Impaired Primary T Cell Responses in L-Selectin–deficient Mice

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Summary

L-selectin is a homing receptor that mediates the selective attachment of leukocytes to specialized high endothelial venules. To study the potential role of L-selectin in immune responses in intact mice, we generated L-selectin–deficient mice by gene targeting. L-selectin–deficient mice are defective in cutaneous delayed-type hypersensitivity (DTH) responses when tested after conventional intervals of immunization (4 d). Primary T cell proliferative responses and cytokine production (interleukin [IL] 2, IL-4, and interferon γ) were also compromised when tested after 5 d of immunization, indicating that L-selectin is important for the immune response to antigens. In contrast, after more prolonged immunization protocols (9 d), normal responses were observed, suggesting that L-selectin–independent compensatory mechanisms exist. Interestingly, humoral responses of L-selectin–deficient mice to keyhole limpet hemocyanin are indistinguishable from wild-type control mice, implying that L-selectin plays no rate-limiting role in T cell help of B cell function. Thus, our results suggest that L-selectin plays an important role in the generation of primary T cell responses but may not be essential for humoral and memory T cell responses. L-selectin does not appear to be rate limiting for the events leading to antigen-driven neutrophil recruitment, since normal DTH responses are obtained at late time points after immunization.

One of the essential components of the immune surveillance process is the circulation of lymphocytes throughout the body into various tissues, where they fight infectious pathogens, their rapid accumulation at sites of injury and infection, and their subsequent return into the circulation. Abnormal accumulation of immune cells in unwanted sites might lead to the development of autoimmune diseases and cell-mediated tissue destruction. To mount a successful immune response, migration of lymphocytes to lymphoid organs and other tissues is a key step, since the initial recognition of foreign antigens and activation of lymphocytes takes place in this organ in most situations (1, 2). Lymphocytes migrate to lymphoid organs such as peripheral lymph nodes (PLN)[1] and Peyer's patches as well as to acute and chronic sites of inflammation by using a number of adhesive mechanisms. One such mechanism is mediated by specific leukocyte interactions with endothelial cells via a family of adhesion molecules called homing receptors (2–5). The role of these interactions has been elucidated in studies using inhibitory mAbs against surface molecules of lymphocytes and high endothelial venules (HEV), where alteration in the recirculation patterns of lymphocyte migration is seen (2). L-selectin is a well-characterized homing receptor, a carbohydrate-binding protein detected by the MEL-14 antibody in mouse (6, 7) that belongs to the three-member (the other two are P- and E-selectin) selectin family of unique adhesion molecules. The selectins are structurally related to each other, and in all three members, the extracellular region includes a C-type lectin domain, an epidermal growth factor–like domain, and a small number of short consensus repeat units. Although these selectins have structural homology, expression of each member is controlled in a unique way on distinct cell types that mediate distinct adhesion functions.

Studies have suggested that L-selectin is required for homing of naive T cells to PLN (8). L-selectin is also expressed on other leukocytes, and blocking of this molecule with the L-selectin antibody appeared to inhibit accumulation of neutrophils at sites of local inflammation produced by nonspecific triggers (9), indicating that L-selectin not only mediates entry of naive T cells to PLN but has more diverse functions. In addition, administration of L-selectin antibody not only prevents homing of naive lymphocytes

[1] *Abbreviations used in this paper:* CS, contact sensitivity; DTH, delayed-type hypersensitivity; ES, embryonic stem (cells); HEV, high endothelial venules; LNC, lymph node cells; PLN, peripheral lymph nodes.

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into PLN but also partially inhibits their entry into Peyer's patches (8), suggesting that L-selectin is not strictly a PLN-specific homing receptor, but may have a more general function for the migration into other lymphoid tissues.

The complex process of naive T lymphocyte homing to PLN is in part initiated by adhesion interactions of tissue-specific ligands on the surface of endothelial tissues with L-selectin on the surface of naive mature T cells. Both in vitro and in vivo studies have indicated that in most cases the lymphocyte–endothelial cell interaction is an active, multistep process (2, 10). Recently, a three-step model of leukocyte localization in the vasculature has been proposed. This model is based on the studies of neutrophil interactions with inflamed vessels but may also be true for lymphocyte–endothelial interactions (2, 11). In this model, the initial interaction of circulating leukocytes with endothelia (rolling) is induced by interactions of certain constitutively expressed leukocyte homing receptors, of which L-selectin is a major contributor. In the second step, the initial binding of leukocytes to endothelial cells brings leukocytes into closer proximity with chemoattractants that are released from the endothelium. Upon binding of chemoattractant molecules to appropriate receptors on leukocytes, activation of certain integrins is induced on the surface of the leukocytes. In the final step, integrins bind to their counterreceptors on endothelium, thus increasing the adhesion and resulting in arrest of the rolling of leukocytes. Leukocytes then follow cues from chemoattractants and, using integrins for traction control, cross the endothelium and enter appropriate tissues (11).

The role of L-selectin in the localization of tissue vasculature was suggested by studies showing blocking of the interaction of L-selectin with its counterreceptor on endothelial cells by MEL-14 antibody (7, 12–14) and by showing the lack of migration of an L-selectin–negative cell line to PLN in vivo (15). Upon antigen exposure and subsequent activation, naive mature T cells shed their surface L-selectin (16–18), suggesting arrest of activated lymphocytes at the site of antigen accumulation in lymphoid organs. Thus, a role of L-selectin in the expansion of specific T cells at the site of antigen exposure is suggested. In the case of memory T cells, there are conflicting reports, some suggesting reexpression of L-selectin, thereby facilitating their migration to other lymphoid organs (19); on the other hand, murine $CD4^+$ T cells of memory phenotype have been shown to lack L-selectin (20, 21).

Until only recently, the role of L-selectin in vivo has been analyzed by injection of MEL-14 mAbs in homing and primary T cell responses (12–14, 22). These studies suggested a role for L-selectin in migration to PLN, Peyer's patches, and sites of inflammation. Using mAbs, however, it was not clear whether impaired migration patterns and reduced T cell–mediated responses were the result of the depletion of L-selectin–expressing lymphocytes, the inhibitory effects of actual migration of lymphocytes to the target tissues, or signaling effects mediated by L-selectin antibody cross-linking. The results of these studies are therefore subject to multiple interpretations.

Recently, Arbones et al. (23) have generated mice lacking L-selectin by using gene targeting and have demonstrated that these mice have a reduced number of lymphocytes in PLN and are defective in lymphocyte homing, leukocyte rolling, and leukocyte migration to site of inflammation. However, these studies did not address the possible role of L-selectin in the immune response. We have independently generated L-selectin–deficient mice and demonstrate here that L-selectin plays an important part in the generation of T cell responses. L-selectin is required for optimal primary T cell responses in the draining PLN upon specific antigen interaction. In addition, impaired delayed-type hypersensitivity (DTH) responses and lack of neutrophil migration to the site of DTH reaction were evident in L-selectin–deficient mice. This alteration is time dependent, and effective T cell priming can occur after longer immunization intervals. Importantly, after T cell priming has occurred, we show that antigen-driven neutrophil recruitment is possible in L-selectin–deficient mice, suggesting that alternative molecules may be involved in extravascular recruitment of functional neutrophils in specific immune responses in vivo. Finally, humoral and memory T cell responses seem to be independent of regulation by L-selectin.

Materials and Methods

L-Selectin Mutant Mice and Embryonic Stem (ES) Cells. ES-D3 cells (10^7) in 0.7 ml of media were transfected with 25 μg of NotI linearized construct (see Fig. 1) with a gene pulser (Bio-Rad Laboratories, Richmond, CA) (25 μF, 0.32 kV). After electroporation, the cells were plated on 10 100-mm plates in media (DME with 15% FCS, 2 mM glutamine, and 0.1 mM 2-ME) supplemented with leukemia inhibitory factor (GIBCO BRL, Gaithersburg, MD). Media with geneticin (170 mg/ml; GIBCO BRL) was added 24 h after transfection. All transfectants were maintained on a monolayer of embryonic fibroblasts during the 10-d drug selection. Genomic DNA was purified from individual clones and analyzed by Southern blotting with a 5′ probe as indicated in Fig. 1 *A*. This probe is a 2.2-kb HincII/HincII fragment from the 5′ end of the L-selectin gene. It hybridizes to an 8-kb XbaII fragment of the endogenous L-selectin gene and to a 6.7-kb fragment of the disrupted allele. Positive clones were found at a frequency of 1/350 G-418–resistant colonies. Blastocyst injection and implantation were performed as described (24). Chimeric male mice were chosen to mate with B6 female mice, and offspring were scored for the 129/SV genotype transmission through the germline by examining coat color. Transmission of the mutated L-selectin allele in Agouti offspring was confirmed by Southern blot analysis. Finally, heterozygous mice were mated to produce homozygotes. L-selectin–deficient mice were maintained in a pathogen-free environment.

Flow Cytometric Analysis. Single-cell suspensions of spleen, PLN, mesenteric lymph nodes, Peyer's patches, blood, and thymus tissue were prepared from 6–8-wk-old L-selectin–deficient or wild-type mice. Cells were stained by incubating for 30 min at 4°C with anti-CD4–PE, anti-CD8–FITC, B220–biotin, CD45R–FITC, Pgp-1-FITC, and L-selectin–biotin. Cells were subsequently washed and fixed in 1% formaldehyde. Samples were analyzed on a FACStar Plus® (Becton Dickinson & Co., Mountain View, CA). A minimum of 10,000 cells were collected for each sample.

Leukocyte Isolation and Counts. Single-cell suspensions from the spleen, thymus, PLN (cervical, axillary, inguinal, and periaortic), and mesenteric lymph nodes were prepared, and the number of cells were determined using a hemocytometer. Blood samples were obtained by puncture of retroorbital plexus of anesthetized mice. $CD4^+$ T cells were purified by a method described earlier (25).

T Cell Proliferation Assay. Mice were immunized with 100 μg KLH in saline in a 1:1 emulsion with CFA containing 1 mg/ml *Mycobacterium tuberculosis* strain H37Ra (Difco Laboratories Inc., Detroit, MI) in the hind footpads. After 5 or 9 d, the popliteal lymph nodes were removed and cell suspensions were prepared. The lymph node cells were cultured in 96-well plates at 5×10^5 cells/well in Bruffs medium supplemented with 5% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and different concentrations of the antigen. Proliferation was measured by addition of 1 μCi of [^{3}H]thymidine (ICN Radiochemicals, Irvine, CA) for the last 18 h of a 5-d culture, and incorporation of radioactivity was assayed by liquid scintillation counting.

Cytokine Assays. Assays for cytokine production by T cells from KLH-immunized mice were conducted by culturing 10^5 purified $CD4^+$ T cells with 5×10^5 irradiated (3,000 rads) splenic cells from unimmunized wild-type mice and different amounts of the KLH in Bruffs medium, supplemented as described above. After 24 h of culture, 100 μl of culture supernatant was removed from each well for IL-2 bioassay. For IL-4 and IFN-γ measurements, supernatants were removed after 4 d of culture. For IL-2 determination, supernatants were added to 10^4 CTLL cells. 24 h later, proliferation of CTLL was measured by addition of 1 μCi of [^{3}H]thymidine per well for the final 6 h of culture. CTLL cells were routinely maintained in DMEM medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% FCS, 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, 10^{-4} M 2-ME, and 50 U/ml rIL-2 (Biogen, Boston, MA). ELISA was used to determine IL-4 and IFN-γ levels in supernatants with a kit from PharMingen (San Diego, CA), using recommended protocols.

Memory T Cell Responses. For generation of memory T cell responses to KLH, mice were immunized with 50 μg KLH in CFA (intraperitoneally or subcutaneously). 8 wk later, animals were killed, and $CD4^+$ T cells were purified from draining lymph nodes, nondraining lymph nodes, and spleen. T cell proliferative responses were analyzed by incubating 10^5 $CD4^+$ T cells with 3×10^5 syngeneic APC and different amount of KLH in vitro, as described above for T cell proliferation assays.

DTH Responses. For KLH-mediated DTH responses, mice were immunized with 100 μg KLH in CFA at two sites on dorsal flanks. 4 d after immunization, the thickness of both ears was measured, and 25 μl of PBS and KLH (1 mg/ml) were injected into the left and right ears, respectively. Ear thickness was measured again 24 h later. Measurement was carried out with a dial thickness gauge (Mitutoyo Corp., Tokyo, Japan).

The kinetics of in vivo T cell–mediated DTH responses were evaluated by using the assay of contact sensitivity ear swelling. Mice were either sensitized epicutaneously with 150 μl of 3% oxazolone in alcohol/acetone (3:1) to the shaved abdomen and four paws or they were sham sensitized. At various time points after sensitization, elicitation of immune response was performed by challenging the ears with 25 μl of 1% oxazolone in oil/acetone (3:1) on each side of each ear. Ear swelling was measured with an engineer's micrometer at various time points after elicitation, and histological evaluations were made by hematoxylin and eosin staining of formalin-fixed ear specimens at matched time points after challenge. Formalin-fixed ear specimens were also stained with GR-1 antibody against neutrophil (PharMingen).

Anti-KLH Antibodies Analysis by ELISA. Mouse anti-KLH IgM, IgG1, IgG2a, IgE, and total Ig levels were determined by isotype-specific ELISA. Briefly, 96-well plates were coated with 10 μg/ml KLH for 24 h. Plates were then washed and blocked with 1% FCS in PBS. Diluted serum samples were then added to triplicate wells, and plates were incubated for 1 h at 37°C. Plates were washed and the antigen-specific antibody titers determined with one of the following biotin-conjugated detection antibodies: goat anti–mouse IgM, IgG1, IgG2a, Ig, or IgE (Southern Biotechnology Associates, Birmingham, AL). All ELISAs were developed by using streptavidin-conjugated horse radish peroxidase

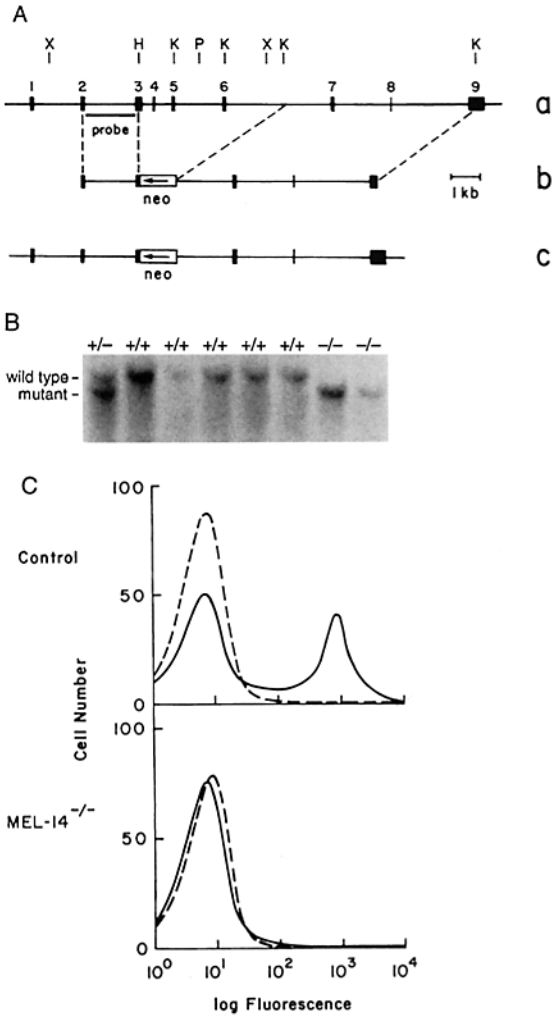

Figure 1. Gene targeting of the mouse L-selectin gene in ES cells. (*A*) Genomic structure of mouse L-selectin. The length of diagnostic restriction fragments and location of probes used for Southern blot analysis are shown. (*a*) B, Bam HI; H, HincII; K, Kpn 1; and X, XbaI. (*b*) Structure of the pMC1neo PolyA targeting vector, which was introduced by electroporation into ES cells. (*c*) Predicted structure of the targeted L-selectin locus. (*B*) Southern blot analysis of representative tail biopsies of L-selectin–mutant mice. Genomic DNA was digested with XbaI and hybridized with the 2.2-kb probe as indicated. (*C*) Leukocytes from L-selectin–deficient mice do not express L-selectin. PBMC from L-selectin–deficient mice and control littermates were stained with biotin-conjugated L-selectin mAb followed by streptavidin–PE staining. Dotted and solid lines represent the background fluorescence level obtained with unstained cells and L-selectin stained, respectively. Results are representative of six such experiments.

and ELISA T-Turbo developing reagent substrate (ICN). Plates were analyzed on an ELISA plate reader (model MR700; Dynatech Laboratories, Inc., Chantilly, VA).

Results

Construction of L-Selectin–deficient Mice. We used gene targeting to create a null mutation in the L-selectin locus of murine ES cells by methods described previously (26). Using L-selectin cDNA as a probe, we first isolated a 16-kb genomic DNA clone containing exons 1–9 from a 129Sv/J mouse genomic library (for details of the genomic structure, see reference 27). To create a targeting construct, we replaced a 5.5-kb HindIII/KpnI fragment encoding the L-selectin domain with the pCM1 *neo* gene in the reverse transcriptional orientation (Fig. 1 *A*). The linearized vector was introduced by electroporation into ES cells, and resistant colonies were selected by culture in media containing G418. ES cell clones carrying the L-selectin mutation through a homologous recombination event were identified by Southern blot analysis. Mutant ES clones were injected into blastocysts of C57BL/6 mice, and the resulting male chimeras were mated to C57BL/6 female mice for germ line transmission of the L-selectin mutation. Offspring derived from ES cells were identified by coat color and tested for the presence of the L-selectin mutation by Southern blotting (Fig. 1 *B*). Heterozygous offspring were then mated to obtain animals homozygous for the mutation. Homozygous mice deficient in L-selectin were produced at the expected Mendelian frequency. These mice were housed in a pathogen-free environment; they did not exhibit any obvious gross physical abnormalities, and they thrived and reproduced normally.

To verify that the L-selectin mutation indeed abolishes L-selectin expression, peripheral blood leukocytes from wild-type and homozygous L-selectin–deficient litter mates were stained with the L-selectin–specific antibody (MEL-14) and analyzed by flow cytometry. Data presented in Fig. 1 *C* show that a large proportion of blood leukocytes have high levels of L-selectin expression in the control wild-type mice, whereas leukocytes from L-selectin–deficient mice do not express L-selectin. We also analyzed expression of L-selectin in cells from thymus, spleen, and lymph nodes from L-selectin–deficient mice and found there was no detectable L-selectin expressed on the surface of these cells (data not shown). We further analyzed the lymphoid organs and blood for absolute number of lymphocytes. Confirming the results of Arbones et al. (23), PLN lymphocyte numbers are dramatically decreased in L-selectin–deficient mice. Likewise, although the PLN in L-selectin–deficient mice were significantly smaller than control PLN, they had normal architecture with primary follicles (data not shown).

L-Selectin Is Important for the Primary T Cell Response. We immunized both L-selectin–deficient and wild-type control mice subcutaneously with KLH/CFA. Visual examination showed much smaller draining lymph nodes in immunized L-selectin–deficient mice than in control littermates, and the number of lymphocytes from draining lymph nodes in L-selectin–deficient mice was only 10% of control littermates (data not shown). However, the gross architecture of draining lymph nodes from primed mice in the L-selectin–deficient mice was indistinguishable from control mice (data not shown). When lymphocytes from KLH-primed mice were stained with CD4, CD8, and B220, the percentage of cells in each compartment was not significantly different between the L-selectin–deficient and control mice (data not shown), indicating that L-selectin did not selectively affect these populations after immunization.

5 d after immunization with KLH in CFA, draining lymph node cells (LNC) were analyzed for proliferative recall responses to KLH in vitro. The KLH-specific T cell proliferative response in draining LNC was dramatically decreased in L-selectin–deficient mice, whereas a good proliferative response was seen in wild-type control mice (Fig. 2 *A*). To examine if the lack of proliferative response seen in L-selectin–deficient mice is caused by lack of CD4 T cell activation or differentiation, we analyzed IL-2, IL-4, and IFN-γ production by purified CD4 cells from draining lymph nodes of KLH/CFA-primed mice in response to an in vitro challenge with KLH (Fig. 2, *B–D*). Our data show a dramatic reduction in the production of IL-2, IL-4, and IFN-γ in L-selectin–deficient mice, whereas wild-type mice produced a significant amount of these cytokines, indicating that the T cells in draining lymph nodes from L-selectin–deficient mice were poorly primed. These data are consistent with a recent study in which Bradley et al. (22) found that in vivo administration of MEL-14 prevented priming of naive CD4 cells for proliferation and cytokine production to KLH in PLN draining the site of antigen injection. In L-selectin–deficient mice, however the T cell proliferative response 9 d after immunization in L-selectin–deficient mice is restored to that of control mice (Fig. 2 *E*). Interestingly, the response of wild-type mice is significantly below the peak response at this time point, so that the overall cumulative response of L-selectin–deficient mice is still reduced. These results suggest that lymphocytes can also enter PLN via an L-selectin–independent mechanism and can be stimulated by KLH/CFA. However, this mechanism presumably provides for a slower entry of these T cells into PLN and therefore permits only delayed immune responses.

DTH Responses in L-Selectin–deficient Mice. DTH is a complex cellular reaction that involves two distinct steps: sensitization and effector function. It has been suggested that naive CD4 T cells differentiate to become sensitized T cells during the sensitization step. When T cells encounter antigen during the effector stage, they are activated to produce proinflammatory cytokines, which serve to recruit inflammatory cells to sites of antigen exposure. L-selectin–deficient and control mice were sensitized by immunizing mice with KLH/CFA; 4 d after immunization, mice were rechallenged with PBS and KLH in the left and right ears, respectively. 24 h later, the swelling of each ear was measured. As shown in Fig. 3, the DTH responses were dramatically reduced when compared with control mice, suggesting that L-selectin is involved in the DTH responses. These results suggest that L-selectin–deficient mice are ei-

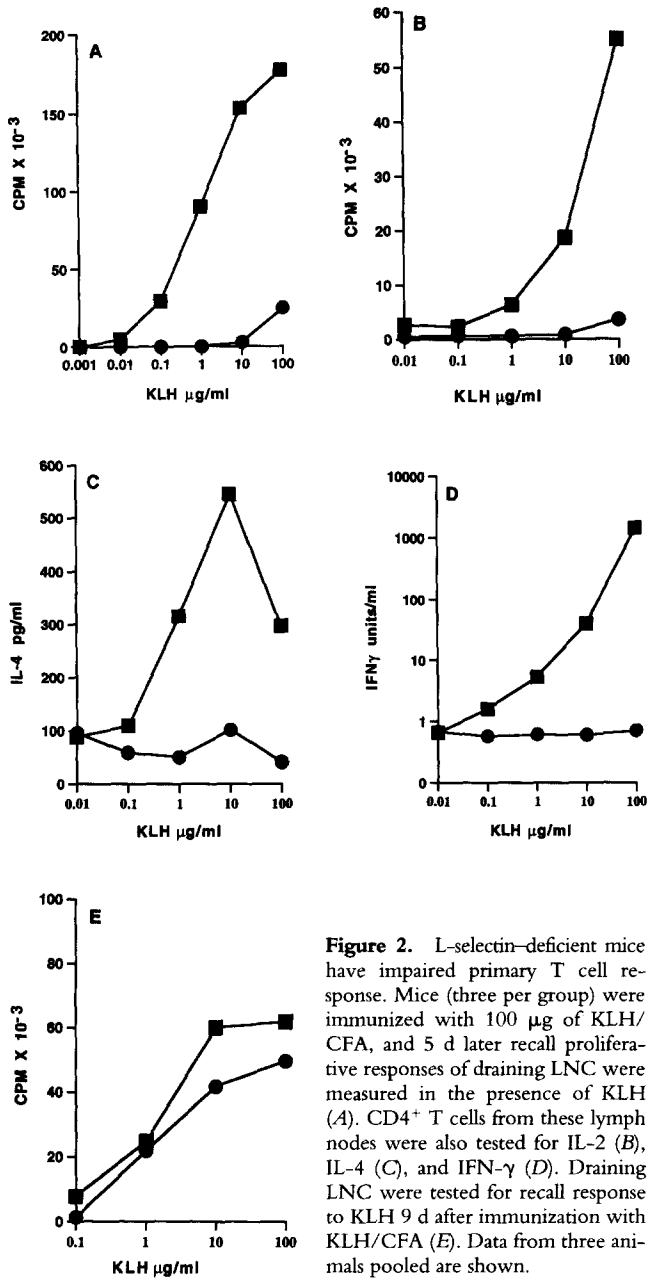

Figure 2. L-selectin–deficient mice have impaired primary T cell response. Mice (three per group) were immunized with 100 μg of KLH/CFA, and 5 d later recall proliferative responses of draining LNC were measured in the presence of KLH (*A*). $CD4^+$ T cells from these lymph nodes were also tested for IL-2 (*B*), IL-4 (*C*), and IFN-γ (*D*). Draining LNC were tested for recall response to KLH 9 d after immunization with KLH/CFA (*E*). Data from three animals pooled are shown.

ther unable to generate recall T cell response or their T cells are impaired in the ability to recruit inflammatory cells to the site of KLH challenge.

To confirm these findings, we used a second assay in which oxazolone-induced elicitation of contact sensitivity (CS) was measured. Results displayed in Table 1 show that L-selectin–deficient mice failed to manifest normal DTH responses after conventional (4-d) sensitization: no significant ear swelling was detected 24 h after elicitation, whereas their congenic littermate controls mounted normal DTH responses at this time point (24 h). To be certain that DTH was not delayed in onset in the L-selectin–deficient strain, ear swelling was also measured at 72 and 120 h after elicitation, confirming the absence of DTH in this strain and the normal resolution of DTH swelling in the wild-type controls. This result is in agreement with a recent report published by Tedder et al. (28) in which they too show deficiencies in the DTH response in their L-selectin–deficient mice, measured at the usual time after priming. Our results implied a deficiency either in the ability to generate recall T cell responses or in the ability of T cells from deficient strains to recruit inflammatory cells to the areas of antigen challenge. To examine this, elicitation of CS was performed 9 d after sensitization of L-selectin–deficient mice. The results presented in Table 1 show that the L-selectin–deficient strain was now able to manifest a DTH response measured 24 h after challenge that was similar in magnitude to the CS response of 4-d immune wild-type mice 24 h after elicitation. Thus, at later time points (9 d after immunization), antigen-specific responses measured both by DTH and by recall responses in vitro were restored to the same levels as wild-type mice. This suggested that with time, L-selectin–independent mechanisms may participate in the development of T cell–mediated immune responses. Finally, since L-selectin may also be involved in vascular adhesion events leading to extravascular neutrophil recruitment (10, 11), we examined the histology of ears (24 h after challenge) after conventional (4 d) and prolonged (9 d) sensitization protocols. These studies showed that L-selectin–deficient strains had diminished neutrophil recruitment to areas of antigen challenge after the 4-d sensitization protocol, but prolonged sensitization allowed normal recruitment of neutrophils to contact challenged ears of immune L-selectin–deficient mice (Fig. 4). This shows that the deficiency in DTH is in T cell priming and not neutrophil extravasation. Interestingly, background ear swelling responses in nonimmune animals challenged with oxazolone were higher in L-selectin–deficient mice than wild-type controls. Histologic analysis showed that this nonspecific response was the result of tissue edema with no cellular infiltrates (data not shown).

Normal Memory Immune Response in L-Selectin–deficient Mice. L-selectin has been implicated as a marker associated with memory lymphocyte function (19). We therefore examined whether L-selectin–deficient mice have impaired memory function. We immunized mice with KLH/CFA (both intraperitoneally and subcutaneously); 8 wk after immunization, T cells were purified from spleens and draining lymph nodes and were analzyed for proliferative responses

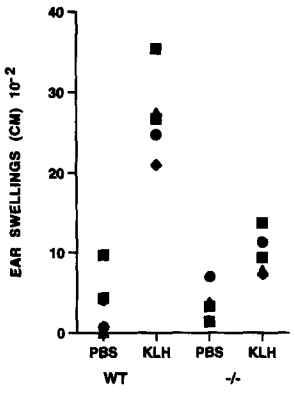

Figure 3. L-selectin is involved in the DTH responses. Mice (three per group) were sensitized with KLH/CFA. 4 d later, PBS and KLH were injected into the left and right ears, respectively. 24 h later, the thickness of ear swelling was measured as described in Materials and Methods.

Table 1. *Oxazolone Contact Sensitivity in L-Selectin–deficient Mice*

Mouse strain	Immune status	Days after immunization	Contact sensitivity ear swelling (U × 10^{-3} cm ± SEM) 24 h	72 h	120 h
+/+	Sham	4	0.0 ± 0.2	2.2 ± 0.7	2.0 ± 0.5
+/+	Oxazolone	4	3.5 ± 0.4*	3.3 ± 0.4‡	4.0 ± 0.9‡
−/−	Sham	4	2.8 ± 0.3	1.7 ± 0.8	1.8 ± 0.8
−/−	Oxazolone	4	2.7 ± 1.0‡	3.0 ± 0.3‡	3.4 ± 0.6‡
−/−	Sham	9	2.8 ± 0.3		
−/−	Oxazolone	9	5.2 ± 0.6§		

Four mice in each group were tested for contact sensitivity as described in experimental procedures.
*$P < 0.005$; ‡not significant; §$P < 0.001$.

to KLH. When animals were immunized with KLH/CFA intraperitoneally, there was no significant difference in the splenic T cell proliferation in response to KLH in L-selectin–deficient mice compared with control littermates (Fig. 5 *A*). Similar results were obtained when mice were immunized subcutaneously (Fig. 5 *B*); moreover, a good recall proliferative response was seen in draining lymph nodes (Fig. 5 *C*). Studies have shown that both the resting and activated murine memory CD4 T cell population from spleen lacks L-selectin (20, 21); presumably, these memory

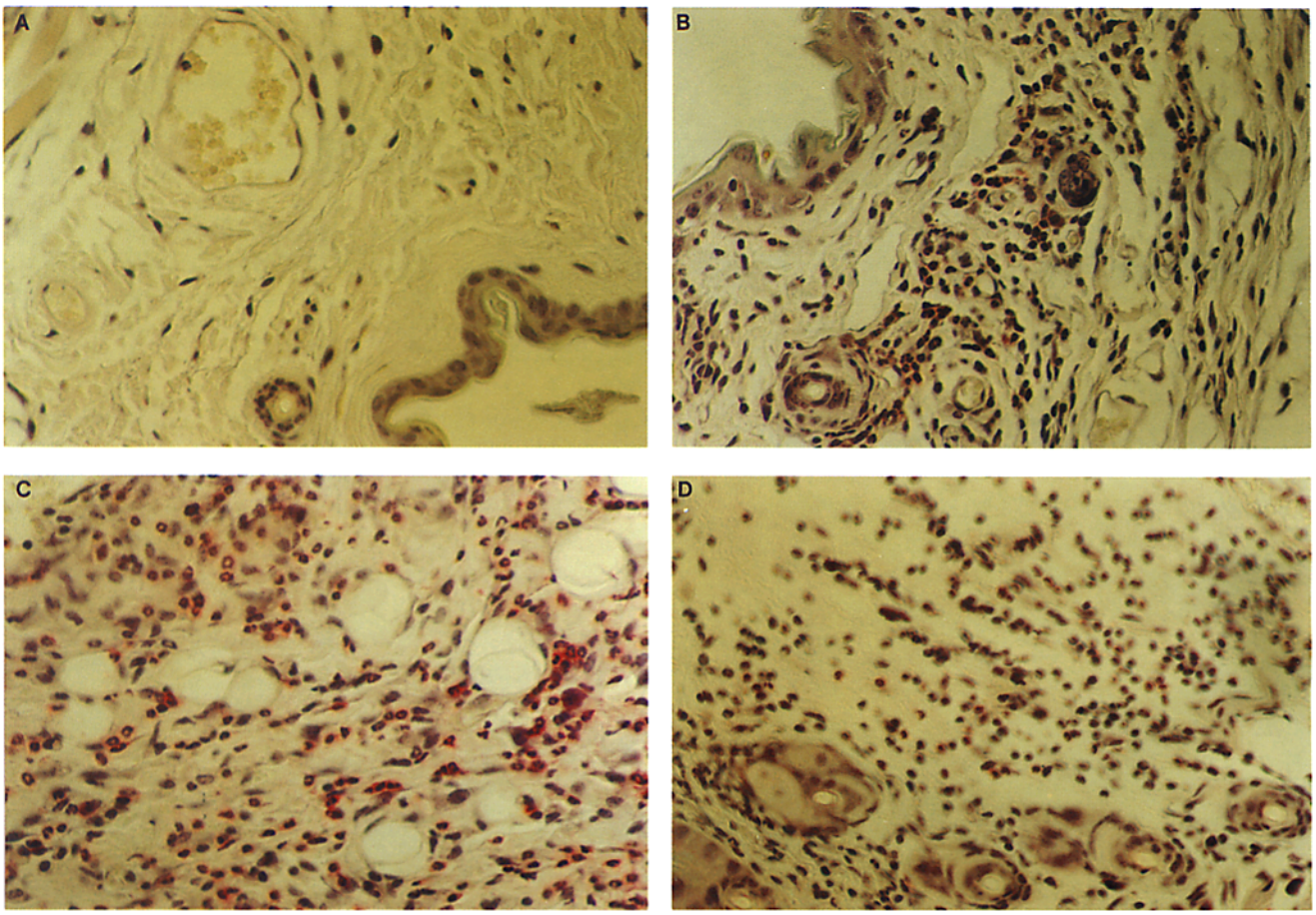

Figure 4. Histological examination of ears. Recruitment of neutrophils to contact challenged ears was examined by staining paraffin-fixed ear tissue sections with mAb specific for neutrophil (GR-1) and counterstaining with hematoxylin. Ear sections from L-selectin–deficient mice at 4 d (*A*) vs 9 d (*C*) after immunization, and from wild-type mice at 4 d (*B*) vs 9 d (*D*) after immunization are shown.

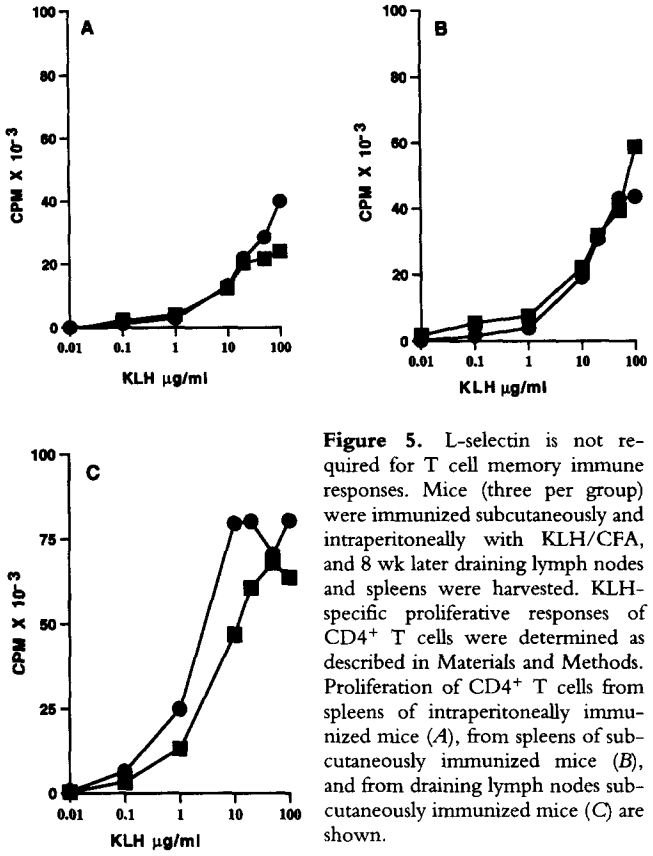

Figure 5. L-selectin is not required for T cell memory immune responses. Mice (three per group) were immunized subcutaneously and intraperitoneally with KLH/CFA, and 8 wk later draining lymph nodes and spleens were harvested. KLH-specific proliferative responses of $CD4^+$ T cells were determined as described in Materials and Methods. Proliferation of $CD4^+$ T cells from spleens of intraperitoneally immunized mice (*A*), from spleens of subcutaneously immunized mice (*B*), and from draining lymph nodes subcutaneously immunized mice (*C*) are shown.

CD4 cells arise from the downregulation or shedding of L-selectin on the L-selectin–positive naive $CD4^+$ T cells.

Normal Humoral Immune Responses in L-Selectin–deficient Mice. To investigate the role of L-selectin in humoral immune responses, we tested the primary antibody response of L-selectin–deficient mice to a protein antigen, KLH. Wild-type and L-selectin–deficient animals were immunized with KLH/CFA, and 7 d later the primary anti-KLH IgM response was determined by ELISA assay. As shown in Table 2, L-selectin–deficient mice produced similar anti-KLH IgM levels when compared with control littermates. This result demonstrates that L-selectin–deficient mice have normal primary antibody responses to KLH. We also examined the humoral immune response by measuring total anti-KLH Ig and other anti-KLH Ig isotypes on day 19. As shown in Table 2, L-selectin–deficient mice produced normal anti-KLH total Ig, IgM, IgG1, IgG2a, and IgE. These results demonstrate that L-selectin–deficient mice can develop normal primary IgM antibodies and switch to other Ig isotypes in response to an antigen challenge.

Discussion

The development of a successful immune response is dependent on the ability of mature lymphocytes to recirculate throughout the secondary lymphoid organs via blood and lymph. This recirculation is essential to allow exposure of the full antigen receptor repertoire to all sites of antigen

Table 2. *L-Selectin–deficient Mice Are Able to Generate Normal Humoral Responses*

Anti-KLH antibody	Days after immunization	L-selectin–deficient mice	Wild-type mice
IgM	7	1.294 ± 0.031	1.334 ± 0.022
IgM	19	0.878 ± 0.248	1.227 ± 0.133
Total Ig	19	1.255 ± 0.008	1.263 ± 0.047
IgG1	19	1.033 ± 0.140	1.188 ± 0.090
IgG2a	19	1.257 ± 0.040	1.302 ± 0.014
IgE	19	0.187 ± 0.040	0.146 ± 0.023

Mice (three per group) were administered 100 μg of KLH/CFA intraperitoneally. The KLH-specific titer of IgM in serum was determined on days 7 and 19, and the total Ig, IgG1, IgG2a, and IgE titers were determined 19 d after immunization by KLH-specific ELISA as discussed in Materials and Methods. Data are presented as mean absorbance at 405 nm ± SEM.

deposition, regardless of its location and site of entry (5). Lymphocyte migration to a particular tissue depends on the interactions between the lymphocytes and specialized venular endothelial cells, which are mediated by specific homing receptors and their ligands. L-selectin is a well-characterized homing receptor that supports the adhesion of lymphocytes to HEV of PLN (7, 12–15). Recently, a role of L-selectin in leukocyte homing, rolling, and migration has been demonstrated in L-selectin–deficient mice (23). However, the possible role of L-selectin in immune response still remains largely unknown. To understand the importance of L-selectin in the in vivo antigen-specific immune response, we generated L-selectin–deficient mice, which provided us with an opportunity to examine the in vivo role of L-selectin in specific immune responses.

We examined L-selectin–deficient mice for subsets of lymphocytes both in the thymus and in the periphery, but no gross abnormalities in the distribution of lymphocytes were seen in these mice, confirming the observations of Arbones et al. (23). Although subset ratios and number of lymphocytes are normal in blood and in other secondary lymphoid organs, reduced numbers of lymphocytes and smaller PLN are seen in L-selectin–deficient mice. These observations are in agreement with previous studies in which MEL-14 antibody was shown to block the migration of lymphocytes to PLN (7, 29–31) and the observations in the L-selectin–deficient mice of Arbones et al. (23). Even after priming with KLH in CFA, our L-selectin–deficient mice again had relatively smaller PLN and slightly larger spleens, thereby strongly supporting a definite role for L-selectin in homing of lymphocytes to PLN. The increased number of lymphocytes present in spleens in L-selectin–deficient mice is consistent with the observations that homing mechanisms to spleen may be different than PLN and may not require the presence of L-selectin on the surface of lymphocytes (2, 31).

The impaired migration of lymphocytes to PLN and the

small size of KLH-primed PLN prompted us to determine whether antigen-specific T cell responses in L-selectin–deficient mice are normal. The results presented here show that L-selectin–deficient mice fail to generate a primary T cell response in lymph nodes draining the site of antigen challenge. These draining lymph node T cells were not only deficient in proliferative response but were also defective in the production of IL-2, IL-4, and IFN-γ in response to KLH. These observations suggest that in L-selectin–deficient mice, draining LNC not only were poorly primed to KLH but also failed to be activated and differentiate to produce cytokines. The lack of primary response in L-selectin–deficient mice may be caused by the failure of migration of naive T cells to PLN. This explanation is in agreement with the small size of KLH-primed lymph nodes and with the studies in which MEL-14 antibody has been shown to block recirculation of naive T cells to PLN (12–14, 22). Another possibility is that L-selectin may modulate functions of T cells directly. Reports have demonstrated association of L-selectin with the TCR–CD3 complex on the T cell surface and suggest that L-selectin modulates signal transduction events in T cells through TCR–CD3 (32). Furthermore, L-selectin has been shown to provide costimulatory signal to T cells (33). However, we do not favor the possibility because in vitro stimulation of naive T cells from L-selectin–deficient mice with anti-CD3 or ConA is normal (Grewal, I.S., J. Xu, and R.A. Flavell, unpublished results), and primary T cell responses at later time points are normal in L-selectin–deficient mice. Taken together, these observations suggest that lack of L-selectin on lymphocyte surfaces slows extravasation of naive and recirculating T cells into PLN, which is responisble for lack of primary T cell response at early time points. At later time points, small numbers of lymphocytes present in PLN expand sufficiently to give good proliferative responses. Alternatively, there must exist a second mechanism of recruitment of naive T cells to PLN, or development of response occurs in the spleen and then memory cells relocate to PLN. Nonetheless, since the number of total T cells per lymph node in L-selectin–deficient mice is only 10% of that wild-type mice, the overall T cell response in L-selectin–deficient mice on a per-animal basis is ~10% of that of wild-type mice, even at later time points.

The migration of lymphocytes to skin is also characterized by tissue-specific homing mechanisms, in a way similar to the homing of lymphocytes to secondary lymphoid organs. Lymphocyte migration to skin has been routinely studied by examination of DTH responses, which serves as an in vivo model of antigen-driven inflammation of skin. In this report, we have examined antigen-specific contact sensitivity responses to oxazolone and a classical DTH response, using KLH as an antigen. As discussed in Results, in the case of both antigens and in contrast to wild-type mice that exhibit peak DTH at 24 h and subsequent resolution of tissue swelling, L-selectin–deficient mice failed to generate a DTH response at the conventionally studied time (4 d) after immunization. However, at later time points after immunization, unequivocal oxazolone contact sensitivity responses were seen. Specifically, when DTH was elicited 9 d after immunization, a response similar in magnitude to 4-d immune wild-type mice was seen. Furthermore, histological studies show decreased migration of neutrophils to the site of inflammation when DTH was elicited 4 d after immunization, which may be the result of lack of effective T cell priming and subsequent cytokine generation required for extravascular recruitment of neutrophils. These results clearly demonstrate that there are fundamental differences in the induction of T cell immunity in L-selectin–deficient and wild-type mice. It is likely that the absence of DTH after 4 d and presence of DTH after 9 d of immunization reflects the retarded kinetics of T cell priming that we observed in primary T cell responses to KLH in L-selectin–deficient mice. This suggests that the in vivo effects of L-selectin may be dominated by its effects on the generation of effective T cell recall events in peripheral lymphoid tissue rather than preventing neutrophil migration to the site of challenge, which is normal after 9 d of immunization. Alternatively, it is possible that there is a reduced recruitment of $CD4^+$ T cells to the challenge site, which is rate limiting. However, the fact that recruitment is better in the L-selectin–deficient mice at day 9 argues against this.

We further examined the memory T cell responses to KLH in L-selectin–deficient mice. Memory T cells are shown to have downregulated their L-selectin and can be distinguished from naive T cells on the basis of their L-selectin expression. L-selectin has been shown to be lost rapidly after activation of T cells (17). These results indicate that L-selectin–deficient mice have normal memory T cell responses to KLH. Our results are consistent with previous studies that demonstrated that naive and memory T cells use different pathways for migration (33). T cells of memory phenotype are found mainly in afferent lymph, whereas naive T cells are usually found in efferent lymph after passing through PLN using HEV–L-selectin interaction (33). The reservoir for memory T cells is the spleen, where these cells lack or have low levels of L-selectin expression. Moreover, L-selectin is not required for the homing of T cells to spleen. These observations can explain the normal memory T cell response shown in our L-selectin–deficient mice, even after intraperitoneal or subcutaneous immunization. In the case of intraperitoneal immunization, T cells are directly activated in the spleen and perhaps expand, and are retained there. In subcutaneous immunization, however, T cells are activated mainly in draining lymph nodes, where naive T cells differentiate into effector and memory cells. As we discussed earlier, there are fewer T cells in PLN of L-selectin–deficient mice, and differentiation of these or any circulating T cells into memory cells is thus possible in these mice. Once these T cells are activated in PLN, perhaps they migrate to spleen and become sequestered there. However, it is possible that some of the T cells either remain in the PLN or recirculate back to the PLN, which can explain the fact that we observe memory T cell responses in the draining lymph nodes of L-selectin–deficient mice upon subcutaneous immunization. In addition, persis-

tence of antigen in lymph nodes over longer periods of time may cause continued recruitment of naive T cells. Overall, our studies suggest that L-selectin does not play a significant part in generation of memory T cells, indicating that L-selectin–independent mechanisms must be operative in generating T cell memory response. Furthermore, our data clearly demonstrated that these functional memory CD4 cells can also be derived from L-selectin–negative cells.

$CD4^+$ T cells play a key role in the development and differentiation of B cells into antibody-secreting plasma cells. Antigen-primed activated T cells and memory $CD4^+$ T cells provide help to B cells to mature and switch IgM class to other isotypes (34). It has been demonstrated that memory T cells devoid of L-selectin migrate to mucosal tissues, whereas memory T cells expressing L-selectin migrate to local lymphoid organs. In these local lymphoid areas, B cells are activated by exposure to antigen. Thus, mice lacking L-selectin might have been expected to have altered antibody responses. To our surprise, however, L-selectin–deficient mice produce normal levels of antibodies, with the exception of IgM, which is slightly lower in L-selectin–deficient mice when tested 19 d after immunization with KLH, whereas switching to other classes was normal when determined by measuring serum levels of other isotypes. It is well established that isotype switching from IgM to other classes in B cells requires cytokines and a direct interaction with T cells. These observations indicate that L-selectin does not influence T cell help for B cell antibody production.

In conclusion, the data presented here show that lymphocytes from mice lacking L-selectin are deficient in homing to PLN, fail to develop a normal primary T cell response to KLH, and are unable to mount a DTH response after conventional immunization intervals. After more prolonged immunization intervals, L-selectin–deficient mice were able to generate contact sensitivity responses and antigen-driven extravascular recruitment of neutrophils. Interestingly, humoral responses to protein antigen (KLH) were unimpaired, with production of normal amounts of KLH-specific antibodies, and memory T cell responses in L-selectin–deficient mice were normal, implying that L-selectin does not play a major role in T cell help for B cell antibody production. Thus, these mice are a valuable tool for studying lymphocyte trafficking and the induction of primary T cell responses to determine possible targets for therapy of autoimmune disease, transplantation, and allergy.

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