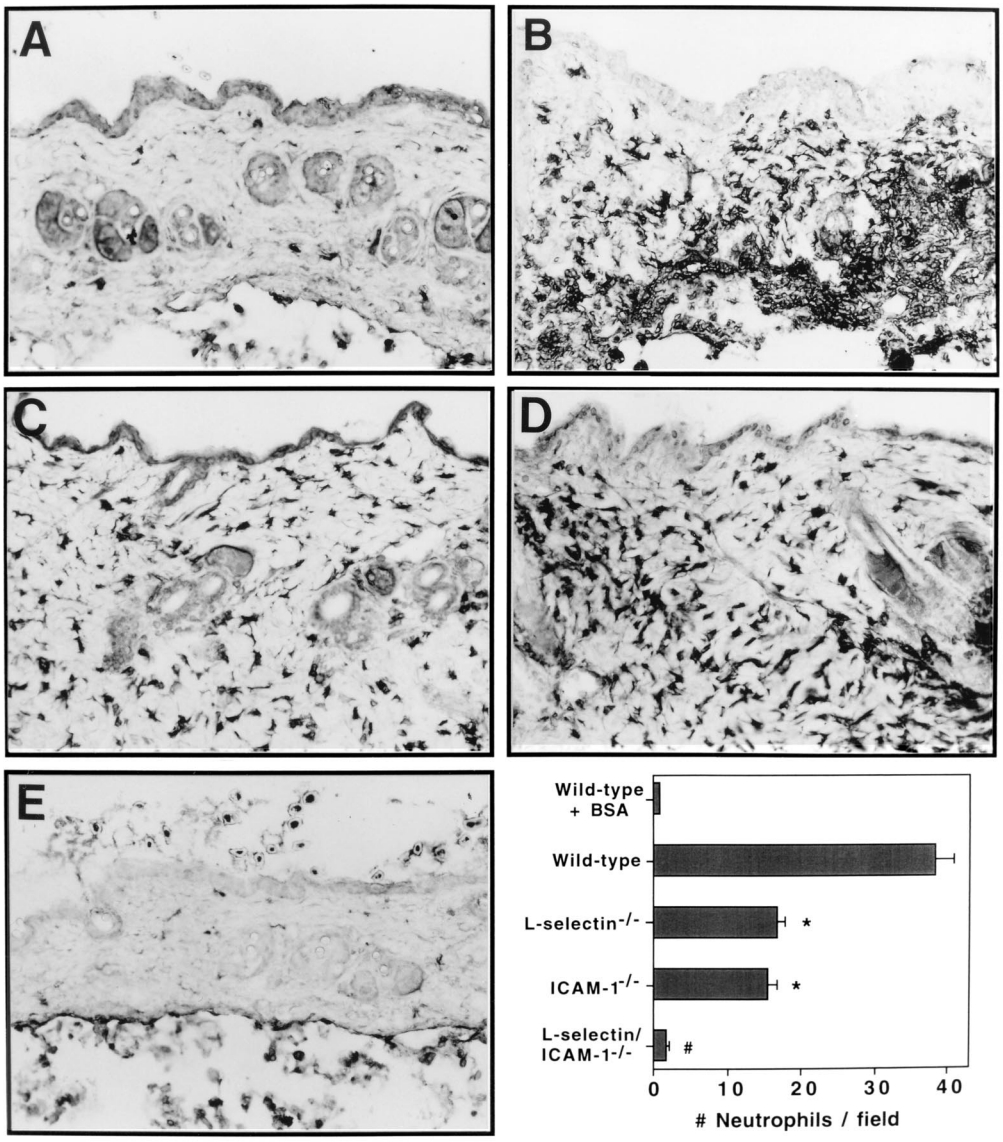

FIGURE 3. IL-8-induced neutrophil migration into the skin of adhesion molecule-deficient mice. Human IL-8 or BSA was injected i.d. into the flank of wild-type or adhesion molecule-deficient mice ($n = 3$). Tissue sections of skin biopsies taken from the injection sites after 4 h were stained with the rat anti-GR1 mAb, a neutrophil marker. Representative sections of skin (*A*) following BSA injection into wild-type mice or following IL-8 injection into wild-type (*B*), L-selectin$^{-/-}$ (*C*), ICAM-1$^{-/-}$ (*D*), and L-selectin/ICAM-1$^{-/-}$ (*E*) mice are shown. The epidermis is at the top of each panel. Positively stained neutrophils appear as darkly stained cells within the dermis (magnification, ×250). The graph shows the numbers of infiltrating GR1^{+} cells present in the dermis quantified by light microscopy. Values represent the mean numbers ± SEM of stained cells per high power (×630) field, with 10 fields assessed in representative tissue sections from each of three mice of each genotype. The number of neutrophils/field for wild-type mice treated with IL-8 is likely to be underestimated because the density of neutrophils was so high and only clearly identifiable cells were counted. *, $p < 0.05$, 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.

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. 3*B*). 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. 3) with minimal evidence of dermal thickening (Fig. 3*E*). Indeed, the IL-8 injection site in L-selectin/ICAM-1$^{-/-}$ mice resembled BSA control injection sites, which consistently contained

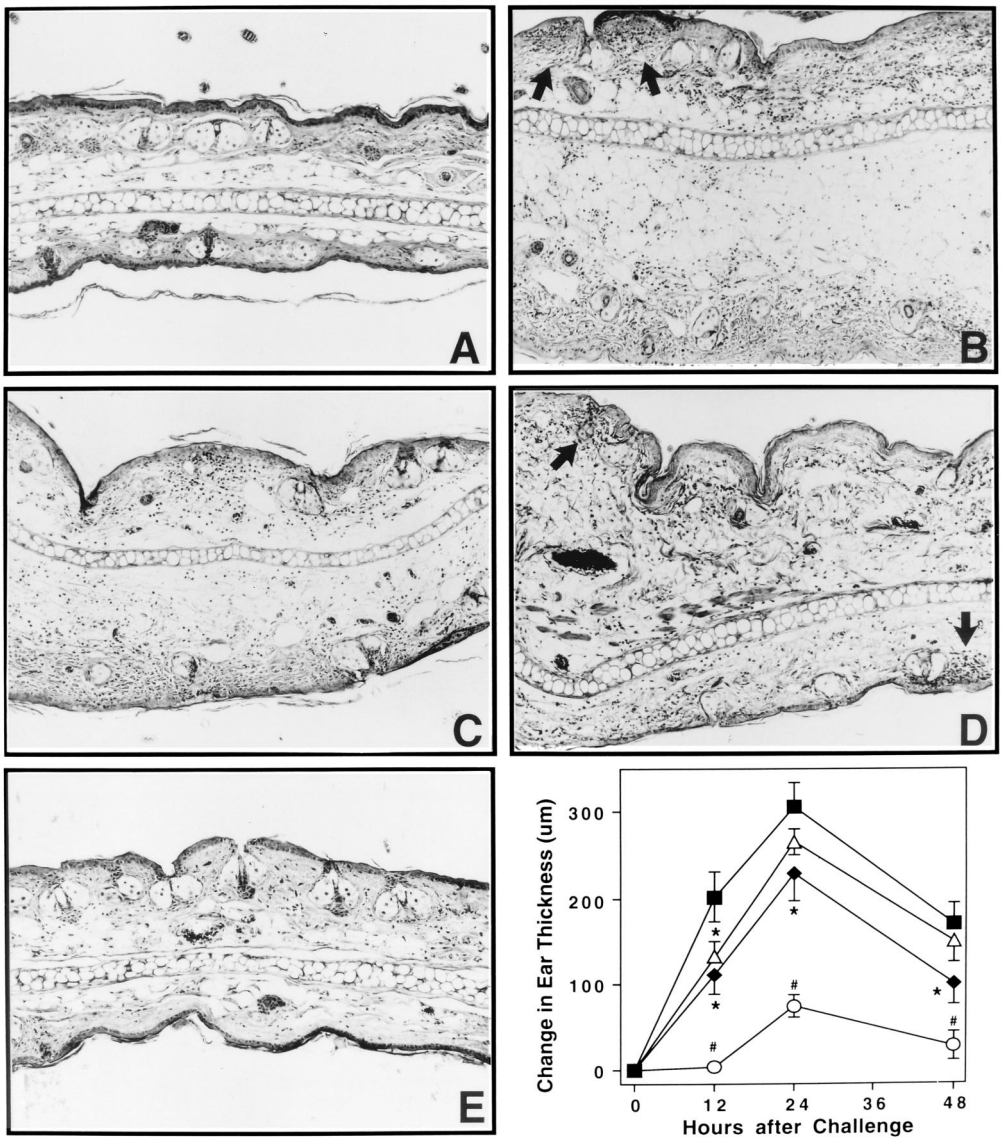

FIGURE 4. Contact hypersensitivity in adhesion molecule-deficient mice. Representative hematoxylin and eosin-stained tissue sections of ear biopsies from oxazolone-sensitized mice undergoing contact hypersensitivity responses 24 h after challenge on the ear pinnae. Shown are biopsies from wild-type mice 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, ×130). The graph shows the mean increases in ear pinnae thickness (±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.

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 taken 24 h after oxazolone challenge 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. 4*B*). 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. 4*E*). 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,

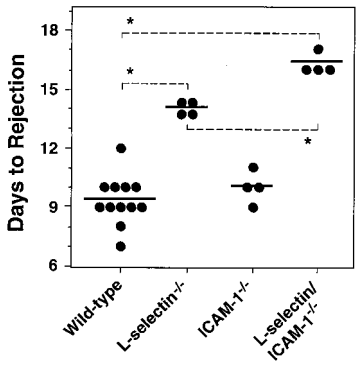

FIGURE 5. Rejection of BALB/c skin grafts placed on adhesion molecule-deficient mice. Grafts were considered to be rejected upon complete conversion to a hard avascular eschar or upon sloughing. Individual symbols represent independent grafts, and bars represent the mean time to graft rejection. Differences between groups of mice were significant, *, $p < 0.001$.

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^d) 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

Table II. *CTL responses in L-selectin/ICAM-1$^{-/-}$ mice following allograft placement*[a]

Genotype	No. of Spleen Cells ($\times 10^{-6}$)	Lytic Units/10^6 Cells	Lytic Units/ Spleen
Wild type	72 ± 9.5	5.1 ± 1.9	328 ± 94
L-selectin$^{-/-}$	128 ± 16.1	3.3 ± 1.8	370 ± 158
ICAM-1$^{-/-}$	187 ± 26.3	5.0 ± 2.4	943 ± 518
L-selectin/ICAM-1$^{-/-}$	167 ± 8.7	5.2 ± 3.8	905 ± 651

[a] CTL activity of spleen cells from mice of each genotype was determined 14 days following placement of BALB/c skin grafts. Lytic units/10^6 effector cells were determined from E:T lysis curves as described in *Materials and Methods.* Total lytic units per spleen were then calculated for each animal using individual spleen counts. Values represent the mean (±SEM) results obtained from three mice in each group.

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 5×10^5 lymphocytes were cultured per well in 96-well tissue culture plates whereas the current study assessed proliferation of 2×10^5 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$^{-/-}$ and ICAM-1$^{-/-}$ mice (13, 18). Most mice succumbed to shock within 3 days. For wild-type mice, the LD_{50} for LPS treatment was $\sim 2 \times 10^5$ endotoxin units (EU)/g of body weight (Fig. 8). By contrast, the LD_{50} for ICAM-1$^{-/-}$ and L-selectin$^{-/-}$ mice was about 10-fold higher, 1.5–2.0 $\times 10^6$ EU/g of body weight. The double adhesion molecule deficiency was slightly more protective with an LD_{50} of $\sim 2.7 \times 10^6$ 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$^{-/-}$ mice (Fig. 1*A*). 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$^{-/-}$ mice (Fig. 1*B*). Similarly, lymphocyte and monocyte migration into the peritoneum during peritonitis was essentially eliminated in L-selectin/ICAM-1$^{-/-}$ 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 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$^{-/-}$ 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$^{-/-}$ 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

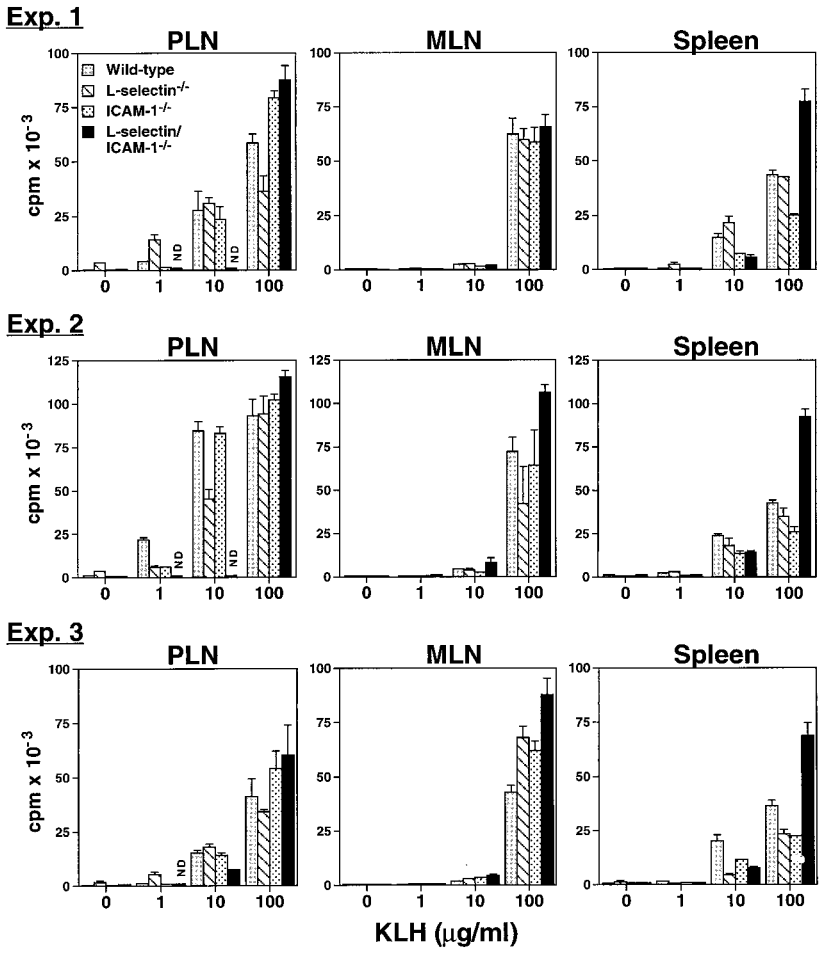

FIGURE 6. T cell proliferative responses in adhesion molecule-deficient mice. Mice were immunized s.c. in the hind limb and at the base of the tail with KLH. Five days later draining PLN, MLN, and spleens were isolated from each mouse. Single-cell lymphocyte suspensions were cultured in vitro with varying amounts of KLH for 5 days. Proliferation was assessed by the incorporation of labeled thymidine added during the last 18 h of culture. Values represent mean thymidine incorporation (±SEM) from triplicate cultures. Results are shown for three independent experiments with one mouse of each genotype. It was not possible to obtain sufficient numbers of lymphocytes from draining PLNs of L-selectin/ICAM-1$^{-/-}$ mice for all KLH concentrations, so some values were not determined (ND).

Table III. *In situ cellular responses in L-selectin/ICAM-1$^{-/-}$ mice following KLH immunization*[a]

Mouse Genotype	Number of Cells ($\times 10^{-6}$) ± SEM		% Increase
	Day 0	Day 5	
PLN[b]			
Wild type	7.2 ± 1.0	45.8 ± 0.9*	641
L-selectin$^{-/-}$	0.6 ± 0.1	2.5 ± 0.5*	455
ICAM-1$^{-/-}$	3.1 ± 0.5	39.1 ± 6.3*	1274
L-selectin/ICAM-1$^{-/-}$	0.3 ± 0.1	1.2 ± 0.1*	408
MLN			
Wild type	12.8 ± 1.0	9.8 ± 3.0	NS[c]
L-selectin$^{-/-}$	6.1 ± 1.0	5.3 ± 1.3	NS
ICAM-1$^{-/-}$	10.3 ± 2.6	9.0 ± 1.9	NS
L-selectin/ICAM-1$^{-/-}$	4.9 ± 0.6	5.4 ± 1.6	NS
Spleen			
Wild type	73.7 ± 3.5	66.1 ± 6.5	NS
L-selectin$^{-/-}$	120.7 ± 12.1	115.4 ± 26.9	NS
ICAM-1$^{-/-}$	56.3 ± 3.3	61.3 ± 7.5	NS
L-selectin/ICAM-1$^{-/-}$	154.5 ± 29.6	122.8 ± 16.6	NS

[a] Values represent the mean number of cells ($\times 10^{-6}$) ± SEM from three age-matched mice of each genotype immunized with KLH as described in Fig. 6.
[b] Values represent results from pooled inguinal, popliteal and para-aortic lymph node pairs.
[c] Not significantly different from day 0.
*, $p < 0.01$; results significantly different from the day 0 value.

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 $\alpha_4\beta_1$ 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

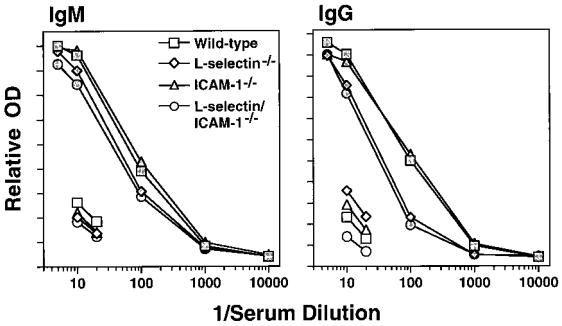

FIGURE 7. Humoral immune responses of adhesion molecule-deficient mice. Serum samples were taken before (open symbols) and 5 days after (filled symbols) KLH immunization of the mice shown in Fig. 6. Values represent the relative levels of Ag-specific IgM or IgG Abs obtained with pooled serum samples as determined by ELISA.

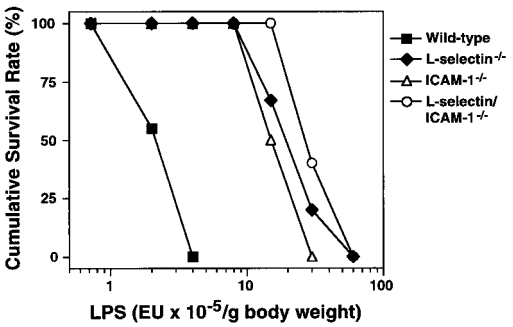

FIGURE 8. Survival of adhesion molecule-deficient mice during LPS-induced septic shock. Mice were given doses of LPS i.p. Survival data are from 3–11 age-matched mice of each genotype. The ICAM-1$^{-/-}$ and L-selectin/ICAM-1$^{-/-}$ mice were ~20% larger and heavier than other mouse strains as previously described (3).

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

a secondary consequence due to disrupted neutrophil entry or immunodeficiency rather than a requirement for lymphocyte expression of L-selectin. To address this directly, the ability of labeled adhesion molecule-deficient lymphocytes to enter the inflamed peritoneum of wild-type mice was assessed. L-selectin deficiency significantly inhibited the ability of lymphocytes to enter the peritoneum between 24 and 48 h after the initiation of inflammation (Fig. 2, Table I). Therefore, there are L-selectin-dependent pathways through which lymphocytes enter sites of inflammation. However, some L-selectin$^{-/-}$ lymphocytes do traffic to sites of peritonitis (Fig. 1*C*, and Ref. 13), DTH reactions (Fig. 4*C*), allogeneic skin transplantation (16), and do enter PLNs through the afferent lymphatics that drain sites of Ag challenge (Table III, and Ref. 24). Therefore, although there are L-selectin–independent pathways through which lymphocytes enter sites of inflammation, a substantial portion of L-selectin-bearing lymphocytes require L-selectin function for effective migration.

Defective effector lymphocyte generation does not explain decreased lymphocyte migration into sites of inflammation in L-selectin$^{-/-}$ mice. L-selectin deficiency did not inhibit the in vivo generation of T cells able to mount in vitro proliferative responses to KLH (Fig. 6) or the generation of CTL responses in skin allograft recipients (Table II, and ref. 16). L-selectin deficiency did not significantly inhibit specific IgM immune responses following immunization with KLH (Fig. 7), and has previously been shown to either augment or delay humoral responses to haptenated Ags depending on the immunization regime (24). In addition, germinal centers are generated in PLNs draining sites of Ag challenge (24). Similarly, L-selectin$^{-/-}$ mice are sensitized normally to OVA during the induction of hypersensitivity responses, although L-selectin deficiency does inhibit inflammation-induced recruitment of leukocytes into tissues during subsequent Ag challenge (28). Current dogma dictates that only memory or activated lymphocytes enter inflamed tissues and thereby enter the draining lymph nodes via the afferent lymphatics while restricting naive lymphocytes to HEV-dependent entry. However, naive T cells migrate into both lymph nodes and extra-lymphoid tissues such as skin to the same extent (29, 30). In addition, at least 10% of the T cells draining chronically inflamed tissue sites have a naive phenotype (31). Therefore, memory and naive lymphocytes each use multiple migration pathways (reviewed in Refs. 32 and 33). Thus, the significant decreases in lymphocyte entry into sites of inflammation in L-selectin$^{-/-}$ mice and in combination with ICAM-1 deficiency are most likely due to interrupted lymphocyte-endothelial interactions rather than a lack of immune response.

Although previous studies have suggested that L-selectin and ICAM-1 are engaged in series by leukocytes, rather than in parallel, the current results are consistent with previous intravital microscopy studies demonstrating that L-selectin and ICAM-1 function synergistically to facilitate leukocyte-endothelial interactions in vivo (3). In the absence of optimal adhesion molecule expression, increases in leukocyte rolling velocities decrease the frequency and efficiency of leukocyte/endothelial interactions and impair leukocyte emigration at sites of inflammation in vivo (8, 11, 34, 35). Thus, when multiple adhesion pathways are blocked, as in the case of L-selectin/ICAM-1 (3) or P-selectin/ICAM-1 deficiencies (4, 5), leukocyte interactions with endothelial cells are sufficiently destabilized so that other adhesion molecule pairs are unable to retard leukocyte rolling velocities enough to facilitate leukocyte entry into sites of inflammation (6). Therefore, leukocyte rolling, firm adhesion, and emigration during inflammation are interrelated events rather than separate processes mediated by interactions between multiple families of adhesion molecules.

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References

1. Tedder, T. F., D. A. Steeber, A. Chen, and P. Engel. 1995. The selectins: vascular adhesion molecules. *FASEB J. 9:866.*
2. Ley, K., and T. F. Tedder. 1995. Leukocyte interactions with vascular endothelium: new insights into selectin-mediated attachment and rolling. *J. Immunol. 155:525.*
3. Steeber, D. A., M. A. Campbell, A. Basit, K. Ley, and T. F. Tedder. 1998. Optimal selectin-mediated rolling of leukocytes during inflammation in vivo requires intercellular adhesion molecule-1 expression. *Proc. Natl. Acad. Sci. USA 95:7562.*
4. Kunkel, E. J., U. Jung, D. C. Bullard, K. E. Norman, B. A. Wolitzky, D. Vestweber, A. L. Beaudet, and K. Ley. 1996. Absence of trauma-induced leukocyte rolling in mice deficient in both P-selectin and Intercellular adhesion molecule-1 (ICAM-1). *J. Exp. Med. 183:57.*
5. Bullard, D. C., L. Qin, I. Lorenzo, W. M. Quinlin, N. A. Doyle, R. Bosse, D. Vestweber, C. M. Doerschuk, and A. L. Beaudet. 1995. P-selectin/ICAM-1 double mutant mice: acute emigration of neutrophils into the peritoneum is completely absent but is normal into pulmonary alveoli. *J. Clin. Invest. 95:1782.*
6. Jung, U., K. E. Norman, K. Scharffetter-Kochanek, A. L. Beaudet, and K. Ley. 1998. Transit time of leukocytes rolling through venules controls cytokine-induced inflammatory cell recruitment in vivo. *J. Clin. Invest. 102:1526.*
7. Dustin, M. L., R. Rothlein, A. K. Bhan, C. A. Dinarello, and T. A. Springer. 1986. Induction by IL-1 and interferon-γ: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). *J. Immunol. 137:245.*
8. Ley, K., M. Allietta, D. C. Bullard, and S. J. Morgan. 1998. The importance of E-selectin for firm leukocyte adhesion in vivo. *Circ. Res. 83:287.*
9. Milstone, D. S., D. Fukumura, R. C. Padgett, P. E. O'Donnell, V. M. Davis, O. J. Benavidez, R. J. Melder, R. K. Jain, and M. A. Gimbrone, Jr. 1998. Mice lacking E-selectin show normal numbers of leukocytes but reduced leukocyte stable arrest on cytokine-activated microvascular endothelium. *Microcirculation 5:153.*
10. Arbones, M. L., D. C. Ord, K. Ley, H. Radich, C. Maynard-Curry, D. J. Capon, and T. F. Tedder. 1994. Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin (CD62L) deficient mice. *Immunity 1:247.*
11. Kunkel, E. J., and K. Ley. 1996. Distinct phenotype of E-selectin-deficient mice: E-selectin is required for slow leukocyte rolling in vivo. *Circ. Res. 79:1196.*
12. Ley, K. E., D. Bullard, M. L. Arbones, R. Bosse, D. Vestweber, T. F. Tedder, and A. L. Beaudet. 1995. Sequential contribution of L- and P-selectin to leukocyte rolling in vivo. *J. Exp. Med. 181:669.*
13. Tedder, T. F., D. A. Steeber, and P. Pizcueta. 1995. L-selectin deficient mice have impaired leukocyte recruitment into inflammatory sites. *J. Exp. Med. 181:2259.*
14. Xu, J., I. S. Grewal, G. P. Geba, and R. A. Flavell. 1996. Impaired primary T cell responses in L-selectin-deficient mice. *J. Exp. Med. 183:589.*
15. Catalina, M. D., M. C. Carroll, H. Arizpe, A. Takashima, P. Estess, and M. H. Siegelman. 1996. The route of antigen entry determines the requirement for L-selectin during immune responses. *J. Exp. Med. 184:2341.*
16. Tang, M. L. K., L. P. Hale, D. A. Steeber, and T. F. Tedder. 1997. L-selectin is involved in lymphocyte migration to sites of inflammation in the skin: delayed rejection of allografts in L-selectin-deficient mice. *J. Immunol. 158:5191.*
17. Sligh Jr., J. E., C. M. Ballantyne, S. S. Rich, H. K. Hawkins, C. W. Smith, A. Bradley, and A. L. Beaudet. 1993. Inflammatory and immune responses are impaired in mice deficient in intercellular adhesion molecule 1. *Proc. Natl. Acad. Sci. USA 90:8529.*
18. Xu, H., J. A. Gonzalo, Y. St. Pierre, I. R. Williams, T. S. Kupper, R. S. Cotran, T. A. Springer, and J.-C. Guiterrez-Ramos. 1994. Leukocytosis and resistance to septic shock in intercellular adhesion molecule 1-deficient mice. *J. Exp. Med. 180:95.*
19. King, P. D., E. T. Sandberg, A. Selvakumar, P. Fang, A. L. Beaudet, and B. Dupont. 1996. Novel isoforms of murine intercellular adhesion molecule-1 generated by alternative RNA splicing. *J. Immunol. 154:6080.*
20. Steeber, D. A., P. Engel, A. S. Miller, M. P. Sheetz, and T. F. Tedder. 1997. Ligation of L-selectin through conserved regions within the lectin domain activates signal transduction pathways and integrin function in human, mouse and rat leukocytes. *J. Immunol. 159:952.*
21. Martin, D. R., and R. G. Miller. 1989. In vivo administration of histoincompatible lymphocytes leads to rapid functional deletion of cytotoxic T lymphocyte precursors. *J. Exp. Med. 170:679.*
22. Butcher, E. C., R. G. Scollay, and I. L. Weissman. 1980. Direct fluorescent labeling of cells with fluorescein or rhodamine isothiocyanate. II. Potential application to studies of lymphocyte migration and maturation. *J. Immunol. Methods 37:109.*
23. Brunner, K. T., J. Mauel, J.-C. Cerottini, and B. Chapuis. 1968. Quantitative assay of the lytic action of immune lymphoid cells on ^{51}Cr labeled allogeneic target cells in vitro: inhibition by isoantibody and by drugs. *Immunology 14:181.*
24. Steeber, D. A., N. E. Green, S. Sato, and T. F. Tedder. 1996. Humoral immune responses in L-selectin-deficient mice. *J. Immunol. 157:4899.*

25. Argenbright, L. W., L. G. Letts, and R. Rothlein. 1991. Monoclonal antibodies to the leukocyte membrane CD18 glycoprotein complex and to intercellular adhesion molecule-1 inhibit leukocyte-endothelial adhesion in rabbits. *J. Leukocyte Biol. 49:253.*
26. Schowengardt, K. O., J. Y. Zhu, S. M. Stepkowski, Y. Tu, M. L. Entman, and C. M. Ballantyne. 1995. Cardiac allograft survival in mice deficient in intercellular adhesion molecule-1. *Circulation 92:82.*
27. Bullard, D. C., L. A. Hurley, I. Lorenzo, L. M. Sly, A. L. Beaudet, and N. D. Staite. 1996. Reduced susceptibility to collagen-induced arthritis in mice deficient in intercellular adhesion molecule-1. *J. Immunol. 157:3153.*
28. Kanwar, S., D. A. Steeber, T. F. Tedder, and P. Kubes. 1998. Overlapping roles for L-selectin and P-selectin in antigen-induced immune responses in the microvasculature. *J. Immunol. 162:2709.*
29. Washington, E. A., W. G. Kimpton, J. E. Holder, and R. N. Cahill. 1995. Role of the thymus in the generation of skin-homing $\alpha\beta$ and $\gamma\delta$ virgin T cells. *Eur. J. Immunol. 25:723.*
30. Kimpton, W. G., E. A. Washington, and R. N. P. Cahill. 1995. Virgin $\alpha\beta$ and $\gamma\delta$ T cells recirculate extensively through peripheral tissues and skin during normal development of the fetal immune system. *Int. Immunol. 7:1567.*
31. Mackay, C. R., W. Marston, and L. Dudler. 1992. Altered patterns of T cell migration through lymph nodes and skin following antigen challenge. *Eur. J. Immunol. 22:2205.*
32. Pabst, R., and R. M. Binns. 1989. Heterogeneity of lymphocyte homing physiology: several mechanisms operate in the control of migration to lymphoid and non-lymphoid organs in vivo. *Immunol. Rev. 108:83.*
33. Westermann, J., and R. Pabst. 1996. How organ-specific is the migration of "naive" and "memory" T lymphocytes? *Immunol. Today 17:278.*
34. Gaboury, J. P., D. C. Anderson, and P. Kubes. 1994. Molecular mechanisms involved in superoxide-induced leukocyte-endothelial cell interactions in vivo. *Am. J. Physiol. 266:H637.*
35. Kanwar, S., B. Johnston, and P. Kubes. 1995. Leukotriene C_4/D_4 induces P-selectin and sialyl Lewis^x-dependent alterations in leukocyte kinetics in vivo. *Circ. Res. 77:879.*