Intravital Microscopy of the Peripheral Lymph Node Microcirculation in Mice

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ABSTRACT

Objective: The purpose of this study was to develop a model for microscopic in situ observation of the murine peripheral lymph node (LN) microcirculation and to characterize the function of the lymphocyte homing receptor L-selectin (CD62L) and the peripheral node addressin (PNAd). The latter is a high-affinity ligand for L-selectin in LN high endothelial venules (HEV).

Methods: The subilac (superficial inguinal) LN was microsurgically dissected in anesthetized adult mice. The nodal microvascular architecture and venular hemodynamics were characterized by bright field and epifluorescence video microscopy. L1-2 pre-B cells that were either mock transfected (L1-2<sup>mock</sup>) or stably transfected to express human L-selectin (L1-2<sup>L-selectin</sup>) were labeled fluorescently and injected into a feeding artery. Cell adhesion in LN venules was studied in both the presence and absence of neutralizing monoclonal antibodies (MAb) to PNAd and L-selectin.

Results: The preparation allowed a detailed analysis of flow dynamics parameters and leukocyte adhesion in LN microvessels. L1-2<sup>mock</sup> cells did not interact with LN microvessels. In contrast, L1-2<sup>L-selectin</sup> cells rolled efficiently in venules but not in arterioles or capillaries. Rolling was most prominent in subcutical HEV (orders III to V) and was less frequent but consistently detectable in downstream medullary and hilus venules (orders I and II). Rolling interactions were abrogated by MAb DREG-56 to the lectin domain of L-selectin and were markedly reduced by the anti-PNAd MAb MECA-79.

Conclusions: The present study develops a new intravital microscopy model for <i>in vivo</i> visualization of leukocyte interactions with microvessels in murine LN. The preparation permitted an analysis of biophysical and molecular mechanisms of leukocyte adhesion to high endothelial cells. The data support the concept that L-selectin and PNAd are the predominant receptor/ligand pair responsible for lymphocyte rolling in HEV. The model will be useful for high-resolution analysis of intra- and extravascular events in living LN.

Key words: Lymph node, high endothelial venule, L-selectin, peripheral node addressin, lymphocyte homing.

INTRODUCTION

An essential function of the immune system is the surveillance of the body by an army of lymphocytes that are constantly searching for foreign antigen (Ag). This is possible because of an intricate network of migratory pathways that have evolved to guide lymphocyte subsets to the tissues that are most likely to contain their cognate Ag (5, 37). Some of the central components of this system are the organized secondary lymphoid tissues that include the spleen, the mucosa-associated lymphoid organs (such as Peyer's patches and appendix), and the large number of lymph nodes (LN). Recent work suggests that lymphocyte migration from the blood compartment may be governed by site-specific multistep adhesion and activation pathways that are critical for the preferential recruitment (homing) of distinct lymphocyte subsets to these organs.
(11, 46). At least in some settings there is evidence that homing requires the adhesion of blood-borne lymphocytes to specialized adhesion molecules, such as the so-called addressins on the luminal surfaces of conspicuous venules within lymphoid organs. These microvessels are lined by high cuboid endothelial cells and are commonly referred to as high endothelial venules (HEV) (20).

Unique information on the contribution of addressins and their lymphocyte counter-receptors (the homing receptors) has been obtained through direct microscopic observation of intravascular lymphocyte interactions with HEV. The power of this experimental approach has become apparent recently in a series of detailed analyses of lymphocyte homing to Peyer's patches in a mouse model (3, 4, 8). In contrast, a similar model has not yet been developed for peripheral LN. This might partially be due to the difficulty of making a physiologically perfused LN accessible to microscopic observation without affecting its function or anatomic integrity. Furthermore, optical problems impose constraints on the visibility of intravascular events, particularly in larger LN. The capsule that covers the node and densely packed layers of cells in the cortex can obstruct the view on HEV in the subcortex, which are thought to be the principal sites of lymphocyte recruitment (33).

Thus, our current knowledge of the adhesion pathways that are involved in lymphocyte homing to LN in vivo is based largely on whole organ studies that use MAb-treated or genetically engineered animals where the accumulation of tagged lymphocytes or the size and cellular composition of LN have been measured (2, 12, 18, 22, 29, 32, 48, 53). A number of in vitro methods have also been developed, most notably the frozen section assay by Stamper and Woodruff (47). Although such models are useful to identify molecules that are directly or indirectly involved in homing, they reveal little regarding their actual function in a physiologic setting. For instance, several in vivo and in vitro studies demonstrate a central role for lymphocyte L-selectin (CD62L) and a number of sulfated sialylated glycoproteins on HEV that are collectively known as the peripheral node addressin (PNAd). Components of PNAd are decorated with an HEV-specific carbohydrate epitope that is recognized by MAb MEC-79 (23, 48) and functions as a potent ligand for L-selectin (7).

Intravital microscopy studies of acutely inflamed non-lymphoid tissues have shown that L-selectin mediates rolling of leukocytes and transfected lymphoma cells by interacting with a venular ligand that is antigenically distinct from PNAd (24, 31, 49, 50). In some settings the accumulation of neutrophils in inflamed tissues requires L-selectin-mediated rolling as a prerequisite first step that must be followed by activation of β2 integrins that mediate subsequent stationary adhesion (51). Consistent with in vivo observations of L-selectin function, in vitro binding of L-selectin to affinity-purified MECA-79-reactive PNAd glycoproteins can also support rolling in flow chamber assays (27, 39, 52). Therefore, it is reasonable to hypothesize that L-selectin binding to PNAd in LN HEV might also support rolling.

The present study explored the suitability of subiliac LN in anesthetized mice for intravital microscopy of the LN microcirculation. This organ can be exposed microsurgically and studied by intravital microscopy while preserving its integrity and physiologic blood supply. We were able to visualize the LN microvasculature and to estimate hemodynamic parameters in all segments of the venular tree by using epifluorescence video microscopy. Moreover, a stably transfected pre-B cell line was used to determine the role of cell surface L-selectin and PNAd in the absence of other adhesion molecules that interact with LN HEV. The data confirm that L-selectin interaction with PNAd is sufficient to support lymphocyte rolling in LN HEV.

**MATERIALS AND METHODS**

**Reagents**

Fluorescein isothiocyanate (FITC)-dextran (150-kD MW) and neuraminidase (vibrio cholerae) were purchased from Sigma (St. Louis, MO). For intravenous applications, FITC-dextran was diluted to 10 mg/ml in sterile physiologic saline and centrifuged at 14,000 g to remove undissolved particles prior to each experiment. 2,7′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) was purchased from Molecular Probes Inc. (Eugene, OR). BCECF stock solution that contained 0.5 mg of the fluorophor in 1 ml dimethyl sulfoxide (DMSO) was prepared on the day of an experiment.

**Monoclonal Antibodies**

FITC-conjugated anti-L-selectin MAb DREG-200 (25) was prepared and used for flow cytometry as described previously (52). Anti-PNAd Mab MEC-79 (rat IgM) (48) and anti-human L-selectin Mab DREG-56 (mouse IgGl) (25) were used for in vivo experiments. These MAbs were purified from tissue culture supernatant and diluted in azide-free phosphate-buffered saline (PBS). Endotoxin levels were determined to be below the detection threshold of the assay system (limulus amebocyte used).
Cell Lines
To assess the ability of LN microvessels to support L-selectin-mediated adhesion we used subcloned L1-2 
selectin cells, a murine pre-B lymphoma cell line that had been stably transfected with a cDNA for human 
L-selectin (7). Mock transfected (L1-2 Vector) cells were used as the control. As reported previously, normal 
L1-2 cells do not express the murine homologues of L-selectin, CD44, CD11b, αβ3 integrin, ICAM-1, or 
CHO ligands for P- or E-selectin. They express little or 
no LFA-1 (CD11a/CD18) but are positive for CD43 and 
VLA-4 (7, 8, 49, 52, and unpublished data). Subcloned 
L1-2 cells were grown in six well plates in RPMI 1640 
culture media that contained standard supplements, 
10% FCS, 250 μg/ml mycophenolic acid (Sigma), 12.5 
μg/ml xanthine, and 1× hypoxanthine/thymidine 
(Gibco, Grand Island, NY). On the day of an exper- 
iment the transfectants were harvested and washed in 
fresh prewarmed (37°C) media. Subsequently cells 
were resuspended to 1 × 10^7/ml and BECF stock 
was added to a final concentration of 2.5 
μg/ml for 30 min. An aliquot of cells was treated 
identically except that no BECF was added and these 
cells were then used for flow cytometry. Labeled cells 
were washed twice in culture media, resuspended in 
RPMI-1640 to 1 × 10^7/ml, and kept at 37°C. Immedi- 
ately before use in intraarterial injections, the solution 
was aspirated in 1-ml plastic syringes (Becton Dickin- 
son). In some experiments cells were treated with 0.1 
U/ml neuraminidase (30 min at 37°C), which does not 
affect L-selectin function but renders cells incapable 
of binding to endothelial selectins (49).

FACS Analysis of L-Selectin Expression
L-selectin expression on L1-2 transfectants was rou- 
tinely assessed on the day of each experiment. Trans- 
flectants were suspended to 1 × 10^7 cells/ml in HBSS 
and kept on ice. Subsequently, 20 μg/ml FITC- 
conjugated MAb DREG-200 was added to 50-μl cell 
suspension that contained L-selectin transfectants or 
control cells. Cells were washed twice after 30 min of 
staining. Mock transfected were used to determine 
light scatter and fluorescence detection thresholds on a 
FACScan flow cytometer (Becton Dickinson, San Jose, 
CA). The mean copy number of human L-selectin per 
cell was estimated using a calibrated microbead sys- 
tem (Quantum Simply Cellular, Flow Cytometry Stan- 
dards Corp.; San Juan, Puerto Rico) following manufac- 
turer instructions.

Animals
Animals used in these studies were male and female 
adult Balb/c or 129SV/G57Bl/6] mice (23–34 g body 
weight). Animals were housed in a viral Ag-free barrier 
facility and kept on standard lab chow and sterile water 
supply ad libidum. All experimental procedures comply 
with National Institutes of Health (NIH) guidelines for 
the care and use of laboratory animals. Procedures 
were also approved by the Standing Committees on 
Animals of Harvard Medical School and The Center for 
Blood Research.

Surgical preparation of the superficial inguinal LN (In. 
subiliacus). Mice were anesthetized by intraperito- 
eal (IP) injection (10 ml/kg) of physiologic saline that 
contained ketamine (5 mg/ml) and xylazine (1 mg/ml). 
During preparation and throughout the experiment, the 
body temperature was monitored and maintained at 
37°C using a radiant tubular heater (Cole Parmer, 
Niles, IL). The hair in the submandibular area of the 
neck, the right and left inguinal area, and the left 
lateral abdominal area was removed using hair removal 
loion (Nair, Carter Products, NY). With the help of an 
operating microscope, PE-10 polyethylene catheters 
were inserted into the right jugular vein for the 
administration of fluids and supplemental anesthetic 
and also in the left carotid artery for arterial blood 
pressure and heart rate monitoring (BPA, Micromed, 
Louisville, KY) and to draw blood samples. In addi- 
tion, a catheter was inserted in the right femoral artery 
and advanced cranially so that its tip was placed in the 
right common iliac artery immediately downstream of 
the aortic bifurcation. This catheter was used for 
retrograde injection of fluorescent cells into the contra- 
lateral iliac artery which gives rise to the left epigastric 
arteries that supply the left subiliac LN.

Following catheterization, the animal was placed in a 
supine position on a plexiglass stage that was specifi- 
cally designed for this model. A semicircular incision 
was made in the abdominal skin extending from the 
proximal left thigh via a region ~0.5 cm laterally to 
the midline to the left lateral costal margin in order to 
prepare the left subiliac LN (Fig. 1). The skin and the 
inguinal fat pad with the embedded LN were gently 
separated from the underlying abdominal wall with 
moistened cotton swaps. Two traction sutures (5-0 silk) 
were attached to the edge of the skin flap and to the 
connective tissue layer that covers the fat pad. The 
preparation was spread over a sterile standard micro- 
scope slide (25 mm × 75 mm) by fixing the sutures 
under slight tension to the plexiglass stage with 
modeling clay. A wall of sterile vacuum grease surround- 
ing the skin flap was built on the microscope slide in 
order to retain saline that was applied throughout the 
surgical procedure to keep the tissue moist. The 
connective tissue and fat cells that obstruct the view on 
the underlying LN were subsequently removed. This
step is critical because the subilia LN partially is covered by branches of the superficial epigastric artery and a parallel vein that give off an anastomosing vessel connected to the dorsal muscular branch that drains venous blood from the dorsal abdominal wall. Surgical damage to these vessels can impede blood flow to or from the LN. Finally, a 24-mm × 50-mm glass cover slip (No. 1; Clay Adams) was placed gently on top of the preparation and supported by vacuum grease. The gap between the cover slip and the lymph node was filled with sterile saline and sealed in order to avoid drying of the tissue.

**Intravital microscopy.** Animals were transferred to an intravital microscope (IV-500, Mikron Instruments, San Diego, CA) that was equipped with infinity-corrected water-immersion optics (Zeiss, Germany), and the preparation was allowed to rest for 30 min. Subsequently, small boluses (~20–50 µl each) of BCECF-labeled L1-2 cells (total volume per injection, 0.3–1 ml) were injected through the catheter into the right femoral artery. In some experiments, the specificity of L1-2L-selectin interactions with LN HEV was tested by adding 20 µg/ml MAb DREG-56 against the lectin domain of human L-selectin 15 min before injection. The functional contribution of MAb MECA-79-reactive PNAD glycoproteins was tested by comparing the intravascular behavior of L1-2L-selectin cells that were injected before or 15 min after intravenous (IV) injection of 10 mg/kg b.w. MAb MECA-79. Fluorescent cells were made visible in arterioles and venules (diameters ranged from 15–120 µm) by video-triggered stroboscopic epi-illumination (Chadwick Helmuth, El Monte, CA) through an FITC filter set. The bright fluorescent label of L1-2 cells allowed the observation of a 1355-µm × 965-µm field of view through a 10× objective (Achromplan, NA 0.3, Water). This frequently permitted the simultaneous recording of cells in all or most branching orders of the nodal venular tree. However, some distal and proximal venules could not be visualized simultaneously because they were too far apart or in different focal planes. In these cases, two or more areas of interest were identified and recorded separately using a 10× or 20× objective. In rare instances it was not possible to determine the exact branching order of HEV in the paracortex. Although fluorescent L1-2 cells were readily visualized in many fourth and fifth order HEV, the borders of deep subcortical vessels were often too blurred to allow accurate measurements of the luminal diameter. Data from these vessels were not included in analyses that compared the role of venular order in rolling and hemodynamics.

At the end of each experiment, 20 mg/kg b.w. FITC-dextran (150 kD) in physiologic saline was administered intravenously. Fluorescent recordings of LN microvessels containing FITC-dextran as an inert plasma marker were taken through a 4× objective (Achromplan, NA 0.16, Water) to visualize the entire intravascular compartment of the LN. These scenes were used for gross analysis of the microvascular LN anatomy and for determination of branching orders. In addition, the 10× and 40× objectives (Achromplan, NA 0.75, Water) were also used to record FITC-dextran–filled microvessels running in the plane of focus for off-line
measurement of their axial length (between upstream and downstream branching points) and luminal diameter, respectively. All scenes were recorded on videotape using a customized low-lag silicon-intensified target (SIT) camera (Dage MTI, Michigan City, IN), a time base generator (For-A), and a Hi-8 VCR (EV C-100, Sony, Japan).

Image Analysis

Video analysis of fluorescent recordings was performed either in real time or at a reduced speed as described (49, 51). Using fluorescent recordings through a 10× objective rolling L1-2 transfectants (i.e., cells that moved much more slowly than the bulk of red blood cells) and freely flowing cells were counted in each microvessel and the rolling fraction was determined as the percentage of rolling cells in the total flux. Recordings of 25 representative venules of all branching orders whose longitudinal axis was within the plane of focus were employed to measure velocities of individual rolling (10 consecutive cells/venule) and noninteracting cells (≥20 consecutive cells/venule) using a PC-based interactive image analysis system for microcirculation research (38). This system was also used to measure the luminal cross-sectional diameter (Dc) of the same set of venules recorded through a 40× objective after labeling the plasma compartment with FITC-dextran. The mean blood flow velocity (Vblood) was estimated from the velocity of the fastest noninteracting L1-2 cell (Vmax) in each venule, according to (30): Vblood = Vmax/(2 - (Dc/Dl)2) where Dl is the mean diameter of L1-2 cells (3.6 μm based on light and scanning electron microscopic measurements of glutaraldehyde-fixed cells). The wall shear rate (γ) was assessed as: γ = Vblood × Dl. Bulk blood flow was calculated according to methods described previously (30). Since the calculation of Vblood, γ, and blood flow are based on the assumption of a simple parabolic flow profile that is not likely to reflect exactly the actual conditions in LN venules, the empirically measured mean velocity of the samples of noninteracting L1-2 cells (V1,1-2) is also provided.

RESULTS

Anatomy of Murine Subiliac LN

In most preparations a single subiliac LN of 1–2 mm diameter was encountered. Paired organs or nodes consisting of two or more distinct lobes with separate microcirculatory networks were found occasionally. Variably large parts of the LN vasculature often could not be visualized because some vessels penetrated the tissue too deeply or were covered by other anatomic structures. However, the visible part of the microcirculatory system consistently displayed a number of characteristic features. Arterial blood was supplied by one or more arterioles that branch from the superficial epigastric artery, which originates from the ipsilateral femoral artery. Some LNs were supplied partially by a dorsal branch of the superficial epigastric artery that anastomoses with the caudal epigastric artery. Blood flow in this anastomosing vessel was reversed occasionally. Thus, segments of LN were at times fed by blood from the caudal epigastric artery. Contrary to the standard models of the vascular architecture in peripheral LN (17, 35) arterioles and venules did not parallel each other. In most preparations, feeding arterioles did not enter the node in the hilus region but penetrated the capsule at one or more locations in the periphery. Most arterial blood was channeled into cortical capillaries that could be visualized after IV injection of FITC-dextran (Fig. 2, top and middle) as dense glomerulus-like networks in areas of the cortex that are thought to contain lymph follicles or germinal centers. Arteriovenous shunts were not detected.

Venous blood converged into one or two large collecting venules (99 ± 27 μm diameter) that drained into the superficial epigastric vein near the hilus of the node. When counting in the upstream direction from the superficial epigastric vein, we could identify up to five successive generations of venules that received blood preferentially or exclusively from the LN (Fig. 2, bottom). First order venules sometimes received additional blood from adjacent connective tissue. Although intranodal venulo-venular anastomoses were rare, they were sometimes detected between second- or third-order venules. Typically, fourth- and fifth-order venules did not extend to the LN capsule but instead originated in the inner cortical/paracortical region. Visible fourth- and fifth-order HEV frequently appeared plump and abrupt diameter changes were often observed at their transition to capillaries. In some cases, HEV appeared to originate from a single capillary whose lumen widened abruptly.

Large numbers of rolling and firmly adherent leukocytes were seen commonly in HEV. This is consistent with the role of LN as lymphocyte recruitment organs. Following IV injection of 150 kD FITC-dextran, adherent leukocytes were detected as dark defects in luminal filling by the fluorescent plasma marker (Fig. 2, middle). Despite the presence of large numbers of adherent and emigrating cells in HEV, there was no evidence of associated disruption of endothelial integrity. FITC-dextran remained strictly confined to the intravascular compartment and no evidence of leakage in the LN parenchyma was detected over at least 2 hours. In contrast, venules in surrounding extralym-
phoid tissues that had been damaged by the surgical procedure sometimes displayed localized FITC-dextran extravasation consistent with a neutrophil-induced increase in macromolecular permeability. This is a hallmark of leukocyte extravasation in inflamed nonlymphoid tissues (10, 42).

**Hemodynamic Parameters in Lymph Node Venules**

Individual velocities of 20 or more consecutive fluorescent L1-2 cells were measured by frame-by-frame analysis of video recordings in 25 LN venules. The lumenal diameter, the axial length, and the order of these vessels was determined after fluorescent labeling of the intravascular compartment with 150-kD FITC-dextran. These measurements were used to analyze venular hemodynamics. As summarized in Table 1, there was considerable heterogeneity among venules of the same order. The wall shear rate, considered an important determinant of leukocyte adhesion in nonlymphoid tissues (16, 43), was lowest in order I venules. However, there was no statistically significant difference (p > 0.05) among the wall shear rates in the different venular branching orders (Kruskal-Wallis test with Bonferroni correction).

The formulas used to estimate $V_{\text{blood}}$ and $\gamma$ are based on the assumption of a parabolic flow profile in a cylindrical tube. However, the flow profile in physiologically perfused HEV is likely to be blunted. This is probably due to the hydrodynamic properties of blood in microvessels (41) and is complicated further by the irregular lumenal surface that owes its shape to high endothelial cells and adherent leukocytes. Therefore, the calculated values given in Table 1 for $V_{\text{blood}}$, $\gamma$, and bulk flow rates are conservative approximations that

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**Figure 2.** Fluorescent micrographs of a subiliac lymph node (LN) after intravenous injection of 150-kD fluorescein isothiocyanate (FITC)-dextran. **Top:** The entire bean-shaped organ embedded in fatty tissue and its relationship to the superficial epigastric artery (a) and vein (v) can be seen using a 4× objective. Cortical borders opposite to the hilus are indicated by *arrowheads*. The bar indicates 500 μm. **Middle:** Higher magnification (40× objective) of a branched high endothelial venules (HEV) in the same preparation (asterisk in Fig. 2 [top]). Note multiple defects in luminal filling, especially in the right (order III) branch, due to adherent lymphocytes and cobblestone-shaped high endothelial cells. The bar indicates 50 μm. **Bottom:** Schematic representation of a typical venular tree in a murine subiliac LN. Order designations were assigned by counting successive generations of venular branches in upstream direction from the large collecting venule that drains into the superficial epigastric vein.
are likely to underestimate the actual hemodynamic conditions encountered by circulating leukocytes. Accordingly, \( V_{L1.2} \) was 15\% higher than \( V_{\text{blood}} \) on average.

### L-Selectin-Dependent Rolling of Transfectants in High Endothelial Venules

BCECF-labeled L1-2 transfectants were injected intraroterally and their behavior in the submucosal LN microcirculation was analyzed. Mock transfectants sometimes slowed briefly in the blood stream. However, L1-2\(^{\text{Vector}}\) cells did not roll continuously along the vascular lining. Occasionally, transfectants got stuck transiently in narrow capillaries both within the LN and in adjacent connective tissue. These cells appeared markedly elongated within the capillary lumen when examined at higher magnification. This phenomenon was not affected by blocking mAbs to L- or P-selectin, PNA\(d\), CD11a, or \(\alpha\)\(\xi\) integrins (unpublished data). This suggests that it reflected capillary plugging due to mechanical properties of L1-2 cells that are somewhat larger in diameter (3.6 ± 1.5 \(\mu\)m) than most endogenous circulating leukocytes (~7 \(\mu\)m).

In contrast to L1-2\(^{\text{Vector}}\) cells, L1-2\(^{\text{Selectin}}\) transfectants rolled efficiently in LN venules (Figs. 3 and 4) but not in arterioles. Rolling fractions correlated inversely with the vessel diameter (Fig. 4, top) but were independent of mean velocity of noninteracting L1-2 cells (Fig. 4, middle) and wall shear rate (Fig. 4, bottom). In contrast, the venular order appeared to be a more important factor since transfectants rolled significantly more frequently in small para- and subcortical HEV (orders III to V) than in collecting venules (orders I and II) in the medulla and hilus (Fig. 5). As discussed above, this difference could not be attributed to differential microhemodynamic factors since the shear rates were lowest in order I venules and there was no statistically significant difference between order II venules and upstream HEV. Generally, rolling fractions of L1-2\(^{\text{Selectin}}\) cells in orders III to V venules were much higher than those found previously for L1-2\(^{\text{Selectin}}\) cells in acutely inflamed rabbit mesentery venules (49). Similarly, the mean rolling velocity determined in a typical order III venule (Fig. 6) was lower than that found previously in nonlymphoid venules (49), which suggests that L-selectin binding to HEV ligand(s) is distinct from other tissues. Consistent with this interpretation, we observed some rolling transfectants in adjacent connective tissue venules but at a lower frequency (<10%) and with a higher rolling velocity than in LN (data not shown).

To demonstrate that rolling of L1-2\(^{\text{Selectin}}\) cells was mediated specifically by L-selectin, rolling fractions in order III to V venules were measured before and after treatment of cells with MAb DREG-56. This MAb to the lectin domain of L-selectin abrogated rolling almost completely (Fig. 7). This suggests that other adhesion molecules did not contribute. Since stably transfected L1-2 cells may overexpress L-selectin at levels that are not encountered on normal circulating leukocytes, we assessed mean copy numbers of sur-
E-selectin ligands. The removal of sialic acid had no effect on rolling fractions confirming that vascular selectins did not play a role here (data not shown). In contrast, IV application of anti-PNAd MAb MECA-79 markedly reduced rolling of L1-2L-selectin cells (Fig. 7) suggesting that their rolling was mediated at least partially by direct interaction of L-selectin and PNAd glycoproteins on HEV.

DISCUSSION

This study reports a new in situ model for the microcirculatory analysis of peripheral LN in mice. The subiliac LN preparation allows the measurement face L-selectin by quantitative flow cytometry immediately before each experiment. On average, unlabeled L1-2L-selectin cells expressed 67,500 ± 17,000 receptors. Assuming that the fluorescent labeling procedure did not affect L-selectin expression, the results suggest that the transfectants used in our experiments expressed L-selectin in the same range as what has been reported for human (40, 45) and rabbit neutrophils (50).

To exclude rigorously a contribution by endothelial selectins to L1-2L-selectin rolling, cells were pretreated with neuraminidase, which destroys sialylated P- and

Figure 3. Fluorescent micrographs of BCECF labeled L1-2L-selectin transfectants in LN venules. (A) A venular tree is shown containing brightly fluorescent cells. Several rolling cells (indicated by asterisks) and one noninteracting transfectant (large arrowhead) can be seen. (B) The same scene 0.3 s later shows that the noninteracting cell has been displaced ~350 μm downstream, whereas the rolling cells have moved with much lower and variable velocities (individual distances traveled are indicated by length of small arrows). The bars indicate 100 μm.

Figure 4. Relationship between L1-2 transfectant rolling and microvascular parameters. Top: L1-2L-selectin cells, but not L1-2Vector cells, roll in LN venules. Each symbol represents the rolling fraction in an individual venule of a given diameter. A regression analysis of L1-2L-selectin rolling fractions and vessel diameter indicates a significant inverse correlation (p < 0.01). In contrast, no significant correlation was found between rolling fractions and V\textsubscript{L\textsubscript{1-2}} (Middle) or rolling fractions and γ (Bottom) in a subset of 25 venules that were sufficiently superficial and whose longitudinal axes were entirely within the plane of focus to allow accurate hemodynamic measurements (see also Table 1).
LN function is enhanced dramatically by several anatomic features that are unique to these organs: afferent lymphatics continuously drain lymph fluid containing Ag from surrounding tissues or from more distal lymphoid organs; and LNs harbor large numbers of tissue-resident professional Ag-presenting cells and other interstitial cells that are thought to have specialized immunomodulatory functions. Thus, lymphocytes with diverse antigenic specificities that enter an LN are exposed to a microenvironment that is uniquely adapted not only to present a concentrated sample of peripheral Ag but also to control the activation and proliferation of those cells that encounter their cognate Ag.

A vital aspect of this intricate system is the efficient and selective recruitment of lymphocytes from the blood stream. This depends on the adhesion of blood-borne cells to tissue-specific molecules on the luminal

Figure 5. Mean rolling fractions of L1-2-L-selectin cells in different generations of LN venules. Venular order numbers were assigned as shown in Figure 2 (Bottom). Rolling fractions in order I and II venules were not statistically different from each other, but they were significantly lower than in order III (p<0.01), IV (p<0.01), and V (p<0.001) venules. Kruskal-Wallis test was used for nonparametric analysis of multiple independent samples followed by Bonferroni correction of p.

Figure 6. Velocity distribution of 111 consecutive L1-2-L-selectin cells (~64,600 L-selectin molecules/cell) in a typical order III HEV (41-μm diameter; γ = 542 sec^-1 corresponding to a wall shear stress of 13.1 dyn/cm^2 assuming a blood viscosity of 0.025 Poise). The fastest cell in the sample was used to determine V_{blood} and the centerline velocity, V_{CL} (4.296 μm/s). Velocities of all cells were normalized to V_{CL}, which was given a value of 1. Cells were pooled in velocity classes from 0 to <0.1, 0.1 to <0.2, and so on. The critical velocity, V_{crit} (i.e., the lowest velocity a noninteracting cell in laminar flow can assume in a cylindrical vessel of this size) (30), is also shown, 40% of all transfectants in this venule moved at a velocity below V_{crit} (501 μm/s) and were thus considered to be rolling. The mean rolling velocity of L1-2-L-selectin cells in this vessel was 171 ± 90 μm/s.
surface of HEV. There is abundant evidence that molecularly distinct interactions of HEV with circulating cells are instrumental in this preferential recruitment of specialized lymphocyte subsets to different sites (12, 20, 36, 37, 46). Moreover, recent studies are beginning to reveal a complex orchestration of adhesion and activation systems that allows particular lymphocyte-HEV interactions to be targeted. For instance, intravital microscopy studies in mice have shown that lymphocyte homing to Peyer's patches follows a multistep adhesion cascade that consists of:

1. primary rolling that requires both L-selectin and α4β7 integrins (4);
2. an activating signal via gαi-coupled signaling proteins (3); and
3. activation-dependent firm adhesion through LFA-1 (CD11a/CD18) and α4β7 (4).

Earlier intravital microscopy investigations (50, 51) as well as in vitro experiments (28) have demonstrated that a three-step scenario consisting of selectin-mediated rolling followed by β2 integrin activation is also essential for neutrophil accumulation in sites of acute inflammation.

In contrast, the molecular mechanisms of lymphocyte homing to peripheral LN have not yet been dissected at a similar resolution. This has been due primarily to a lack of adequate animal models that allow in situ investigations as employed in PP and several nonlymphoid tissues. The model presented here may fill this gap. We demonstrate that L1-2 pre-B lymphoma cells that were transfected with human L-selectin, but not control transfectants, roll in HEV and this involves binding to MECA-79-reactive PNAd epitopes. These findings are consistent with earlier in vitro studies that
have shown that PNAd and L-selectin are a receptor-ligand pair (7). Their interaction under flow in vitro supports rolling, but not firm adhesion, of neutrophils, lymphocytes (15, 27, 39), and L-2 selectin transfectants (52). L-selectin also mediates rolling of neutrophils (31, 50) and transfected pre-B cell lines expressing human (49) or murine (24) L-selectin in nonlymphoid vessels through binding to an endothelial ligand that is distinct from PNAd. Furthermore, L-selectin is involved in rolling of lymphocytes on MAdCAM-1 but only if MAdCAM-1 is decorated with the MECA-79 epitope (4, 6).

We observed that L-2 cells did not arrest or emigrate in HEV despite the apparently high efficiency of L-selectin in initiating adhesive interactions in LN HEV. In contrast, numerous mouse leukocytes adhered firmly to HEV suggesting that selectin-PNAd interactions may be required but not sufficient for homing to LN. These findings are in agreement with recent kinetic studies that indicate that selectin-carbohydrate bonds are highly efficient in tethering flowing cells to a stationary ligand in the presence of fluid shear, but that the lifetime of the bond is too short to permit stationary adhesion (1). In fact, L-selectin can mediate rolling only above a minimum shear threshold (15). These data are thus consistent with the hypothesis that a multistep adhesion cascade is required for lymphocyte homing to peripheral LN. Primary adhesion is initiated through L-selectin whose engagement seems both required and sufficient to mediate rolling. A subsequent activation event may then trigger the engagement of secondary adhesion molecules. Indeed, recent in vivo studies in mice suggest that the β2 integrin LFA-1 (CD11a/CD18) is required for efficient lymphocyte homing to PLN (22). As mentioned previously, L-l-2 cells are essentially negative for LFA-1. They may also lack the appropriate chemoattractant receptors that may be required to activate integrins on rolling cells. L-2 cells express the γδ integrin VLA-4, (α4β1) which can be utilized by these cells during rolling on immobilized VCAM-1 in vitro (8). However, L-2 bone marrow cells did not roll in LN microvessels suggesting that VLA-4-dependent adhesion pathways are not a factor here. This is consistent with earlier studies that have demonstrated that Mabs to L-selectin and LFA-1, but not to α4 integrins, block lymphocyte homing to LN in mice (22). However, although the present experiments with transfected L-1-2 cells confirm the function of L-selectin as a rolling receptor and demonstrate that L-selectin-mediated adhesion is remarkably efficient in LN HEV, they do not exclude the possibility that other adhesion molecules may participate in rolling of endogenous lymphocyte subsets.

Based on electron microscopy studies, Marchesi and Gowans observed that lymphocytes enter the nodal tissue preferentially through HEV within the paracortical area (33). We found that this region contains orders III-V venules that supported L-selectin-dependent adhesion more efficiently than order I and II venules in the medulla and hilus despite similar wall shear rates. In fact, hemodynamic factors such as mean blood flow velocity and wall shear rate had no significant influence on the frequency of rolling in LN HEV at least within the range observed here. This contrasts with microvascular beds in nonlymphoid organs (such as the mesentery) where hemodynamic parameters were shown to be significant determinants of leukocyte margination (16, 43). However, endothelial factors appear to be more important in determining the frequency and strength of rolling interactions even in the mesentery (30, 34). The preference of rolling in orders III to V HEV may be partially explained by the ratio of endothelial surface area to intravascular volume. This ratio is inversely proportional to the vessel diameter and may thus allow more frequent collisions of circulating cells with the walls of small HEV. This may be aided by the unique shape of the cuboid high endothelial cells that may be more bumpy and thus may experience more frequent contact with passing lymphocytes than squamous endothelial cells in medullary venules (14). Alternatively, the preference of L-selectin-dependent rolling in orders III to V venules might reflect a differential expression of PNAd. High densities or a specialized presentation of L-selectin ligands in combination with the conspicuous anatomy and ultrastructure of paracortical HEV could be responsible for the segmental differences in rolling and may obscure subtle influences by hemodynamic parameters.

Following adhesion and diapedesis through the HEV wall, different lymphocyte subsets are thought to migrate to distinct regions within the node and, eventually, most cells leave the lymph compartment via efferent lymphatics and thoracic duct to return to the blood. This recirculation phenomenon is remarkably effective. For instance, the number of lymphocytes that home to a single unstimulated prefemoral LN in a sheep may be as high as 7 × 10⁷ per hour and recruitment can increase by more than threefold upon antigenic stimulation (13). The total flux of lymphoid cells through the thoracic duct of rats and mice was reported as 1.9 × 10⁸ × kg⁻¹ × hr⁻¹ (21) and 2.3 × 10⁷ × kg⁻¹ × hr⁻¹ (19), respectively. Data from the present study suggest that the mean blood flow through a venular tree in a subiliac LN preparation can exceed 1 µl/min. Thus, assuming a concentration of naive
lymphocytes in whole blood of 1–2 × 10⁷/µl, a single subiliac LN may recruit up to 3 × 10⁹ cells per day via its HEV, or ~2% of the recirculating pool that can be harvested from the thoracic duct (which also receives large numbers of cells from mucosa-associated lymphoid tissues). Naturally, this estimate is only based on blood flow calculations and does not take into account other variables such as recruitment efficiency. We also cannot exclude possible hemodynamic changes due to the tissue preparation. Such changes could result from subtle obstructions of feeding or draining blood vessels in which case flow rates in untouched subiliac LN may be expected to be somewhat higher than those encountered here.

In conclusion, an intravital microscopy model has been established that can be used to study the biophysical and molecular mechanisms of leukocyte trafficking in physiologically perfused subiliac LN of anesthetized mice. To characterize this new model, this study has documented the hemodynamics and branching pattern of the venular tree in subiliac LN. Since L-selectin and PNAld are known to be critical for lymphocyte migration to LN, we have determined the in vivo correlate of their interaction. Using stably transected lymphoma cells expressing physiologic L-selectin levels, this study demonstrates that L-selectin is highly efficient at mediating lymphocyte rolling, but not firm adhesion, through binding to PNAld. While the frequency of L-selectin mediated rolling was independent of wall shear rate and blood flow velocity, it was significantly more pronounced in paracortical HEV than in medullary and hilus venules. The mechanisms underlying this spatial preference remain to be identified.

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