GlyCAM-1, a Physiologic Ligand for L-Selectin, Activates β2 Integrins on Naive Peripheral Lymphocytes

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Summary

Naive T cells are selectively recruited from the blood into peripheral lymph nodes during lymphocyte recirculation. L-selectin, a lectin-like receptor, mediates the initial attachment of lymphocytes to high endothelial venules (HEV) in lymph nodes. A subsequent step involving the activation of β2 integrins has been proposed to facilitate firm adhesion, but the activating signals are poorly understood. We report here that either antibody-mediated cross-linking of L-selectin on human lymphocytes or treatment of the cells with GlyCAM-1, an HEV-derived, secreted ligand for L-selectin, stimulates their binding to ICAM-1 through the β2 integrin pathway. Furthermore, GlyCAM-1 causes the rapid expression of a neoepitope on β2 integrins associated with a high-avidity state. Naive (CD45RA$^+$), but not memory (CD45R0$^+$) lymphocytes, respond to L-selectin cross-linking or GlyCAM-1 treatment. Thus, the complexing of L-selectin by specific ligands may provide key signals to naive lymphocytes, contributing to their selective recruitment into peripheral lymphoid organs.

The widely accepted cascade model of leukocyte recruitment from the blood (1, 2) proposes that selectins mediate tethering and rolling of leukocytes along the endothelium. Leukocyte integrins, upon rapid activation, are responsible for slowing and arresting the cells through interactions with specific counter-receptors displayed on the endothelium. Arrest is followed by diapedesis into the tissue. In the case of lymphocyte homing to lymph nodes, L-selectin on the lymphocytes mediates the initial tethering and rolling of the cells along the specialized endothelial cells of HEV[1] (3, 4). LFA-1 (αLβ2) appears to be the most relevant integrin, since specific mAbs against αLβ2 effectively block lymphocyte migration to LN (5, 6) and the ICAM counter-receptors are expressed on LN HEV (7). An unsolved problem is the identity and nature of the signals (possibly chemokines) that are responsible for the triggering of αLβ2 from a conformation or distribution that manifests weak affinity for the ICAMs to a high affinity state (8, 9).

GlyCAM-1 (10, 11), in contrast to the two other molecularly described HEV-ligands for L-selectin (MAdCAM-1 and CD34), is a secreted protein with no known association with the endothelial surface (12). It is present in mouse serum at a concentration of ∼1.5 μg/ml and the isolated molecule retains L-selectin binding activity (11). These characteristics raised the possibility that GlyCAM-1 might serve as a soluble signaling molecule acting through cell surface L-selectin. In the present study, we show that ligation of L-selectin via either GlyCAM-1 or antibody cross-linking enhances β2 integrin function in naive, but not memory, T cells.

Materials and Methods

Antibodies and Cells Lines. mAb24 (mouse IgG$_1$) was a kind gift of Dr. N. Hogg (Imperial Cancer Research Fund, London, UK). E3 is a mouse melanoma cell line transfected with human ICAM-1 and was provided by Dr. L. Graf, Jr. (University of Chicago, IL) (13). IB4 (mouse IgG$_1$) was a gift of Dr. T. Tednock (Athena Neurosciences, S. San Francisco, CA). Humanized DREG55 and DREG200 mAbs (human IgG$_4$) were generously provided by Dr. E. Berg (Protein Design Labs, Mountain View, CA), and mouse DREG55 and DREG56 mAbs (IgG$_1$) were donated by Dr. T.K. Kishimoto (Boehringer-Ingelheim, Ridgefield, CT). Other mAbs used were 18D3 and TS1/6 (Endogen, Cambridge, MA) and anti-CD45RA/RO (K. Soo, DNAX Corp., Palo Alto, CA).

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[1]*Abbreviation used in this paper:* HEV, high endothelial venules.

Isolation of GlyCAM-1. GlyCAM-1 was isolated from mouse serum as described by Singer and Rosen (11). The yield of Gly-CAM-1 was ~0.5 μg per ml of starting serum. GlyCAM-1 thus isolated was >98% pure as determined by amino-terminal microsequencing and Coomassie staining.

Adhesion Assays. Human mononuclear cells were isolated from normal adult volunteers by centrifugation of Heparinized or EDTA-treated venous blood over Histopaque 1077 (Sigma Chem. Co., St. Louis, MO). After washing in cold Buffer A (Ca^{2+}/Mg^{2+} Free Hank's Balanced Salt Solution with 0.125% human serum albumin), peripheral blood lymphocytes (PBL) were isolated by negative selection with anti-CD14 (monocyte) coupled magnetic beads (Dynal, Lake Success, NY). Typically, >95% of monocytes could be removed from the mononuclear cells as detected by size/granularity parameters in a flow cytometer. Memory and naive populations were isolated by appropriate incubation with anti-CD45RO, anti-CD45RA, anti-CD19 (B cell), and anti-CD14 mAbs (Caltag, South San Francisco, CA) for 30 min on ice followed by negative selection using sheep anti-mouse IgG-coated magnetic beads (Dynal). Typically, 95% of monocytes and 97% of B cells were removed by this method. After isolation, lymphocytes were labeled with BCECF/AM (Calbiochem-Novabiochem, La Jolla, CA) (14) for 25 min at 37°C before further treatment as detailed in the figure legends. 75 μl of cells (10^5 cells) were transferred to triplicate wells of a 96-well plate containing confluent E3 cells and allowed to settle for 20 min at 22°C before stimulation. After stimulation (see figure legends) for 20 min at 37°C, the plates were washed four times with Buffer B (Buffer A plus 0.6 mM $Ca^{2+}/0.2$ mM Mg^{2+}) and cell binding was measured with a Millipore Cytofluor fluorescence plate reader. Unless otherwise indicated, the error bars shown in the figures represent the standard errors of the mean (SEM) based on three determinations. All experiments were repeated at least three times with different blood donors. The data shown are representative results from one donor.

For cross-linking studies (Figs. 1 and 6), total (unfractionated), memory, and naive T lymphocytes were isolated and labeled as described above. In Fig. 1, unfractionated T cells were either untreated or treated with 5 μg/ml humanized (IgG$_4$) anti-L-selectin mAb with low affinity for human Fc receptors (DREG55, reference 15) at 22°C for 20 min in buffer A. In Fig. 6, total, memory, and naive lymphocytes were treated with 2.5 μg/ml biotinylated humanized DREG200 at 22°C for 20 min in buffer A. In both experiments, the treated cells were pelleted and resuspended in Buffer B (Buffer A + 0.6 mM $Ca^{2+}/0.2$ mM Mg^{2+}) to a final concentration of 1.3 × 10^6 cells/ml and then distributed to wells as described above. To achieve conventional cross-linking, a cross-linking goat F(ab')$_2$ anti–human IgG (Fig. 1) was added to the indicated final concentration (μg/ml). For cross-linking of a biotinylated L-selectin mAb (DREG200), avidin was added at the indicated concentrations (see Fig. 6). Data are expressed as percent of cells bound relative to the total number of cells added.

Flow Cytometry. For real-time measurement of mAb24 binding (16), 5 × 10^5 PBL (isolated as above) in 200 μl buffer B were preincubated with 15 μg/ml Cy3-conjugated mAb24 for 10 min at 22°C. Cells were warmed to 37°C, and stimulant was added immediately at time zero. Samples were continuously mixed and drawn into a Becton-Dickinson FACScan®. 3,000 events were collected in 10 s for each time point.

Cell surface staining was performed on the isolated populations with FITC-conjugated anti-L-selectin (DREG56, 0.3 μg/ml; Immunotech), anti-CD45RA (MEM56, 4 μg/ml; Caltag), anti-CD45RO (UCHL1, 4 μg/ml; Caltag), or anti-CD18 (MEM48,

4 μg/ml; Caltag) antibodies at the indicated concentrations and analyzed by flow cytometry. Similar staining patterns were observed with at least five different donors using two different antibodies for L-selectin and CD18.

For routine mAb24 binding assays (see Fig. 5), isolated naive and memory T cell lymphocyte populations were resuspended in buffer B at 1.0 × 10^5 cells in 50 μl buffer containing either 1 μg/ml FITC-mAb24 or non-immune class-matched FITC-IgG$_1$ (Caltag) as a control for nonspecific binding of IgG to cells. Gly-CAM-1 or PMA was added to the indicated final concentrations for 20 min at 37°C. After pelleting, the cells were then resuspended in 50 μl buffer B containing 20 μg/ml rabbit F(ab')2 anti–mouse IgG (Zymed, South San Francisco, CA) for 10 min at 22°C to stabilize the mAb24 signal, which tended to decrease in the absence of the stimulus. For flow cytometric analysis, the cells were pelleted and resuspended in buffer B. mAb24-positive cells (>10 fluorescence units) are defined as cells exhibiting fluorescence above that observed with unstimulated PBL stained with a FITC-IgG$_1$ isotype control antibody. Control FITC-IgG$_1$ binding in the presence of GlyCAM-1 or PMA was the same as in unstimulated cells.

Results and Discussion

Previously, Simon et al. (16) demonstrated that antibody-mediated cross-linking of L-selectin on neutrophils upregulates the function of the β2 integrin, Mac-1 (αMβ2). We asked whether cross-linking L-selectin on lymphocytes could also stimulate adhesion through a β2 integrin pathway. As shown in Fig. 1, treatment of human PBL with an L-selectin mAb plus a secondary cross-linking antibody stimulated adhesion to ICAM-1 transfected melanoma cells about twofold. The stimulated adhesion was blocked by a β2-specific mAb but not by a function-blocking, β1-directed mAb. Treatment of lymphocytes with the L-selectin mAb alone or the cross-linking antibody had no effect.
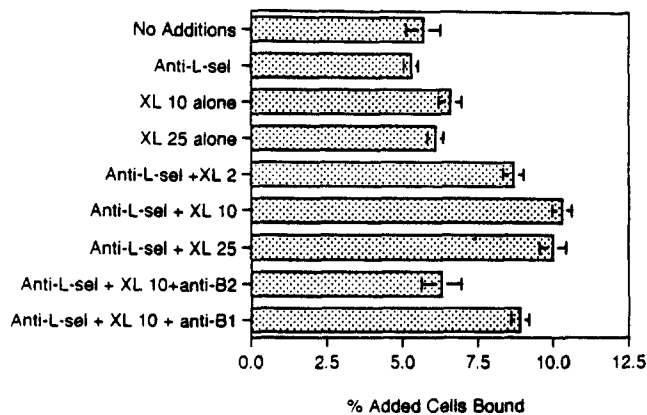

Figure 1. Cross-linking L-selectin stimulates adhesion of peripheral blood lymphocytes to ICAM-1. Peripheral blood lymphocytes were isolated, labeled, and treated with humanized anti-L-selectin antibodies (*Anti-L-sel*) in an adhesion assay as described in Materials and Methods. Where indicated, lymphocytes were pretreated for 10 min at 22°C with either 10 μg/ml anti-β2 antibody (anti-B2) (TS1/16) or a function blocking anti-β1 antibody (anti-B1) as a control (18D3) before adding the cross-linking antibody (XL, in μg/ml). Cross-linking had no effect when untransfected melanoma cells were used as the substratum (not shown).
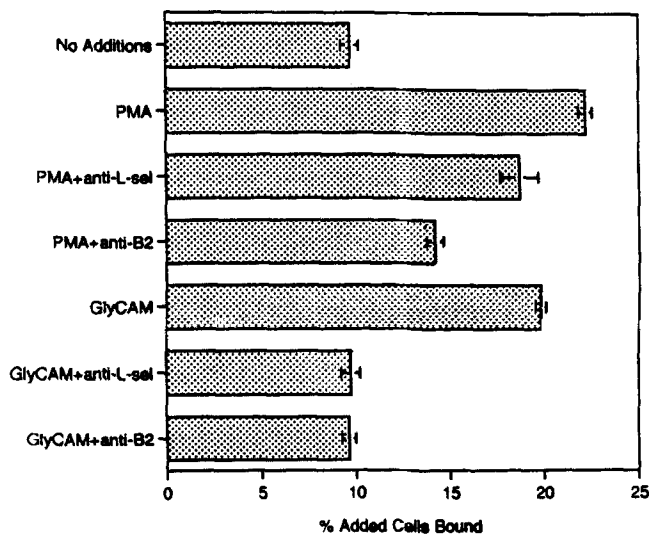
Figure 2. GlyCAM-1 stimulates the adhesion of peripheral blood lymphocytes to ICAM-1 in an L-selectin and β2 integrin dependent manner. Human PBLs were isolated, labeled with BCECF/AM, and used in an adhesion assay as described in Materials and Methods. Cells were pretreated (where indicated) with 10 μg/ml of either a function-blocking anti-β2 antibody (anti-B2, IB4) or a function-blocking humanized anti-L-selectin antibody (anti-L-sel, DREG55) for 10 min at 22°C before adding stimulants. Pretreatment with an anti-β1 antibody did not block GlyCAM-1 stimulated adhesion (not shown). Phorbol 12-myristate 13-acetate (PMA) was used at final concentration of 100 nM. GlyCAM-1 was added to 4 μg/ml. GlyCAM-1 did not stimulate lymphocyte binding to the non-transfected melanoma line.

Figure 3. GlyCAM-1 rapidly upregulates expression of the mAb24 neoepitope associated with β2 integrin activation. Cells were warmed to 37°C and stimulant added just after time zero (Mn^{2+}, 3 mM, ◆ or GlyCAM-1, 2.5 μg/ml, ■). In the blocking studies, the L-selectin mAbs DREG200 (▲) and DREG56 (✕) were preincubated with the cells for 10 min before the addition of GlyCAM-1. Data are expressed as the percent mAb24 expression at a given time point relative to unstimulated cells at time zero for each experimental condition (set at 100%). In the absence of stimulation, the mAb24 signal remained at the $t = 0$ control baseline level (not shown).

To determine whether a physiologic ligand of L-selectin could also stimulate adhesion to ICAM-1, we employed GlyCAM-1 purified from mouse serum (11). GlyCAM-1 cross-reacts with human L-selectin, since preincubation of PBL with 4 μg/ml of the ligand blocked 90% of the binding of a FITC-labeled L-selectin mAb (DREG56) but not that of a control mAb (Leu4).

As shown in Fig. 2, GlyCAM-1 stimulated the adherence of unfractionated human PBL to ICAM-1 transfected melanoma cells to the same extent as a phorbol ester. Pretreatment of the cells with an L-selectin mAb specifically blocked the stimulation by GlyCAM-1 while minimally affecting PMA-stimulated binding. Both the PMA- and GlyCAM-1-stimulated adhesion of PBL to ICAM-1 were strongly inhibited by a β2-specific mAb but not by an anti-β1 specific mAb (not shown). Thus, antibody cross-linking of L-selectin or GlyCAM-1 stimulates β2-dependent adhesion of lymphocytes to the endothelial counter-receptor, ICAM-1.

mAb24 recognizes a β2 integrin neoepitope associated with high-affinity ligand binding (16, 17). In a real-time assay, GlyCAM-1 rapidly increased the expression of the mAb24 neoepitope on PBL (Fig. 3), while the binding of a conventional β2 mAb was not affected (not shown). The response was detected at 30 s, the earliest time point examined. The kinetics of the neoepitope induction by GlyCAM-1 paralleled those seen with manganese, a direct integrin activator (17). The GlyCAM-1 response was abolished
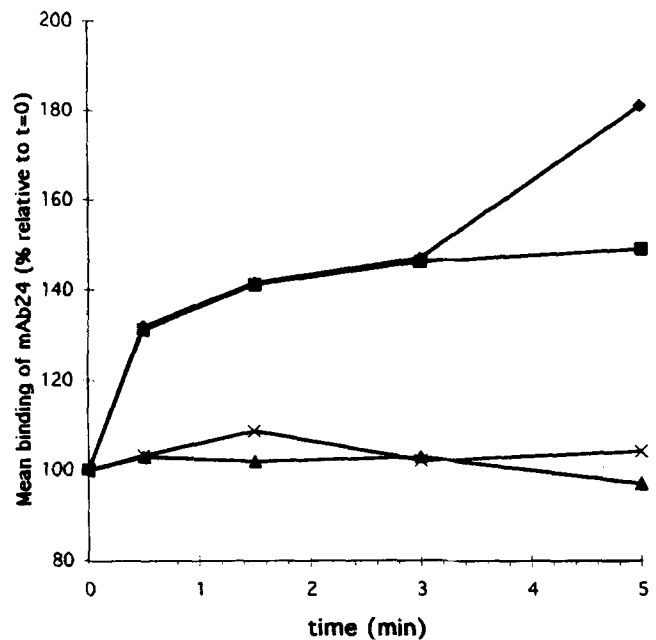
by pretreating the PBL with L-selectin mAbs. Desialylated GlyCAM-1 was unable to stimulate the mAb24 epitope (not shown), thus demonstrating the critical importance of carbohydrates for the activity of GlyCAM-1 (18). Finally, a dot-plot analysis showed that only a portion of the total lymphocytes increased binding of the mAb24 antibody in response to GlyCAM-1 (not shown), which suggested that a subpopulation of lymphocytes was responsive to GlyCAM-1.

Naive T cells are preferentially recruited into peripheral lymph nodes from the blood (21) compared to memory T cells. To determine whether there might be a differential response of naive vs. memory T cells to L-selectin ligation, we isolated these populations of T cells from peripheral blood based on their expression of CD45RA (naive) and CD45RO (memory) (Fig. 4). The isolated populations were either uniformly CD45RA^{hi} or CD45RO^{hi} and exhibited distinct integrin profiles. The CD45RO^{hi} population was divided into two equal populations of L-selectin^{hi} and L-selectin$^{lo/null}$ cells, while naive lymphocytes were 60–80% L-selectin^{hi} and 20–40% L-selectin^{lo}. At a concentration (1.5 μg/ml) which approximated its mean systemic level (11), GlyCAM-1 did not affect mAb24 binding. However, at 6 and 15 μg/ml, GlyCAM-1 significantly augmented the mAb24 epitope on naive cells, producing a 2.5✕ increase in the number of positive cells (Fig. 5). In an experiment

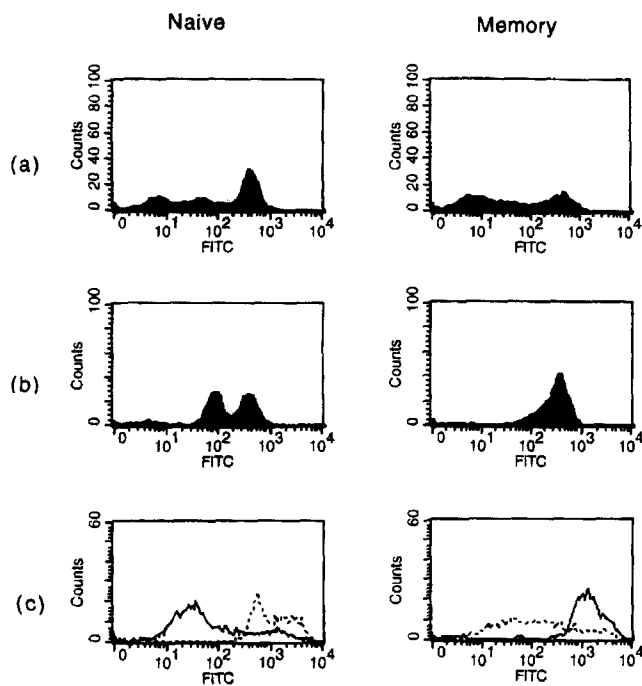**Naive**   **Memory**

(a) (b) (c)

Figure 4. L-selectin, CD18, and CD45RA/RO surface staining on isolated memory and naive T cells. Lymphocyte populations were isolated and stained with (*a*) anti-L-selectin, (*b*) anti-CD18, (*c*) anti-CD45RA (*dashed lines*) and anti-CD45RO (*solid lines*) as described in Materials and Methods. Unstained cells or cells stained with an irrelevant class-matched control antibody had an average fluorescence of 4–5 fluorescence units on the scales shown.

Figure 5. GlyCAM-1 enhances the expression of the mAb24 neo-epitope on naive, but not memory, T lymphocytes. Isolated lymphocyte subpopulations were treated with GlyCAM-1, PMA, and Mn^{2+} in the presence of FITC-mAb24 as described in Materials and Methods. The cells were gated by forward and side scatter parameters and then analyzed for FITC fluorescence. Data is shown as percentage of mAb24-positive cells (see Materials and Methods for definition) in the presence of an increasing concentration of GlyCAM-1 for naive (□) and memory (△) T cell populations. PMA stimulation resulted in the conversion of naive T cells to 88% positive (mean channel fluorescence [MCF] = 18) and of memory T cells to 94% positive (MCF = 35). Mn^{2+} stimulation resulted in the conversion of >95% of both memory and naive cells with MCF of 274 and 271 units, respectively.

with a different donor, GlyCAM-1 at 9 μg/ml enhanced the percent positive cells by 3.5× (from 20–70%), whereas at 3 μg/ml, an intermediate degree of stimulation was seen (45% positive cells). In contrast, memory cells exhibited a higher basal level of mAb24 expression, but were completely refractory to stimulation by GlyCAM-1 (Fig. 5). Importantly, both populations of cells were responsive to PMA or manganese (see Fig. 5 legend), establishing that the β2 integrins on memory cells could be activated by other stimuli. Although NK cells (~10% of peripheral blood lymphocytes) were not specifically removed by our isolation technique, they could not, by themselves, account for a significant proportion of the mAb24 response to Gly-CAM-1 since we have observed up to 70% of naive lymphocytes responding to GlyCAM-1. We cannot, however, formally exclude the possibility that NK cells partially contribute to the GlyCAM-1 response in our assays.

Consistent with these neoepitope studies, cross-linking of L-selectin via antibodies stimulated adhesion of naive cells by 3-fold to the ICAM-1 substratum, whereas memory cells showed no response (Fig. 6). Cross-linking of CD5 using biotinylated anti-CD5 antibodies by this technique did not lead to enhanced adhesion compared to antibody-treated cells alone. Thus, the activation of β2 integrins produced by ligation of L-selectin with either antibodies or GlyCAM-1 occurs preferentially on naive cells.

Previous studies have explored the physiological factors that might activate LFA-1 on intravascular lymphocytes.
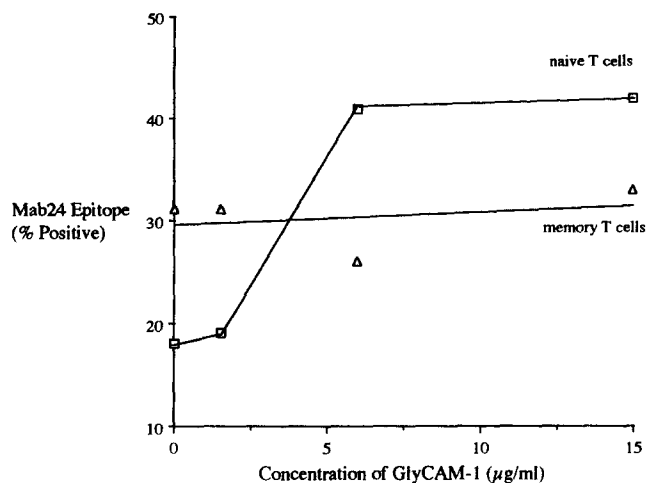
However, the results have been conflicting (20, 21). One study (20) found that several chemokines can augment PBL adhesion to ICAM-1, but the time periods of the assay were relatively long (>15 min). Another study failed to observe chemokine stimulation of PBL binding to ICAM-1 under a variety of assay conditions (21).

The present results demonstrate that GlyCAM-1 satisfies a number of important criteria for an intravascular trigger of lymphocyte integrins: (*i*) it is synthesized by the specialized endothelial cells of HEV and is secreted into the blood (10–12); (*ii*) it activates β2 integrins within seconds, compatible with a lymphocyte's limited transit time through HEV (2); (*iii*) the activation is selective for naive lymphocytes, providing a potential explanation of their preferential recruitment into lymph nodes. The multivalency of Gly-CAM-1, intrinsic to its mucin-like structure (22), may explain why it is active without additional cross-linking, while the L-selectin mAbs require a second antibody.

GlyCAM-1 is detectable in LN and Peyer's patch HEV (10) and in HEV-like vessels induced at sites of chronic inflammation (23). Thus, the integrin triggering effects of GlyCAM-1 or potentially of other HEV-associated ligands for L-selectin (CD34, MAdCAM-1, Sgp200) (22) may be pertinent at multiple sites of leukocyte trafficking. A previous study in a parallel plate flow chamber failed to detect activation of LFA-1 on PBL that were rolling on surface of immobilized "peripheral lymph node addressin" (mixed population of HEV-ligands from human tonsils) (24). Thus, the ligation of a small percentage of the L-selectin at focal
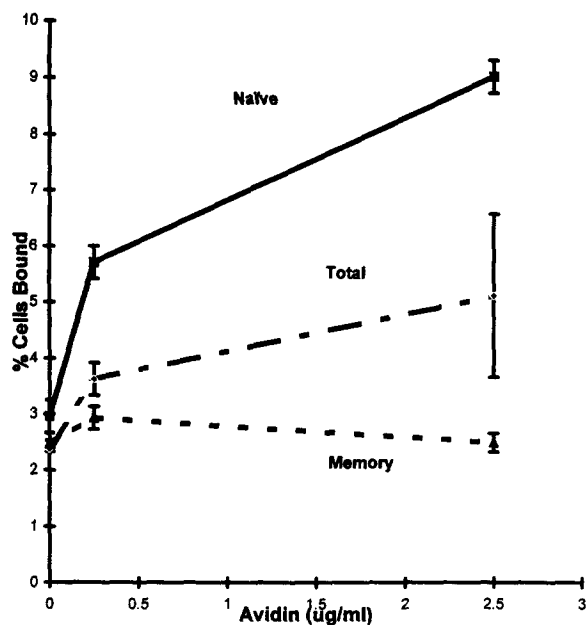
Figure 6. Cross-linking L-selectin stimulates the adhesion of naive, but not memory lymphocytes, to ICAM-1. Isolated lymphocyte populations were labeled, treated with biotinylated anti-L-selectin antibody, and cross-linked with varying concentrations of avidin as described in Materials and Methods. There was no effect of DREG200 treatment alone on adhesion (not shown). Error bars indicate half-range variation for duplicate determinations.

sites of adhesion may not be sufficient to activate LFA-1, whereas the more global effects of soluble GlyCAM-1 on the entire lymphocyte surface are activating. It should be noted that all of our effects were seen at a concentration of 3 μg/ml or higher of GlyCAM-1 (2× serum levels) which is predicted to ligate the majority of cell surface L-selectin based on competition of DREG56 binding (see above).

GlyCAM-1 has been reported to be down-modulated in draining LN (mouse) after antigen challenge (25). In sheep, antigen stimulation of LN results in a dramatic increase in memory T cell recruitment, which correlates with VCAM-1 induction on HEV (26). Thus GlyCAM-1 may be a specific modulator of naive lymphocyte migration into LN, whereas after vigorous antigen challenge, other triggering molecules (perhaps affecting β1 integrin function) (27) may furnish the signals for memory cell entry (26).

Several studies have demonstrated signal transduction functions for L-selectin in leukocytes (16, 28–30), induced by antibody cross-linking or artificial carbohydrate-based ligands. This report provides the first evidence that a physiologic L-selectin ligand can affect integrin activity on a subset of lymphocytes and provides a plausible mechanism to explain the selective recruitment of naive vs. memory T cells to peripheral LN. The mechanisms of rapid integrin triggering through an L-selectin pathway, now documented for both neutrophils and lymphocytes, remain to be elucidated.

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