Anti-CD43 Inhibition of T Cell Homing

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Summary

The homing of lymphocytes from the blood is controlled by specialized processes of lymphocyte–endothelial cell interaction. Interference with these processes offers the potential to manipulate lymphocyte traffic, and thus to modulate normal and pathologic immune and inflammatory responses. We selected antilymphocyte monoclonal antibodies (mAbs) for inhibition of lymphocyte binding in vitro to lymph node high endothelial venules (HEV), specialized vessels that support lymphocyte recruitment into lymph nodes. mAb L11 blocks T cell binding to lymph node and Peyer's patch HEV and inhibits T cell extravasation from the blood into organized secondary lymphoid tissues. In contrast, L11 has no effect on lymphocyte binding to purified vascular ligands for L-selectin, $\alpha4\beta7$, or LFA-1, suggesting that it inhibits by a novel mechanism. The L11 antigen is CD43, a sialomucin implicated in vitro in regulation of lymphocyte activation, whose expression is often dysregulated in the Wiskott-Aldrich syndrome. CD43 represents a novel target for experimental and therapeutic manipulation of lymphocyte traffic and may help regulate T cell distribution in vivo.

Regulated trafficking of lymphocytes through secondary lymphoid tissues is key to systemic immunity and is controlled by active mechanisms of lymphocyte–endothelial cell (EC) recognition (1, 2). Recruitment of lymphocytes from the blood has been separated into multiple sequential steps characterized as contact initiation ("tethering"), rolling, pertussis toxin-sensitive $G\alpha i$-mediated activation, and activation-dependent integrin triggering and arrest (1–4). Each step may be mediated by different adhesion or activation receptors allowing specificity through use of unique combinations of receptors to create specific homing pathways (1–4).

A number of adhesion molecules involved in lymphocyte homing via high endothelial venules (HEV) have been identified. In Peyer's patches (PP), the adhesion cascade for naive lymphocytes appears to involve a series of overlapping adhesion events with L-selectin, and to a lesser extent $\alpha4\beta7$, initiating interaction, L-selectin and $\alpha4\beta7$, both participating in rolling, and $G\alpha i$-linked activation-triggered arrest that requires both $\alpha4\beta7$ and LFA-1 (5). L-selectin, but not $\alpha4$ integrins, are also implicated in lymphocyte homing to LN; in this site, L-selectin appears critical in targeting the entry of most lymphocytes and LFA-1 participates in activation-dependent arrest as well (6, 7). However, additional molecules may be involved even in these relatively well-studied models. For example, recent studies (Salmi, M., E.L.

Berg, E.C. Butcher, and S. Jalkanen, personal communication) raise the possibility that vascular adhesion protein 1 (VAP1) may play an important role in primary (activation-independent) lymphocyte interactions with HEV in human LN, perhaps acting in sequence with or, for some lymphocyte subsets, as a substitute for L-selectin–initiated interactions. Moreover, the molecules involved in activation events during lymphocyte–HEV recognition have not been identified. In addition to its importance for understanding the physiology of lymphocyte trafficking, identification of molecules involved in or capable of modulating lymphocyte–EC recognition may reveal novel targets for the therapeutic regulation of pathological inflammatory and immune responses.

Lymphocyte–HEV recognition is readily studied in vitro in analyses of lymphocyte binding to HEV in frozen tissue sections in an assay developed by Stamper and Woodruff (8). In this assay, the molecular elements involved both in primary adhesion and activation-dependent interactions have been identified or shown to participate; thus, it represents a powerful tool for dissecting this cellular event in its molecular basis (9; see Discussion). Therefore, to identify novel molecular targets for controlling lymphocyte homing, we selected mAbs for their ability to block lymphocyte binding to HEV in frozen sections. We describe here an mAb, L11, that inhibits lymphocyte–HEV interaction in vitro

and lymphocyte recruitment to LN, PP, and spleen in vivo. Inhibition is selective for T cells, suggesting an experimental and therapeutic approach and potentially a physiologic mechanism for differential control of T versus B cell homing. We demonstrate that the L11 antigen is CD43, a major membrane sialoglycoprotein of hematopoietic cells (10, 11), implicated in the regulation of T cell activation and adhesion in vitro.

Materials and Methods

Antibodies. mAb L11 was produced by immunizing Fisher 344 rats four times at 3-wk intervals with the monocytoid cell line WEHI78/24 (12; gift of R. Coffman, DNAX, Palo Alto, CA). Spleen cells were fused with SP2/0 myeloma cells (American Type Culture Collection; Rockville, MD) using traditional polyethylene glycol fusion methods. Hybridoma supernatants were screened for their ability to block binding of peripheral lymph node (PLN) and mesenteric lymph node (MLN) lymphocytes to PLN HEV in Stamper-Woodruff frozen section assays (described below). L11 hybridoma was cloned three times by limiting dilution. The isotype (IgG2a) was determined by Ouchterloney analysis (ICN Biomedicals, Inc., Costa Mesa, CA). FITC-labeled Thy1.2, anti-CD43 mAb S7, and FITC-labeled S7 were purchased from PharMingen (San Diego, CA) and PE-conjugated goat anti–rat IgG was purchased from Jackson ImmunoResearch Labs. (West Grove, PA). RA3-6B2 (anti-B220; gift of R. Coffman; 13) was produced, purified, and FITC-conjugated. MECA367 (anti–mouse mucosal addressin [MAdCAM-1]; 14), MJ64, anti–mouse CD44 (15), and rat IgG negative control mAbs 9B5 (16) and 30G12 (anti-T200) were prepared in the laboratory.

Stamper-Woodruff Frozen Section Assays. Modified Stamper-Woodruff frozen section assays were performed (8, 17) using isolated BALB/c PLN and MLN lymphocytes (2×10^7/ml) mixed 1:1 with tretramethylrhodamine B isothiocyanate (TRITC)-labeled rat MLN lymphocytes in DMEM (BioWhittaker, Walkersville, MD) without bicarbonate containing 20 mM Hepes, pH 7.0, and 0.1% (vol/vol) fetal bovine serum (assay buffer). TRITC labeling of rat internal standard cells (not recognized by L11) was performed as previously described using 1.5 μg TRITC/ml of assay buffer for 20 min at 37°C (18). Cells were preincubated with isotype-matched control mAb (30G12) or with L11 (0.25 μg/10⁶ cells) for 20 min on ice and added to freshly cut PLN frozen sections (100 ml/section) and allowed to bind for 30 min with constant rotation (76 rpm) at 4°C. Slides were gently placed in 1.5% gluteraldehyde in PBS containing 2 mM Ca^{2+} and 2 mM Mg^{2+}. The number of mouse and rat lymphocytes bound to >30 HEV on each of the quadruplicate sections was determined by fluorescence microscopy. The ratio of the number of L11-treated cells bound/rat internal standard to the number of control antibody–treated cells bound/rat internal standard was calculated and multiplied by 100 to express sample cell binding as percent of control cell binding. T cells were isolated by negative selection on anti–B cell and anti-macrophage columns (R & D Sys., Inc., Minneapolis, MN) and were >97% Thy1⁺. B cells were identified in mixed populations by prestaining cell suspensions with FITC-conjugated anti-B220. Data represents the mean and standard error of four experiments.

Adhesion Assays. The ability of L11 to inhibit lymphocyte binding to purified vascular ligands was assessed as previously described (19, 20).

Flow Cytometry. BALB/c MLN and PLN lymphocytes were immunostained with L11 (1 μg/10⁶ cells) or isotype-matched con-

trol mAb, PE-conjugated mouse anti–rat IgG followed by FITC-conjugated anti–Thy-1. Cells were analyzed on a FACScan® (Becton Dickinson, San Jose, CA). Cross-blocking experiments were performed by preincubating lymphocytes with 9B5, MJ64, S7, or L11 (5 μg/10⁶ cells) for 20 min and then adding FITC-labeled S7, MJ64, or 30G12. After an additional 20 min, incubation cells were washed and analyzed as above.

In Vivo Homing. Lymphocytes from BALB/c MLN, PLN, and spleens were labeled with 10 mM Cell-Tracker Orange (Molecular Probes Inc., Eugene, OR) according to the manufacturer's instructions or with TRITC, washed, and treated with L11 or isotype-matched negative control mAb (1 μg/10⁶ cells) for 20 min. Cell suspensions were diluted and centrifuged. The supernatant was then removed and 5×10^7 cells were resuspended in 0.5 ml of HBSS (BioWhittaker) and injected via the tail vein. In other experiments, excess antibody (50 μg) was co-injected with cells. At the appropriate time point, peripheral blood was collected by heart puncture and lymphocyte suspensions prepared from lymphoid organs. Cells were stained with FITC-conjugated anti–Thy-1 and analyzed by two-color flow cytometry.

Transfection and Immunofluorescence. Chinese hamster ovary P (CHO-P) cells were transfected with mouse CD43 or mouse MAdCAM-1 cDNA as described (21) and stained with L11 mAb or control mAb MECA367 (anti-MAdCAM-1) followed by PE-conjugated goat anti–rat IgG and visualized by fluorescence microscopy.

Results

mAb L11 Inhibits T Cell Binding to HEV In Vitro and In Vivo. To identify antibodies capable of inhibiting lymphocyte trafficking, mAbs were generated and screened for their ability to block lymphocyte binding to HEV in LN frozen sections, using a modified Stamper-Woodruff assay (8, 17). One antibody isolated in this screen, L11 (isotype IgG2a), blocks T cell binding to normal and inflamed PLN HEV by >80% (Fig. 1). Interestingly, it has much less effect on LN B cell binding. Two-color flow cytometric analysis of LN lymphocytes reveals that L11 antigen is
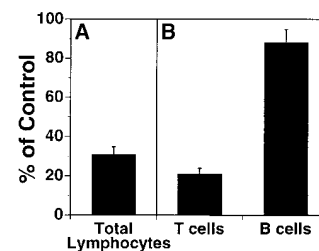
Figure 1. mAb L11 inhibits T cell binding to HEV in vitro. (*A*) L11 blocks binding of total LN lymphocytes to PLN HEV in modified Stamper-Woodruff frozen section assays performed using isolated BALB/c PLN and MLN lymphocytes mixed 1:1 with TRITC-labeled rat MLN cells (internal standard cells not recognized by L11). Cells were preincubated with isotype-matched control mAb or with L11, added to freshly cut PLN frozen sections, and incubated for 30 min with constant rotation (76 rpm) at 4°C. The number of mouse and rat lymphocytes bound to >30 HEV on each of quadruplicate sections was determined by fluorescence microscopy. The ratio of the number of L11-treated cells bound/rat internal standard to the number of control antibody treated cells bound/rat internal standard was calculated and sample cell binding expressed as percent of control cell binding. (*B*) Blocking of T versus B cells was assessed as described for total lymphocytes using T cells isolated by negative selection (>97% Thy 1⁺). B cells were identified in mixed populations by prestaining cell suspensions with FITC-conjugated anti-B220.
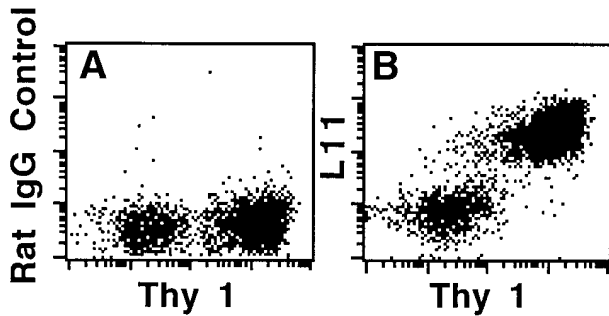
Figure 2. Two-color flow cytometric analyses of expression of L11 antigen by LN T cells (Thy 1^+) and weak expression by Thy 1^- cells (predominantly B cells). MLN and PLN lymphocytes were immunostained with isotype-matched control mAb (*A*) or L11 (*B*), PE-mouse anti–rat IgG and finally FITC-conjugated anti–Thy-1. Cells were analyzed using a FACScan® and CellQuest© software; x- and y-axis are $\log_{10}$ fluorescence.

highly expressed by T cells but also weakly by B lymphocytes (Fig. 2), correlating with the predominant inhibitory effect of L11 on T cells.

Inhibition of binding to HEV suggests involvement of the L11 antigen in T cell homing. We therefore assessed the effect of L11 on short-term localization of intravenously injected lymphocytes in vivo. Pretreatment of syngeneic LN cells with L11 results in significant inhibition of 1-h homing of T cells, but not B cells, to PLN, MLN, PP, and spleen (Fig. 3 *A*). Although similar inhibition of T cell trafficking is observed whether L11-pretreated lymphocytes are washed before injection or are co-injected with excess mAb, if lymphocytes are injected with excess antibody, significant reduction ($\leq 30\%$ inhibition) of B lymphocyte homing is also observed (data not shown). Importantly, L11-treated cells were significantly enriched in the blood of recipients, consistent with mAb inhibition of EC interaction and recruitment rather than nonspecific or toxicity-induced clearance of injected cells from the circulation. Fluorescence microscopic examination of frozen sections of LN following in vivo homing reveals decreased accumulation of L11-treated cells in HEV compared to control cells, in spite of enhanced blood levels, suggesting that the antibody inhibits events involved in lymphocyte–HEV recognition in vivo.

To further assess the fate of antibody-treated cells and the possibility of cell damage or toxicity caused by L11, longer-term redistribution studies were performed. Lymphocytes were labeled with 10 μM Cell-Tracker Orange, preincubated with saturating levels of L11 or control antibody, washed, and injected into syngeneic recipients. Animals were killed at 1, 24, and 48 h. As above, 1-h homing was blocked by L11, but by 48 h, the distribution and number of L11-treated cells in lymphoid tissues was similar to that of control cells (Fig. 3 *B*).

mAb L11 Does Not Inhibit Binding of T Cells to Purified Vascular Ligands. In situ lymphocyte interactions with HEV involve sequential overlapping engagement of L-selectin, $\alpha 4\beta 7$, and LFA-1 (5). To determine if L11 was directly inhibiting engagement of these adhesion receptors, the
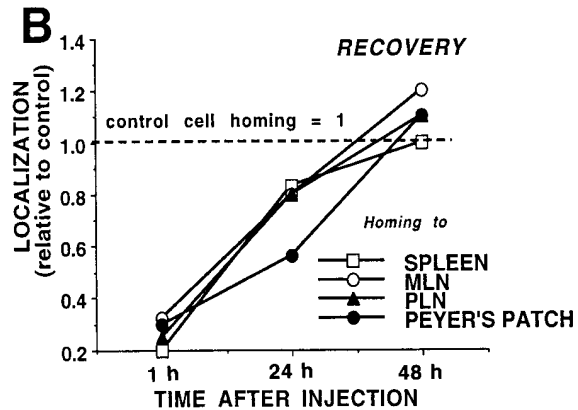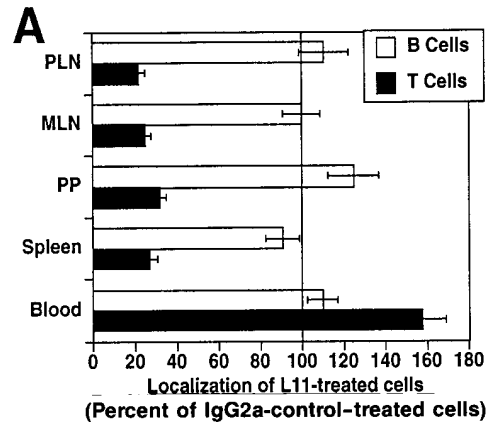
Figure 3. mAb L11 inhibits T cell binding to HEV in vivo. (*A*) L11 pretreatment inhibits T (*closed bars*) but not B (*open bars*) cell localization to PLN, MLN, PP, and spleen 1 h after intravenous injection. TRITC-labeled lymphocytes were treated with L11 or isotype-matched negative control mAb for 20 min, washed, and 5×10^7 cells injected via the tail vein. After 1 h, peripheral blood was collected by heart puncture and lymphocyte suspensions prepared from lymphoid organs were stained with FITC-conjugated anti–Thy-1, spiked with internal standard fluorescent beads for enumeration, and analyzed by two-color flow cytometry. (*B*) Homing of L11-pretreated T cells returns to that of control cells over 48 h. Redistribution studies were performed as described for *A* using cells labeled with 10 mM Cell-Tracker Orange.

ability of L11 to inhibit lymphocyte binding to purified vascular ligands was assessed. L11 had no effect on T cell rolling on the L-selectin ligand peripheral node addressin (PNAd; data not shown) or on binding to the $\alpha 4\beta 7$ ligand MAdCAM-1 (Fig. 4 *A*). As described (22), lymphocytes require activation to bind the LFA-1 ligand intracellular adhesion molecule 1 (ICAM-1); L11 had no effect on this interaction when integrins were triggered by replacement of Mg^{2+} with Mn^{2+} in the binding buffer (Fig. 4 *B*). These results suggest that the L11 antigen is not acting as a receptor for known vascular adhesion ligands, and that L11 does not directly inhibit engagement of these receptors.

L11 Antigen is CD43. Immunofluorescence flow cytometry reveals that L11 antigen is highly expressed by bone marrow neutrophils as well as T cells (not shown). This expression pattern is similar to that of CD43 (10). In addition, the relative molecular mass of the L11 antigen, 100 kD in
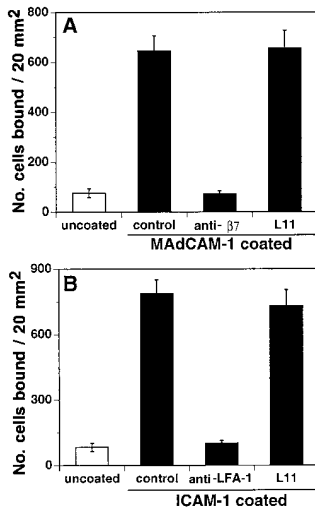
Figure 4. L11 fails to block binding of lymphocytes to MAd-CAM-1 or ICAM-1. (*A*) anti-β7 mAb cocktail containing FIB504, FIB30, and DATK32 (26) blocks binding of MLN lymphocytes to purified MAd-CAM-1 and (*B*) anti–LFA-1 mAb blocks binding of Mn^{2+}-treated MLN lymphocytes to purified ICAM-1; however, L11 fails to block binding to either ligand.

Western blots (not shown), is also similar to that reported for mouse CD43 (11). Therefore, we next assessed reactivity of L11 with CHO-P cell transfectants expressing mouse CD43 (11). L11 (Fig. 5, *left*) but not control mAb (Fig. 5, *right*), stained CD43-transfected CHO-P cells, as did anti-CD43 mAb *S7* (not shown). L11 failed to stain mock-transfected CHO-P cells or CHO-P cells expressing the irrelevant antigen MAdCAM-1. Thus, L11 can recognize CD43.

To determine if the T cell antigen recognized by L11 is CD43, LN lymphocytes were stained (at subsaturating concentrations) with L11 or S7 (detected with PE-conjugated anti–mouse IgG) and FITC-labeled S7 or FITC-labeled Thy-1. As shown above, double staining with FITC–Thy-1
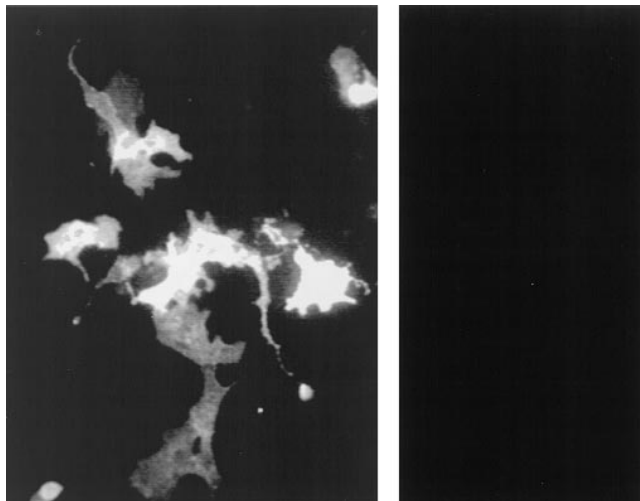
Figure 5. L11 antigen is CD43. L11 (*left*), but not control mAb (*right*), stained CD43-transfected CHO-P cells. CHO-P cells were transfected with mouse CD43 or mouse MAdCAM-1 cDNA and stained with L11 mAb or control mAb MECA367 (anti–mouse MAdCAM-1). MECA367 recognized MAdCAM-1 transfectants (not shown) but failed to stain CD43 transfectants (*right*). L11 stained CD43 transfectants (*left*), but not MAdCAM-1 transfectants (not shown).
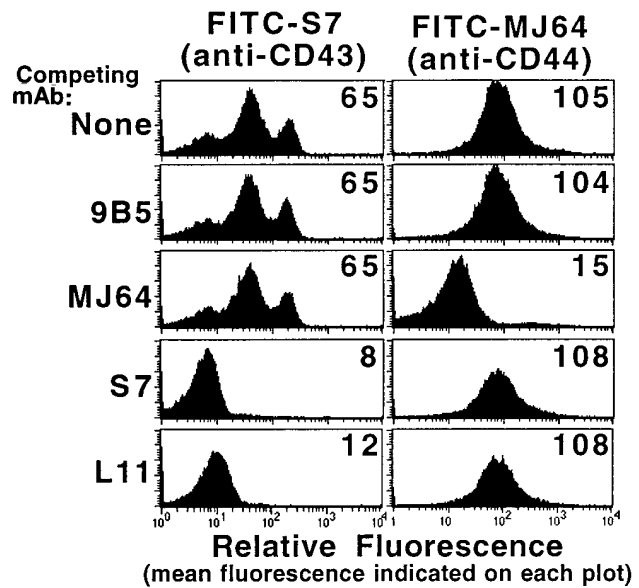
Figure 6. L11 antigen on T cells is CD43. LN lymphocytes preincubated with L11, anti-CD43 mAb S7, anti-CD44 mAb MJ64, control mAb 9B5, or buffer were stained with FITC-labeled S7 or MJ64 and their fluorescence measured by FACS® analysis. The x-axis is $\log_{10}$ fluorescence; mean fluorescence is indicated on each histogram. L11 blocked FITC-S7 binding but not control mAb (FITC-MJ64) binding to T cells.

and L11 (Fig. 2 *B*) yielded double-positive cells, indicating that these molecules are both expressed by T cells. In contrast, double staining with L11 and FITC-S7 yielded a linear relationship (not shown) indicating that the molecule recognized by these mAbs are expressed equivalently on individual T cells, a pattern suggesting that the mAbs recognize a common antigen. Finally, the epitopes recognized by L11 and S7 appear to be close or overlapping as L11 (and S7) blocks binding of FITC-labeled S7 (Fig. 6) but not binding of FITC-MJ64 (anti-CD44), whereas MJ64 blocks binding of FITC-MJ64 but not FITC-labeled S7. Together these data indicate that the antigen defined by L11 on T cells is CD43.

Discussion

We have shown that anti-CD43 mAb L11 is a potent inhibitor of lymphocyte homing from the blood into lymphoid organs, including the LN, PP, and spleen. Consistent with the preferential expression of CD43 on T cells, the effect is selective for T cell homing, although when the mAb is co-injected in excess some redistribution in B cell trafficking can also be observed. Inhibition is not due to toxicity since there is a concurrent increase in blood levels of T cells when homing is blocked and the distribution of L11-treated cells returns to normal after 48 h.

The selective effect on T cells is unusual since most previously identified lymphocyte receptors involved in homing are shared by subsets of B cells as well, reflecting the common mechanisms of B and T cell traffic at least to organized lymph tissues. Moreover, molecules previously im-

plicated in homing generally influence traffic in a tissue-selective fashion (2), whereas the effect of anti-CD43 shown here influences trafficking to LN, PP, and even spleen to a significant extent. This suggests an effect on an event common to T cell trafficking to diverse sites.

CD43 is an abundant leukocyte surface sialomucin that has been implicated both in antiadhesive roles, acting as a passive barrier to engagement of surface adhesion receptors, and in proadhesive activities, mediated, for example, through intracellular signaling and activation of leukocyte integrins (e.g., LFA-1) (23–25). In this context, the failure of L11 to inhibit lymphocyte rolling on PNAd or lymphocyte binding to MAdCAM-1 or ICAM-1 in vitro renders it unlikely that it is inducing a passive barrier activity of CD43. On the other hand, L11 might inhibit a normal proadhesive signaling activity in the context of lymphocyte homing.

However, our results are also consistent with involvement of a previously unidentified vascular ligand for CD43, with CD43–endothelial interaction representing an additional step in the multistep processes of lymphocyte–HEV (and spleen marginal sinus lining cell) interactions in vivo. In this context it is relevant that VAP-1 has recently been implicated in lymphocyte–HEV interactions in the PLN and its role in the multistep process of HEV interaction appears to precede that of LFA-1.

In conclusion, anti-CD43 mAb L11 inhibits lymphocyte recruitment to lymphoid organs, acting at the level of lymphocyte–EC recognition. Inhibition is selective for T cells, suggesting an experimental and therapeutic approach, and potentially a physiologic mechanism, for differential control of T versus B cell homing.

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