

Entry of Naive CD4 T Cells into Peripheral Lymph Nodes Requires L-Selectin

By Linda M. Bradley,* Susan R. Watson,† and Susan L. Swain*

From the *Department of Biology and the University of California, San Diego, Cancer Center, University of California, San Diego, California 92093; and the †Department of Immunology, Genentech, Inc., South San Francisco, California 94080

Summary

Binding of L-selectin expressed on lymphocytes to carbohydrate ligand(s) on lymph node high endothelial venules is thought to initiate lymphocyte extravasation from blood to lymph during recirculation and localization to sites of antigen (Ag) exposure. Previous studies have shown that treatment of lymphocytes with antibody to L-selectin (MEL-14) ablates trafficking to peripheral lymph nodes (PLN). In mice, naive but not memory CD4 cells express L-selectin. To examine the role of L-selectin in helper T cell migration, we studied the effects of in vivo administration of MEL-14 on CD4 cell responses. Systemic exposure of mice to MEL-14 depleted CD4 cells expressing a naive phenotype (CD45RB^{hi}, CD44^{lo}) from PLN but not from spleen. The majority of residual lymph node CD4 cells exhibited the reciprocal, memory phenotype (CD45RB^{lo}, CD44^{hi}). MEL-14 treatment prevented priming of naive CD4 cells for proliferation and cytokine production (IL-2 and IL-4) to keyhole limpet hemocyanin in PLN draining the site of Ag injection, but not in the spleen. The results suggest that naive cells were not depleted, but rather diverted to other sites where priming occurred. The data demonstrate that L-selectin mediates extravasation of naive CD4 cells into PLN and that its function cannot be replaced by other homing receptors.

One of the characteristic features of the immune system is the continuous recirculation of small lymphocytes from blood to lymph that is thought to be essential both to immunologic surveillance for infectious pathogens and the rapid localization of Ag-specific lymphocytes to sites of Ag exposure. Lymphocyte extravasation between apposed endothelial cells into peripheral lymphoid tissues is initiated by adhesive interactions of tissue-specific homing receptors with ligands on the luminal surface of vascular endothelium (1). Adhesion to high endothelial venules of peripheral lymph nodes (PLN) in vitro is primarily mediated by the lymph node homing receptor, L-selectin, a carbohydrate-binding protein (2) detected in the mouse by the MEL-14 antibody (3). L-selectin is initially acquired by mature thymocytes before exit to the periphery and entrance into the recirculating lymphocyte pool (4, 5). It was initially postulated that expression of L-selectin was required for lymphocyte recirculation to PLN on the basis of studies showing that lymphocytes treated with MEL-14 antibody (3) and L-selectin negative cell lines (6) are unable to migrate into lymph nodes in vivo. L-selectin is shed after lymphocyte activation (7–10) but can be reexpressed upon return to a resting state (8) suggesting that loss of L-selectin permits localization of responding cells in sites of Ag exposure and that its reexpression coincides with development of a memory state (11). However, we have shown that murine memory CD4 cells lack L-selectin (12, 13). In addition, CD4 cells expressing a naive phenotype (high

levels of CD45R, low levels of adhesion molecules) appear to be the major population to recirculate to PLN via high endothelial venules, while CD4 cells of the reciprocal, memory phenotype principally enter lymph nodes via the afferent lymph (14). Because of the importance of understanding lymphocyte trafficking to develop strategies for immune intervention in a variety of disease processes, we examined the in situ role of L-selectin in the migration of murine naive CD4 cells during primary immune responses. The data demonstrate that L-selectin is required for entry of naive CD4 cells into PLN and thus for development of primary CD4 responses in those sites.

Materials and Methods

Mice. C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Antibodies. Antibodies were isolated by protein G purification (Pharmacia Fine Chemicals, Piscataway, NJ). Rat IgG2a was obtained from normal sera. MEL-14 was purified from ascites. Anti- α 4 integrin (PS/2.3) was a gift of Sherman Fong (Genentech, Inc., S. San Francisco, CA). Antibodies for depletion of T and B cells have been described (15) and include anti-Thy 1.2 (F7D5 and HO.13.14), anti-CD4 (RL.172.4), anti-CD8 (HO.2.2, AD4), anti-class II (D3.137), and anti-HSA (J11d). Antibodies for staining included: CD44 (Pgp-1) IM7.8.1), L-selectin (MEL-14), and CD45RB (23G2). Rat IgG2a, and IgG2b isotype controls were from PharMingen (Sorrento Valley, CA). Anti-rat κ antibody (RG7/9.1) was conjugated to FITC. PE-anti-CD4 (GK1.5) was

from Becton Dickinson & Co. (Mountain View, CA). The following anti-cytokine or anti-cytokine receptor antibodies were used: 7D4 and PC.61 (anti-IL-2R); 11B11 (anti-IL-4); and XMG1.2 and R46A2 (anti-IFN- γ). Hamster anti-mouse CD3 (2C11) was from culture supernatants. Peroxidase-labeled goat anti-rat IgG was from Caltag Laboratories (San Francisco, CA).

CD4 Cell Preparation. Primary effector cells were induced by s.c. injection of mice with 200 μ g KLH (Calbiochem Corp., La Jolla, CA) in CFA at the base of the tail, 5 d before use (16). CD4 cells were enriched to >80% from periaortic lymph nodes and/or pooled inguinal and brachial lymph nodes, or spleens by depletion of CD8 cells and B cells with anti-CD8, anti-class II, and anti-HSA antibodies, and rabbit and guinea pig C (GIBCO BRL, Gaithersburg, MD).

APC Preparation. Normal spleen cells, depleted of T cells with a mixture of anti-Thy.1.2, anti-CD8, and anti-CD4 antibodies and C were used as APC. For anti-CD3 responses, APC were treated for 30 min at 37°C with 25 μ g/ml mitomycin C (Sigma Chemical Co., St. Louis, MO). For KLH-specific responses, APC were cultured for 18 h in 100-mm petri dishes (Fisher Scientific, Pittsburgh, PA) at 5×10^6 ml in RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 7% FCS (Hyclone Laboratories, Logan, UT), 100 μ g/ml penicillin, 100 U/ml streptomycin, 4 mM L-glutamine, 10 mM HEPES, and 5×10^{-5} M 2-ME and with 100 μ g/ml KLH before treatment with mitomycin C.

Flow Cytometry. Expression of CD44, L-selectin, and CD45RB was analyzed as described (12). CD4⁺ cells were stained with the indicated monoclonal antibodies, followed by FITC-anti-rat κ antibody, and PE-anti-CD4.

In Vitro Restimulation of CD4 Cells. For KLH responses, CD4 cells were cultured at the indicated concentrations in triplicate in 200 μ l supplemented RPMI with 2×10^5 Ag-pulsed APC in 96-well flat-bottomed plates (Costar Corp., Cambridge, MA). For anti-CD3 stimulation, wells were pretreated with 10 μ g/ml 2C11 for 2 h at 37°C and CD4 cells were cultured with 10^5 APC. Supernatants were harvested after 48 h. To assess proliferation, CD4 cells cultured under the above conditions were pulsed with 1 μ Ci [³H]thymidine at 48 h and harvested 18 h later.

Cytokine Assays. The cytokine assays have been previously described (13). Cytokines in culture supernatants were referenced to rIL-2, rIL-4, and rIFN- γ from X63.Ag8-653 cells transfected with murine cDNA for the respective cytokines (17). IL-2 and IL-4 were detected by proliferation of the NK cell line (18) which responds to both cytokines, but is specific for IL-2 when IL-4 activity is blocked by 11B11 antibody, and specific for IL-4 when IL-2 activity is blocked by the anti-IL-2R antibodies, 7D4 and PC.61. Data are half-maximum units where 1 U of IL-2 equals 14 pg protein, and 1 U of IL-4 equals 0.7 pg protein. IFN- γ was detected by ELISA using R46A2 as coating antibody and biotinylated-XMG1.2 as the second step reagent. Data are units per milliliter where 1 U of IFN- γ equals 0.1 ng protein.

Serum MEL-14. The presence of MEL-14 in sera was measured by ELISA as described (19) using L-selectin-Ig for capture and purified MEL-14 antibody as the standard.

Results

To assess the role of L-selectin in lymphocyte migration to PLN, groups of four to six C57BL/6 mice were injected i.v. with a single dose of 250 μ g MEL-14 antibody, or of rat IgG2a as an isotype control. 18 h later, mice were immunized by s.c. injection of 200 μ g KLH in CFA. The half-

life of MEL-14 in the serum was 5–6 d, at which time circulating levels of MEL-14 ranged from 33 ± 5 to 51 ± 7 μ g/ml. At day 6, a 10–50-fold reduction in PLN cellularity (pooled inguinal, brachial, and periaortic lymph nodes) was observed in mice treated with MEL-14 as compared with isotype control-treated animals (Fig. 1). In contrast, there was a 22% increase in the spleen cell number after MEL-14 treatment. These results suggest that most of the cells in the PLN are in the continuously recirculating pool and depend upon L-selectin for entry into PLN, but not spleen. Similar changes were seen in five additional experiments, irrespective of whether KLH was injected.

In parallel experiments (not shown), PLN draining the site of KLH injection (periaortic lymph nodes) from control mice exhibited histologic changes typically associated with a primary immune response including marked lymph node enlargement due to lymphoid proliferation and prominent germinal centers in the lymphoid follicles. In contrast, PLN from MEL-14-treated animals were atrophied, lacked germinal centers and zones of lymphoid expansion, and contained only mature resting lymphocytes. Thus, treatment with MEL-14 prevented induction of histological changes associated with a primary immune response in lymph nodes. Despite the large decrease in the lymph node cellularity in MEL-14-treated animals, the proportions of CD4⁺ and CD8⁺ T cells, and B220 bearing B lymphocytes were similar to those of PLN from mice treated with irrelevant IgG2a (not shown), indicating that MEL-14 antibody did not selectively affect recirculation of these populations and that approximately equal proportions of each cell type were excluded by MEL-14 treatment.

MEL-14 Blocks Priming of CD4 Cells in Lymph Nodes. To evaluate effects of MEL-14 treatment on CD4 cell function, primary effector cells were generated by immunization with KLH as above, 1 d after i.v. injection of MEL-14 or IgG2a antibody. CD4 cells were isolated from periaortic lymph nodes draining the site of Ag injection and from spleen, 5 d after

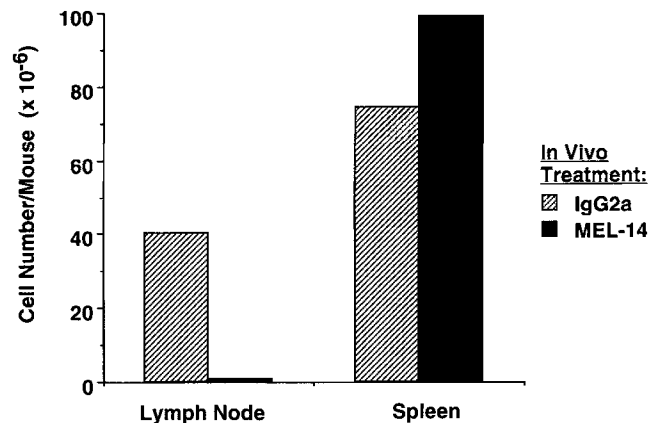


Figure 1. Effects of in vivo treatment with MEL-14 antibody on peripheral lymph nodes. A single dose of 250 μ g MEL-14, or of rat IgG2a was administered i.v. to groups of four to six mice 18 h before s.c. injection of 200 μ g KLH/CFA. 5 d later, the total numbers of nucleated cells from PLN (pooled inguinal, brachial, and periaortic lymph nodes) and spleen were determined.

priming, at the peak of the primary CD4 cell response (16). KLH-specific proliferation (Fig. 2 A), and IL-2 and IL-4 production (Fig. 2, B and C) by CD4 cells from MEL-14-treated animals were negligible compared with the responses obtained from control CD4 cells. In contrast, Ag-specific responses of splenic CD4 cells were unaffected by MEL-14 treatment (Fig. 2, A-C). Similar results were obtained in three additional experiments.

As a specificity control, we compared the *in vivo* effects of MEL-14 to an identical dose of an antibody to $\alpha 4$ integrin (PS/2) (20), an IgG2b antibody which binds to 90% of CD4 cells and blocks homing to Peyer's patches but not lymph nodes *in vivo* (21). In two experiments, anti- $\alpha 4$ had no effect on cell recovery from PLN or spleen and, by comparison with an isotype-matched control, did not diminish development of KLH-specific primary effector CD4 cells (assessed by proliferation and cytokine production) in either site (not shown). In other studies, an anti-CD44 antibody of the IgG2a isotype, IRAWB14.4 (22), which binds to nearly all lymphocytes from PLN and spleen blocked contact sensitivity responses but not homing to PLN (23). We conclude that the *in vivo* effects of MEL-14 treatment are due to the capacity to bind L-selectin rather than some nonspecific mechanism.

To evaluate the functional potential of the CD4 popula-

tion that remained in the lymph nodes after MEL-14 treatment, we examined the response to plate bound anti-CD3. Proliferation (Fig. 3 A), as well as IL-2, IL-4, and IFN- γ secretion (Fig. 3 B) comparable to that of control CD4 cells was elicited from residual lymph node CD4 cells. Thus, the few remaining lymph node CD4 cells were not adversely affected by the MEL-14 antibody. Comparable results were obtained in two additional experiments.

Surface Phenotype of CD4 Cells After *In Vivo* MEL-14 Treatment. We analyzed the surface phenotype of lymph node and splenic CD4 cells 6 d after exposure to MEL-14 (Fig. 4 A). As expected, lymph node CD4 cells from control animals had a predominantly naive phenotype: L-selectin^{hi}, CD45RB^{hi}, and CD44^{lo} (1, 24, 25). In contrast, CD4 cells from MEL-14-treated mice lacked L-selectin, a majority were CD45RB^{lo}, and at least half were CD44^{hi}, a phenotype associated with memory/Ag-primed CD4 cells (1, 12, 13, 24). These data, representative of four experiments, suggest that MEL-14 treatment *in vivo* selectively depleted naive CD4 cells from PLN. Splenic CD4 cells from MEL-14-treated mice also did not express L-selectin (Fig. 4 B), but in contrast to PLN, CD4 cells of otherwise naive phenotype (CD45RB^{hi}, CD44^{lo}) predominated. Although MEL-14 was present in serum (as indicated above), no background staining of CD4 cells with anti-rat κ secondary antibody was observed (not

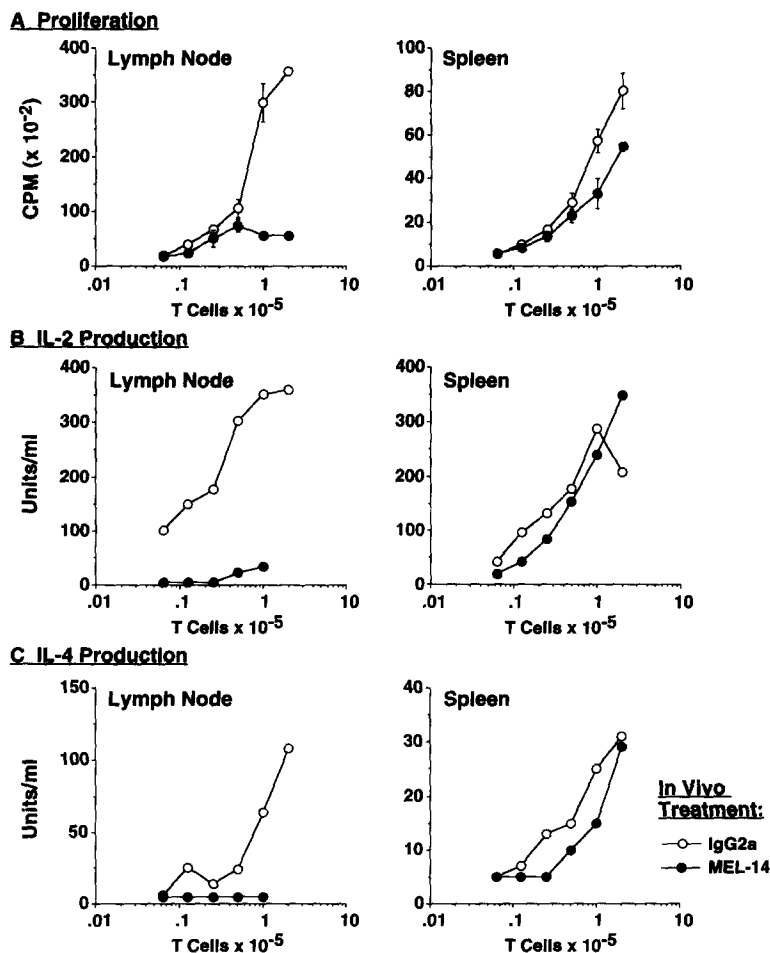


Figure 2. Failure to prime lymph node but not spleen CD4 cells in MEL-14-treated mice. Periaortic lymph node and splenic CD4 cells were isolated from mice treated 6 d previously with either IgG2a or MEL-14, 1 d before immunization with KLH as in Fig. 1. Varying numbers of CD4 cells were cultured with 2×10^5 KLH-pulsed APC. Proliferation (A) was measured at 72 h. Supernatants were tested after 48 h for IL-2 (B) and IL-4 (C).

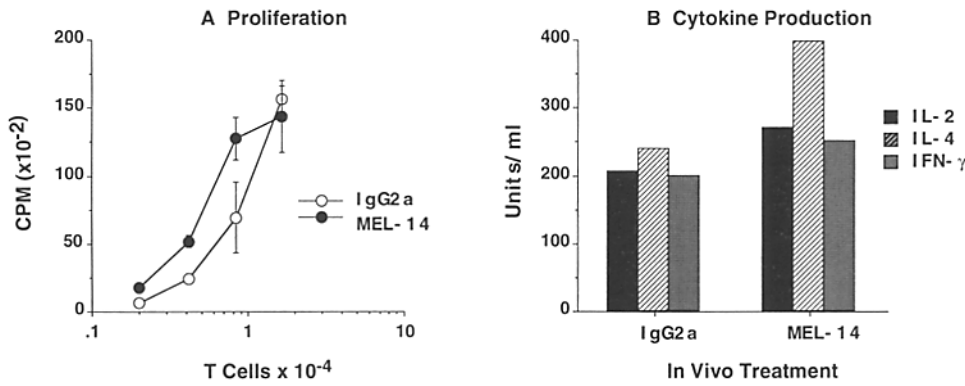


Figure 3. Response of CD4 cells to anti-CD3 after in vivo MEL-14 treatment. CD4 cells isolated from PLN (inguinal, brachial, and periaortic) of mice 6 d after treatment with rat IgG2a or MEL-14 were tested for (A) proliferation and (B) cytokine production in response to platebound anti-CD3. In (A), CD4 cells were cultured at the indicated numbers with 10^5 splenic APC and were tested for proliferation at 72 h. In (B), 3.3×10^4 CD4 cells were cultured with 10^5 APC, and supernatants were tested at 48 h for cytokines.

shown). The data suggest that L-selectin was lost from the surface of cells rather than masked. Moreover, since expression of other surface markers was unaffected, we conclude that the cells were not depleted by MEL-14 treatment, but were instead diverted to the spleen and other sites.

Discussion

The results of this study demonstrate that when L-selectin is blocked by treatment with MEL-14 antibody in vivo, there is a profound depletion of nucleated cells from PLN and a failure to generate primary immune responses in the lymph nodes draining the site of Ag injection (Fig. 1). Since CD4 cells from PLN of MEL-14-treated mice are unable to generate primary effector cells, as measured by a complete lack of priming for proliferation or cytokine secretion in response

to KLH (Fig. 2), the loss of responsiveness is in part due to a blockade of naive CD4 cell migration from the blood and potentially also to depletion of naive CD4 cells as well as other recirculating lymphocytes from lymph nodes. The data demonstrate that L-selectin is required for recirculation of naive CD4 cells to PLN, confirming its function in situ as the peripheral lymph node homing receptor. When lymphocyte trafficking via L-selectin is blocked during a primary response, neither naive CD4 cells nor responding, potentially circulating CD4 cells, are able to use alternative adhesion receptors to migrate into PLN.

Although naive CD4 cells are not primed in PLN in the presence of circulating MEL-14, the finding that normal KLH-specific primary effector CD4 cells are generated under the same conditions in the spleen (Fig. 2) indicates that L-selectin does not play a role in trafficking to the spleen. Despite the lack

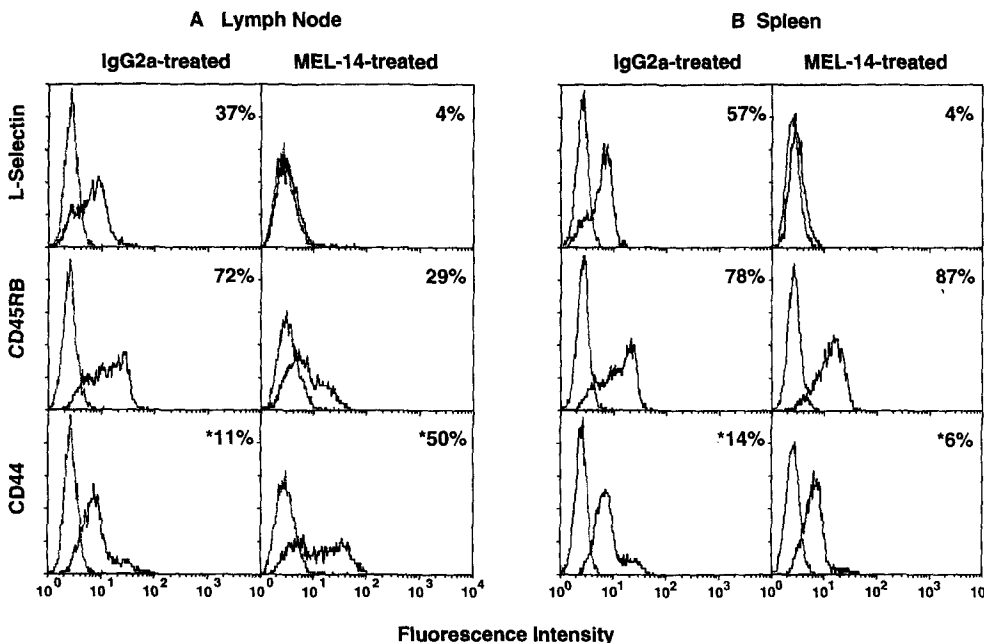


Figure 4. MEL-14 treatment depletes naive CD4 cells from lymph node but not spleen. CD4 cells from periaortic lymph node (A) and spleen (B) of mice treated 6 d previously with IgG2a or MEL-14, and 5 d previously with KLH/CFA were stained with the indicated antibodies, followed by FITC anti-rat κ (RG7/9.1) antibody, and PE-anti-CD4. Shown are histograms generated by gating on 5,000 CD4⁺ cells. Percent positive cells was determined on the basis of staining greater than that of isotype control antibodies, with the exception of CD44 where high versus low expression distinguishes CD4 subsets. The percent CD44^{hi} staining was determined with cursors set in (A) at 18 and 12 for lymph node CD4 cells from IgG2a-, and MEL-14-treated mice, respectively, and in (B) at 16 for splenic CD4 cells from IgG2a- and MEL-14-treated mice. No binding of RG7/9.1 was obtained in the absence of primary antibody (not shown), indicating that the rat antibodies that were present in vivo were not adhering to the CD4 population at the time of isolation.

of surface L-selectin on CD4 cells from MEL-14-treated mice, splenic CD4 cells exhibit typical levels of CD45RB and CD44 expression, with a substantial proportion of the cells bearing a naive phenotype (Fig. 4). Together, the functional and phenotypic data demonstrate that naive, L-selectin-bearing CD4 cells are not depleted by systemic exposure to MEL-14, but rather that L-selectin is shed from the surface or downregulated. When trafficking to PLN is blocked by MEL-14, naive CD4 cells can apparently localize and respond in other lymphoid sites such as the spleen. This conclusion is strengthened by the finding that the primary anti-KLH serum antibody response is delayed, but not blocked by MEL-14 treatment and that the development of KLH-specific memory CD4 cells is unaffected (not shown).

In contrast to the spleen, PLN from MEL-14-treated mice are depleted of CD4 cells bearing a naive phenotype (CD45RB^{hi}, CD44^{lo}) and the residual CD4 population is comprised of small resting cells that express a memory phenotype (CD45RB^{lo}, CD44^{hi}) (Fig. 4). Previously, we showed that both resting and activated murine memory CD4 cells from the spleen lack L-selectin (12, 13). In addition, although circulating CD4 cells in both the blood and the thoracic duct lymph are predominantly L-selectin positive, KLH-specific memory is exclusively contained in the minor, L-selectin negative subset (L. M. Bradley, unpublished observations). Since CD4 cells of memory phenotype remain in the PLN of MEL-14-treated animals, it appears that memory CD4 cells either do not use L-selectin for migration into PLN or only tran-

siently express and use L-selectin to enter PLN during a secondary response. It is also possible that the CD4 subset that remains in PLN after exposure to MEL-14 is nonrecirculating.

It is probable that recirculation of naive CD4 cells is regulated by a multistep process that involves combinations of adhesion molecules and their ligands. The paradigm for extravasation of cells across endothelium (reviewed in reference 1) is based on neutrophil migration where cells are initially retarded in the blood flow by the transient binding of selectins to their carbohydrate ligands. The arrest of leukocytes is mediated by integrins subsequent to activation of the binding avidity by chemokines, such as IL-8, which bind to G-protein-coupled receptors. Integrin activation leads to strong adhesion to endothelium and transmigration into tissue. As both L-selectin and LFA-1 have been shown to play a role in the trafficking of lymphocytes to PLN (3, 26, and S. R. Watson, unpublished observations), and G-protein receptors are required for lymphocyte recirculation (27), the model developed for neutrophil trafficking may also be valid for lymphocyte recirculation. Thus, L-selectin expressed on naive CD4 cells would mediate initial attachment to high endothelial venules of PLN. The results of our study indicate that it is possible to dramatically and selectively alter lymphocyte recirculation by blocking a single adhesion pathway *in vivo*. The results underscore the therapeutic potential of selective modification of lymphocyte trafficking by the use of antibodies to selectins or their carbohydrate ligands.

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Address correspondence to Linda M. Bradley, Ph.D., Department of Biology 0063, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0063.

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