

The Cutaneous Lymphocyte Antigen Is an Essential Component of the L-selectin Ligand Induced on Human Vascular Endothelial Cells

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Summary

L-selectin mediates leukocyte rolling on vascular endothelium during inflammation. Although vascular endothelium can be activated with inflammatory cytokines to express functional L-selectin ligands, these ligands have not been well characterized. In this study, fucosyltransferase VII cDNA (Fuc-TVII) transfection of the EA.hy926 human vascular endothelial cell line (926-FtVII) induced functional L-selectin ligand expression and expression of sialyl Lewis^x (sLe^x), as defined by HECA-452 (cutaneous lymphocyte antigen; CLA) and CSLEX-1 mAbs. Cytokine activation of human umbilical vein endothelial cells (HUVEC) also induced functional L-selectin ligand expression, with increased CLA expression and Fuc-TVII transcription. The majority of L-selectin-dependent lymphocyte attachment to activated HUVEC and 926-FtVII cells was blocked specifically by treating the endothelial cells with the HECA-452 mAb, but not the CSLEX-1 mAb. CLA-bearing ligands on vascular endothelium also required sulfation and appropriate molecular scaffolds for functional activity, but were distinct from the L-selectin ligands previously identified by the MECA-79 mAb. These findings demonstrate that the HECA-452-defined antigen, CLA, is an essential carbohydrate component of vascular L-selectin ligands.

Key words: L-selectin • cutaneous lymphocyte antigen • endothelium • human leukocytes • adhesion

L-selectin mediates lymphocyte binding to high endothelial venules (HEV)¹ of peripheral lymph nodes, and facilitates leukocyte rolling along vascular endothelium during immune or inflammatory responses (1, 2). L-selectin is constitutively expressed by most leukocytes, whereas other members of the selectin family, P- and E-selectin, are expressed by activated vascular endothelium. The selectins bind carbohydrate determinants displayed in the proper context on a limited number of glycoproteins or proteoglycans, which serve as specific scaffolds for the display of oligosaccharides recognized by the selectin lectin/epidermal growth factor-like domains. L-selectin binds to at least four different heavily glycosylated mucin-like proteins expressed

by HEV: GlyCAM-1 (3), CD34 (4), MAdCAM-1 (5), and a 200,000 *M_r* ligand (6). L-selectin also binds P-selectin glycoprotein ligand-1 (PSGL-1) expressed by leukocytes (7–10). Although the complete repertoire of L-selectin ligands is yet to be defined, most bear sulfated, sialylated, and fucosylated O-linked carbohydrate side chains which are essential for L-selectin binding (2).

Prototype carbohydrate ligands for the selectins include the sialyl Lewis^x (sLe^x) tetrasaccharide. Lymph node HEV express sLe^x (11, 12), although sLe^x expression alone is generally not sufficient for L-selectin binding (13). L-selectin ligands expressed by HEV also characteristically require sulfation (6, 14) and bear a sulfate-dependent carbohydrate epitope identified by the MECA-79 mAb that inhibits lymphocyte binding to peripheral lymph nodes (15, 16). Sialylation and fucosylation of appropriate carbohydrate determinants are also critical for L-selectin ligand generation (17). Fucosyltransferase-VII (Fuc-TVII) is dominant in generating selectin ligands as illustrated by Fuc-TVII-deficient mice which are characterized by a severe selectin ligand deficiency resulting in blood leukocytosis, impaired

L. Tu and M.D. Delahunty contributed equally to this work.

¹Abbreviations used in this paper: CLA, cutaneous lymphocyte antigen; Fuc-TVII, fucosyltransferase-VII; 926-FtVII, EA.hy926 cells stably transfected with Fuc-TVII cDNA; HEV, high endothelial venules; HUVEC, human umbilical vein endothelial cells; L'IgM, human L-selectin-mouse IgM fusion protein; OSGE, O-sialoglycoprotease; PSGL-1, P-selectin glycoprotein ligand-1; sLe^x, sialyl Lewis^x.

leukocyte extravasation during inflammation, and faulty lymphocyte migration (18). The specific localization of Fuc-TVII expression to HEV also correlates with L-selectin ligand expression (19). A specific subset of anti-sLe^x mAbs, including HECA-452, 2F3, and 2H5, but not other anti-sLe^x mAbs such as CSLEX-1 and FH6, recognize sLe^x determinants and putative L-selectin ligands found on HEV (12, 20). The 2H5 mAb blocks L-selectin-dependent binding of leukocytes to HEV and inflamed skin venules in rats (21), and the HECA-452 mAb identifies a sLe^x-like determinant termed the cutaneous lymphocyte antigen (CLA) (22–25). Antigen binding by the 2H5 and HECA-452 mAbs is independent of sulfation, but requires both sialic acid and fucose. By contrast, the MECA-79 mAb defines sulfate-dependent, but Fuc-TVII- and sialic acid-independent, carbohydrate antigens. Although some structures recognized by the HECA-452 and 2H5 mAbs have been identified (20), the precise structural specificity of their ligands has not been defined. Therefore, L-selectin ligands on HEV are likely to be synthesized through the concerted action of sialyltransferases, Fuc-TVII, and appropriate sulfotransferase(s).

A critical issue remaining is the identity of the vascular endothelial L-selectin ligand expressed at sites of inflammation. A cytokine-induced ligand for L-selectin is expressed by cultured human umbilical vein endothelial cells (HUVEC) and microvascular endothelial cells (26–29). The ligand is not CD34, and cultured HUVEC do not express PSGL-1 or MAdCAM-1 (30, 31). L-selectin binding to HUVEC is sialic acid-dependent (26), but is resistant to cell treatment with *O*-sialoglycoprotease (OSGE) (30), an endopeptidase specific for glycoproteins containing closely spaced *O*-linked sialic acid residues. Vascular endothelial cells express sulfotransferases that may contribute to L-selectin ligand generation (32). Cultured HUVEC also express sialyltransferases and fucosyltransferases, and upregulate fucosyltransferase activity after cytokine activation (33). Likewise, inflammation induces increased expression of 2H5 mAb-defined determinants on vascular endothelium (21). Thus, it is possible that L-selectin ligands expressed on vascular endothelium are sLe^x-like determinants displayed by OSGE-resistant cell surface molecules that are distinct from the MECA-79 mAb-defined peripheral node addressins. Therefore, functional L-selectin ligands expressed by human vascular endothelium were further characterized in this study using HUVEC, an endothelial cell line derived from HUVEC, EA.hy926 (926 cells), and 926 cells transfected with human Fuc-TVII cDNA (926-FtVII cells). 926 cells maintain most of the characteristics of primary HUVEC (34, 35). These studies revealed that the CLA determinant appears to be a critical component of the L-selectin ligand that mediates lymphocyte tethering and rolling on activated vascular endothelium.

Materials and Methods

L-selectin-IgM Fusion Protein. A human L-selectin-mouse IgM fusion protein (L'IgM) was generated by fusing cDNAs encoding

the extracellular domains of human L-selectin with the CH2-CH4 domains of mouse μ heavy chain. The L-selectin cDNA contained a membrane-proximal seven amino acid deletion, Δ K-S, which prevents endoproteolytic cleavage as described (36). The L-selectin-IgM cDNA was used to transiently transfect COS cells by the DEAE-dextran method. After 1 wk, the culture supernatant fluid was concentrated by ultrafiltration (membrane XM300; Amicon Inc.) and L'IgM concentrations were measured by ELISA as described (37).

Antibodies. The anti-L-selectin (LAM1-3 and LAM1-14) mAbs were made as described (38, 39). The HECA-452 (anti-CLA), CSLEX-1 (anti-sLe^x), and MECA-79 hybridomas were obtained from the American Type Culture Collection. The anti-PSGL-1 (PL-1; Dr. Kevin Moore, University of Oklahoma Health Science Center, Oklahoma City, OK), CD57 (HNK-1; Dr. Max Cooper, University of Alabama in Birmingham, Birmingham, AL), anti-P-selectin (G1; Dr. Bruce Furie, Beth Israel Medical Center, Boston, MA), anti-VAP1 (TK8-14; Dr. Shirpa Jalkanen, University of Turku, FIN-20520 Turku, Finland) mAbs were gifts. The mouse mAbs to E-selectin (HAE-1f), VCAM-1 (HAE-2d), ICAM-1 (HAE-4b), and CD20 (HB13b) were produced as described (26, 40). The anti-CD34 (My10; Becton Dickinson), anti-mouse CD25 mAb (rat IgM; Southern Biotechnology Associates), FITC-conjugated goat anti-rat IgM and anti-mouse IgM antibodies (Southern Biotechnology Associates), and FITC-conjugated goat anti-mouse IgG (CALTAG Laboratories) were obtained commercially.

Cell Lines and Lymphocyte Isolation. 926 cells were a gift from Dr. Cora-Jean Edgell (University of North Carolina at Chapel Hill, Chapel Hill, NC). 926 cells expressing α 1,3 fucosyltransferase (926-FtVII) were generated by transfecting 926 cells with Fuc-TVII cDNA (from Dr. Brent Weston, University of North Carolina at Chapel Hill). Experiments were carried out using either two different uncloned polyclonal populations of 926-FtVII cells, lines of HECA-452⁺926-FtVII cells cloned from these parental populations of transfected cells, or oligoclonal populations of HECA-452⁺ bright cells isolated by fluorescence-based cell sorting. Similar, if not identical, immunofluorescence staining and cell-binding results were obtained with each cell population. COS cells were similarly transfected with Fuc-TVII cDNA with subsequent selection of clonal transfected cell lines.

Heparinized blood of normal volunteers was isolated using protocols approved by the Human Use Committee of Duke University (Durham, NC). PBLs were isolated by density gradient centrifugation using Lymphoprep (Nycomed Pharma As). HUVEC were isolated and cultured as described (26). In some experiments 926-FtVII cells or HUVEC were incubated with OSGE (80 and 160 μ g/ml, respectively; Accurate Chemical and Scientific Corp.) or *Vibrio cholerae* neuraminidase (0.1 U/ml; Calbiochem) in HBSS with Ca²⁺ and 10 mM Hepes buffer for 1 h at 37°C before the adhesion assays were carried out. To reduce protein sulfation, 926-FtVII cells and HUVEC were grown in medium containing 10 mM NaClO₃ (Sigma Chemical Co.) for 24 h before functional assays. HL-60 cells and the mouse pre-B cell line, 300.19, were cultured as described (8). 300.19 cells transfected with a nonshedding form of L-selectin (LAM-N) were generated as described (36) and are termed 300.19-L'.

Immunofluorescence Staining and Flow Cytometry Analysis. Lymphocytes or single-cell suspensions of 926 cells, HUVEC, or COS-FtVII cells were washed twice, and 10⁶ cells were incubated with primary antibodies diluted to optimal concentrations for immunostaining on ice for 30–40 min. After washing, secondary antibody diluted to the appropriate concentration for op-

timal staining was added to the cells for 30 min. The cells were washed and immunofluorescent staining was analyzed on a FAC-Scan® flow cytometer (Becton Dickinson). For staining with the L'IgM fusion protein, 10⁶ cells were incubated with L'IgM (10 µg/ml) for 40 min on ice, then diluted FITC-conjugated goat anti-mouse IgM antibody was added for 40 min, followed by addition of PBS-2% Nu-serum for immediate analysis by flow cytometry.

Nonstatic Cell Binding Assays. An in vitro adhesion assay similar to the Stamper-Woodruff frozen section assay (41) was used as described (8, 26). 926 cells and 926-FtVII cells were cultured overnight on glass microscope slides. In some experiments, COS or COS-FtVII cells were transiently transfected with PSGL-1 (pPL85, from Dr. Dale Cumming, Genetics Institute, Cambridge, MA) or CD34 (from Dr. Daniel Tenen, Beth Israel-Deaconess Hospital, Boston, MA) cDNAs using the DEAE-dextran method as described (8). 24 h later, transfected COS or COS-FtVII cells were transferred onto glass slides and were cultured overnight to between 25 and 50% confluence. Lymphocytes or 300.19 cells were washed twice in cold DMEM with high glucose plus 5% FCS and then resuspended in the same medium. In mAb blocking experiments, cells were preincubated in 200 µl medium with or without the appropriate mAb for 20 min on ice before the binding assays were initiated. Lymphocytes or 300.19 cells were added (10⁶ in 200 µl) to the slides with horizontal rotation (64 rpm) at 4°C. After 30 min, the medium was tipped off the slide, and the slides were placed vertically in fixative (PBS, 2.4% [vol/vol] glutaraldehyde, pH 7.4) overnight at 4°C. The slides were evaluated by phase-contrast microscopy, and representative fields were photographed (final magnification: ×400). The number of adherent cells was counted for at least 10 random microscopic fields (0.16 mm²) on each of three slides.

For assessing lymphocyte interactions with HUVEC, endothelial cells were grown to 50% confluence on 0.1% gelatin-coated glass slides and were stimulated with 100 U/ml of recombinant human TNF-α (Genzyme Corp.) for 6 h. Lymphocytes (10⁶ in 200 µl) resuspended in M199 medium containing 20% FCS were overlaid onto each washed slide. Subconfluent monolayers of HUVEC were used in these assays to allow accurate determinations of the numbers of leukocytes bound with similar results obtained when confluent monolayers of HUVEC were used. The use of subconfluent endothelial cell monolayers did not promote leukocyte binding due to matrix interactions under rotation, as the leukocytes did not bind to spaces between HUVEC. Nonstatic binding assays were carried out as above.

Endothelial-Leukocyte Adhesion Under Defined Flow Conditions. Lymphocyte-endothelial cell interactions under physiologic flow conditions were assessed using an in vitro flow chamber as described (42). 926 or 926-FtVII cells grown to confluence on glass coverslips were placed in a parallel-plate flow chamber. Lymphocytes (10⁶ cells/ml) resuspended in PBS containing 0.75 mM CaCl₂, 0.75 mM MgCl₂, and 0.5% (wt/vol) BSA were perfused through the chamber for 10 min at a calculated shear stress of 1.85 dyn/cm² via a syringe pump (Harvard Apparatus). Lymphocyte-endothelial interactions were videotaped using an inverted phase-contrast microscope with 10× or 40× objective power (Olympus Corp.) and a CCD video camera (Hitachi Denshi, Ltd.). After a 10-min perfusion period, multiple fields were videotaped for at least 30 s each over the next 4 min of perfusion. In some cases, composite digitized images of single video tape frames were generated using commercial software (Optimus version 5.0; Optimus Inc.) with contrast and brightness optimized using Adobe Photoshop (Adobe Systems). Rolling velocities were measured by determining the average

time it took 100 lymphocytes to roll a 400-µm distance over the endothelial cell monolayer in multiple fields for each experiment. Rolling lymphocyte numbers represent the number of rolling lymphocytes that crossed a 400-µm line over a 10-s time period in at least 10 random fields for each experiment.

RNA Isolation and PCR Amplification. Total cellular RNA isolated from cell pellets of 926-FtVII cells and HUVEC was used for cDNA synthesis as described (43). PCR amplification of Fuc-TVII cDNA used sense 5'-TCA GCC ACC TCC GAG GCA TCT TCA ACT G-3', and antisense 5'-CGT TGG TAT CGG CTC TCA TTC ATG CCA GTG A-3' primers to amplify a 500-bp fragment. PCR amplification was carried out at 94°C for 5 min, then 30 cycles of 94°C for 1 min, 66°C for 1 min, 72°C for 1 min, followed by 72°C for 5 min. Primers for actin were sense 5'-ATG TTT GAG ACC TTC AAC AC-3', and antisense 5'-CAC GTC ACA CTT CAT GAT GG-3', which generate a 495-bp fragment with PCR amplification as follows: 94°C for 5 min, then 30 cycles of 94°C for 1.5 min, 55°C for 2 min, 72°C for 1 min; followed by 72°C for 5 min. The PCR products were electrophoresed, transferred to nitrocellulose, and then hybridized with radiolabeled Fuc-TVII cDNA probe. Blots were exposed to x-Omat AR film (Kodak) or quantified using a Storm 680 PhosphorImager (Molecular Dynamics).

For semiquantitative analysis of Fuc-TVII mRNA levels, cDNA generated as described above was diluted over a broad range of concentrations (neat to 1:5,000). The relative intensity of Fuc-TVII and actin PCR bands generated from each cDNA aliquot was then determined by gel analysis to generate dose-response curves. The intensity of PCR products at a dilution of 1:50 to 1:100 was in the midpoint of the linear range where band intensity was proportional to the amount of input cDNA. For HUVEC, this translated into ~4 × 10⁵ starting cell equivalents for cDNA synthesis and Fuc-TVII amplification, and ~2 × 10⁵ starting cell equivalents for actin cDNA synthesis and amplification.

Statistical Analysis. Data are expressed as means ± SEM unless indicated otherwise. The Student's *t* test was used to determine the significance of differences in sample population means.

Results

Vascular Endothelial Cells Express L-selectin Ligands. L-selectin ligands expressed on vascular endothelium were characterized using the chimeric fusion protein, L'IgM, a method originally described by others (7). Immunofluorescent staining of HUVEC-derived 926 cells demonstrated low levels of L'IgM reactivity that were inhibited to background staining levels by the presence of EDTA (Fig. 1, top). Treatment of 926 cells with cytokines or agents previously shown to induce L-selectin ligands on endothelial cells (26) did not affect L'IgM reactivity (data not shown). 926 cells stably transfected with a cDNA encoding Fuc-TVII (926-FtVII) reacted more intensely with L'IgM than untransfected 926 cells (Fig. 1, middle). L'IgM binding to 926-FtVII cells was completely inhibited by EDTA (Fig. 1, middle) and was decreased by 65–76% in the presence of the LAM1-3 mAb, which blocks L-selectin function (Fig. 1, bottom). Therefore, 926 endothelial cells express L-selectin ligands which are augmented by increasing Fuc-TVII expression.

Lymphocyte-Endothelial Interactions Are Mediated by L-selectin and Its Ligands. Whether L-selectin ligands expressed

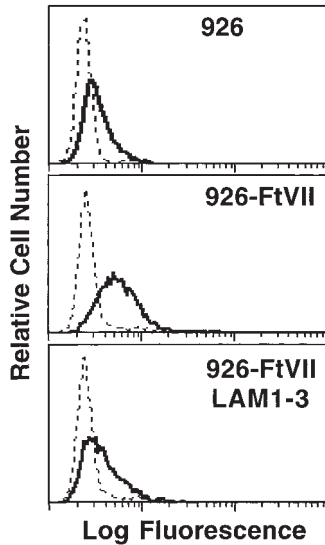


Figure 1. L-selectin binds ligands expressed on (top) 926 and (middle) 926-FtVII cells. Cells were stained in indirect immunofluorescence assays with L'IgM (10 μ g/ml) and FITC-labeled goat anti-mouse IgM antibody, and analyzed by flow cytometry. (Bottom) 926-FtVII cells were stained with L'IgM (10 μ g/ml) that was preincubated with a function-blocking anti-L-selectin mAb (LAM1-3, 50 μ g/ml) for 20 min. The dotted lines represent background staining with an unreactive mouse IgM antibody (HB13b, 10 μ g/ml), with secondary antibody alone, or with L'IgM in the presence of 5 mM EDTA. Results represent those obtained in three experiments and with two independent lines of 926-FtVII cells.

on 926-FtVII cells were functionally active was assessed using 300.19-L'. In a nonstatic cell binding assay developed to assess leukocyte-endothelial cell interactions (26), 300.19-L' cells bound to 926-FtVII cells at significant levels (15.6 ± 0.9 cells/0.16-mm² field; $P < 0.001$), whereas 300.19 cells

did not bind (0.3 ± 0.1 cells/field; Fig. 2 A). Furthermore, human lymphocytes bound to 926-FtVII cells at significant levels but did not bind to 926 cells (Fig. 2, B and C). Lymphocyte binding to 926-FtVII cells was blocked by lymphocyte pretreatment with the LAM1-3 mAb (99%), but not by the non-function-blocking LAM1-14 mAb (Fig. 2, B and C). Thus, lymphocyte interactions with 926-FtVII cells were mediated specifically by L-selectin binding its endothelial ligands.

L-selectin also mediated lymphocyte binding to 926-FtVII cells under in vitro conditions of physiologic shear flow. High numbers of lymphocytes attached to 926-FtVII cell monolayers at a shear force of 1.85 dyn/cm² and rolled at velocities as slow as 100 μ m/s, which were significantly lower than the theoretical velocity of a lymphocyte not interacting with the endothelial cell surface (526 μ m/s) (44). Mean rolling velocity at this shear force was 226 ± 77 μ m/s (\pm SD, $n = 200$, Fig. 3 A). As expected for a totally L-selectin-dependent adhesive interaction, 926-FtVII cells supported significant L-selectin-dependent rolling at shear stresses between 0.75 and 3.0 dyn/cm² with maximal rolling between 1 and 2 dyn/cm² as described (45). In addition, lymphocytes rolled into the field of view, indicating that cells had attached upstream of the field under observation. The majority of lymphocytes in contact with 926-

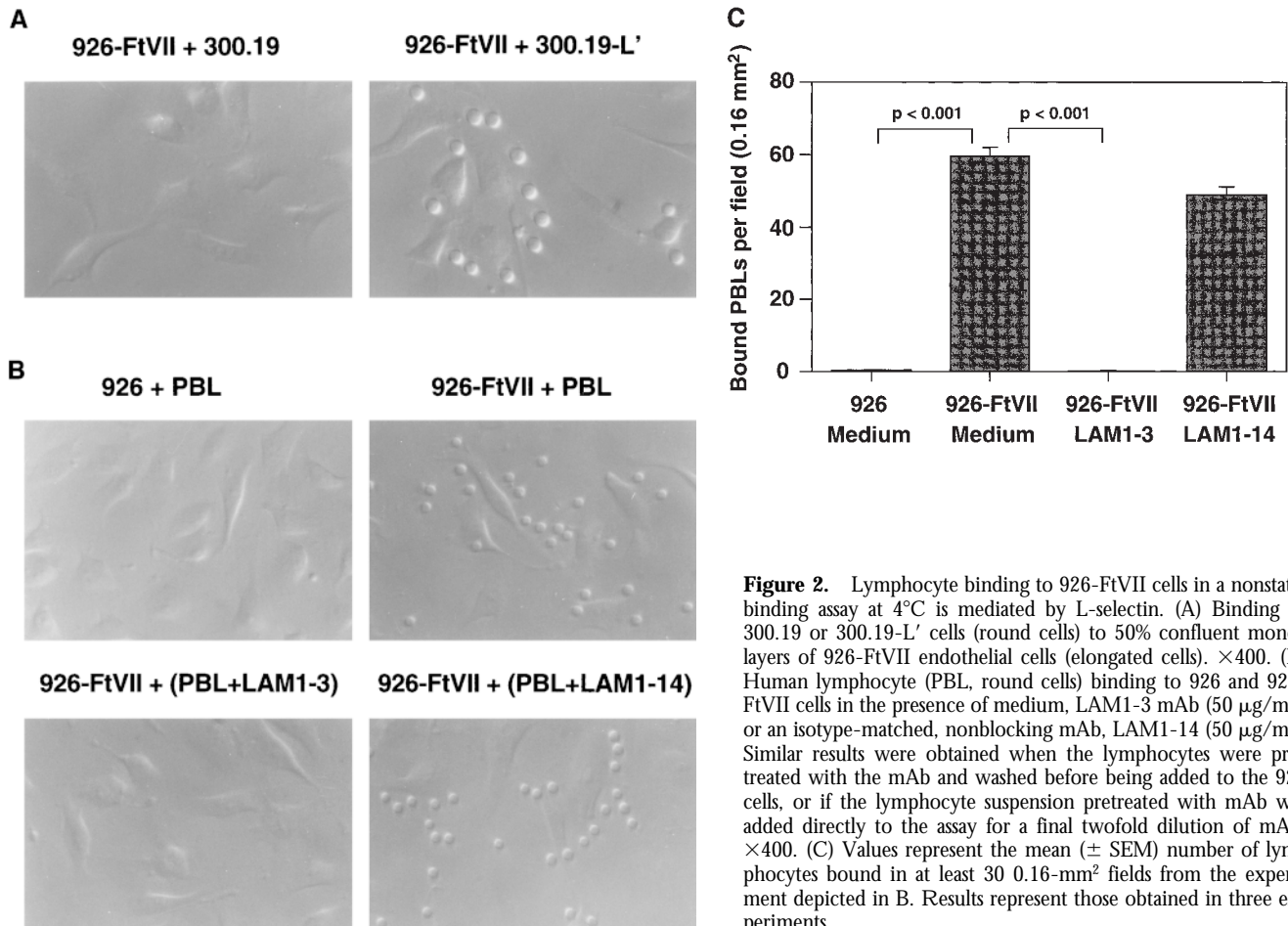
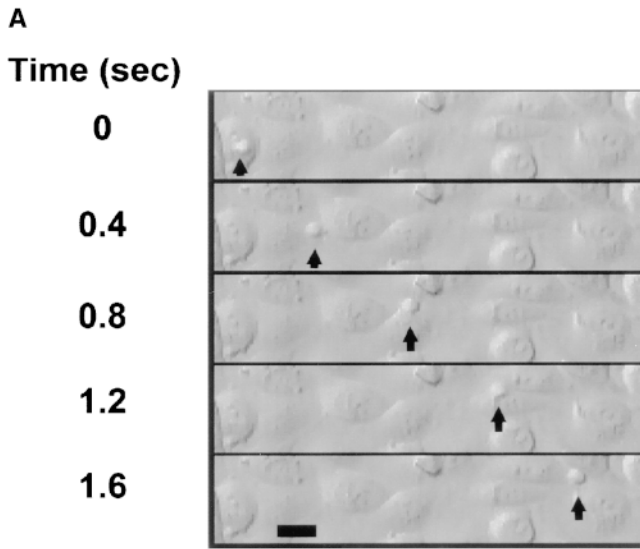


Figure 2. Lymphocyte binding to 926-FtVII cells in a nonstatic binding assay at 4°C is mediated by L-selectin. (A) Binding of 300.19 or 300.19-L' cells (round cells) to 50% confluent monolayers of 926-FtVII endothelial cells (elongated cells). $\times 400$. (B) Human lymphocyte (PBL, round cells) binding to 926 and 926-FtVII cells in the presence of medium, LAM1-3 mAb (50 μ g/ml), or an isotype-matched, nonblocking mAb, LAM1-14 (50 μ g/ml). Similar results were obtained when the lymphocytes were pretreated with the mAb and washed before being added to the 926 cells, or if the lymphocyte suspension pretreated with mAb was added directly to the assay for a final twofold dilution of mAb. $\times 400$. (C) Values represent the mean (\pm SEM) number of lymphocytes bound in at least 30 0.16-mm² fields from the experiment depicted in B. Results represent those obtained in three experiments.



layers of 926 or 926-FtVII cells. Lymphocytes preincubated with either medium, the LAM1-3, or LAM1-14 mAbs (50 $\mu\text{g}/\text{ml}$) for 20 min were diluted fivefold and perfused through the flow chamber. 926 or 926-FtVII cells grown on slides were overlaid with medium alone, or containing the HECA-452 (HECA, 10 $\mu\text{g}/\text{ml}$) or CSLEX-1 (CSLEX, 50 $\mu\text{g}/\text{ml}$) mAbs for 20 min immediately before the assays. Values represent the mean (\pm SEM) number of rolling lymphocytes that entered a 400- μm -wide field over a 10-s time period in 10 randomly chosen fields near the centerline of the flow chamber after a 10-min time period. Results represent those obtained in three experiments, except for HECA and CSLEX results, which represent two experiments.

FtVII monolayer remained in contact for their entire transit across the field of view, although there was no stationary adhesion of lymphocytes to 926-FtVII cell monolayers. By contrast, lymphocytes did not interact with untransfected 926 monolayers at detectable levels (Fig. 3 B). Preincubation of lymphocytes with the LAM1-3 mAb reduced the frequency of rolling lymphocytes by 94–97%, whereas the LAM1-14 mAb had no effect (Fig. 3 B). Thus, lymphocyte tethering and rolling on 926-FtVII cells is mediated by L-selectin interacting with its endothelial ligands.

926-FtVII Cells Express the CLA and sLe^x Antigens. The expression of L-selectin ligands by 926 and 926-FtVII cells was assessed using a panel of mAbs reactive with characterized adhesion molecules. 926-FtVII cells expressed sLe^x antigens at high levels as defined by both the CSLEX-1 and HECA-452 mAbs (Fig. 4 A). Untransfected 926 cells expressed HECA-452-defined epitopes at low levels, but it was not possible to detect CSLEX-1 mAb-defined antigen (Fig. 4 A). 926 and 926-FtVII cells did not express detectable levels of the antigens identified by the MECA-79 or HNK-1 mAbs and did not express detectable levels of P-selectin, E-selectin, CD34, PSGL-1, VCAM-1, VAP-1, or ICAM-1 as described previously in some cases (46, 47). The virtual absence or low expression of adhesion molecules other than L-selectin ligands may explain the relatively fast rolling velocities of leukocytes on 926-FtVII cells and is consistent with the complete deficit in lymphocyte firm adhesion on 926-FtVII cells during in vitro flow chamber assays. Consistent with this observation, a lack of P-selectin expression in vivo increases leukocyte rolling from ~ 49 $\mu\text{m}/\text{s}$ in wild-type mice to ~ 129 $\mu\text{m}/\text{s}$ in P-selectin-deficient mice under conditions where L-selectin pre-

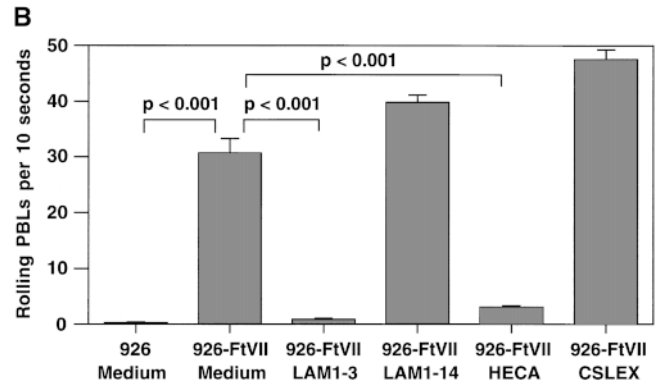


Figure 3. L-selectin mediates lymphocyte tethering and rolling under physiologic shear flow through CLA expressed by 926-FtVII cells. (A) Representative lymphocyte rolling on 926-FtVII monolayers in a parallel-plate flow chamber ($\times 40$ objective) at a shear force of 1.85 dyn/cm^2 . Single frames of video tape at 0.4-s intervals were digitized to generate a composite image of a rolling lymphocyte (arrowheads). Flow is from left to right. Bar, 20 μm . (B) Numbers of lymphocytes rolling across mono-

layers of 926 or 926-FtVII cells. Lymphocytes preincubated with either medium, the LAM1-3, or LAM1-14 mAbs (50 $\mu\text{g}/\text{ml}$) for 20 min were diluted fivefold and perfused through the flow chamber. 926 or 926-FtVII cells grown on slides were overlaid with medium alone, or containing the HECA-452 (HECA, 10 $\mu\text{g}/\text{ml}$) or CSLEX-1 (CSLEX, 50 $\mu\text{g}/\text{ml}$) mAbs for 20 min immediately before the assays. Values represent the mean (\pm SEM) number of rolling lymphocytes that entered a 400- μm -wide field over a 10-s time period in 10 randomly chosen fields near the centerline of the flow chamber after a 10-min time period. Results represent those obtained in three experiments, except for HECA and CSLEX results, which represent two experiments.

dominantly mediates rolling (48). In addition, ICAM-1 deficiency also results in leukocytes rolling at faster velocities in vivo (49). Therefore, increased sLe^x and CLA expressions were the only detectable differences between 926 and 926-FtVII cells.

CLA Is the L-selectin Ligand on Vascular Endothelium. Whether or not sLe^x expression contributed to lymphocyte attachment on 926-FtVII cells was assessed using the HECA-452 and CSLEX-1 mAbs. Preincubation of 926-FtVII cells with the HECA-452 mAb blocked most lymphocyte attachment (94–95%; $P < 0.001$), whereas the CSLEX-1 mAb did not significantly inhibit lymphocyte binding in the nonstatic binding assay (Fig. 4 B). Pretreatment of lymphocytes with the HECA-452 mAb before the binding assay did not significantly affect lymphocyte binding to 926-FtVII cells (Fig. 4 B). Under defined shear flow conditions using a parallel-plate flow chamber, preincubation of 926-FtVII cells with the HECA-452 mAb significantly ($>90\%$) reduced the number of lymphocytes rolling at 1.85 dyn/cm^2 of shear stress (Fig. 3 B). By contrast, the CSLEX-1 mAb did not inhibit lymphocyte rolling. Thus, the CLA subset of sLe^x antigens is a critical component of the L-selectin ligands expressed on 926-FtVII cells.

Sialylation and Sulfation Are Important Features of Vascular L-selectin Ligands. Whether sialylation or sulfation influenced L-selectin-mediated lymphocyte attachment to 926-FtVII cells was assessed. Pretreatment of 926-FtVII cells with neuraminidase abolished ($>98\%$ decrease) lymphocyte attachment to 926-FtVII cells in both nonstatic binding assays (Fig. 5 A) and under defined flow conditions (Fig. 5 B). Similarly, culturing 926-FtVII cells in the presence of sodium chlorate (NaClO_3) for 24 h significantly in-

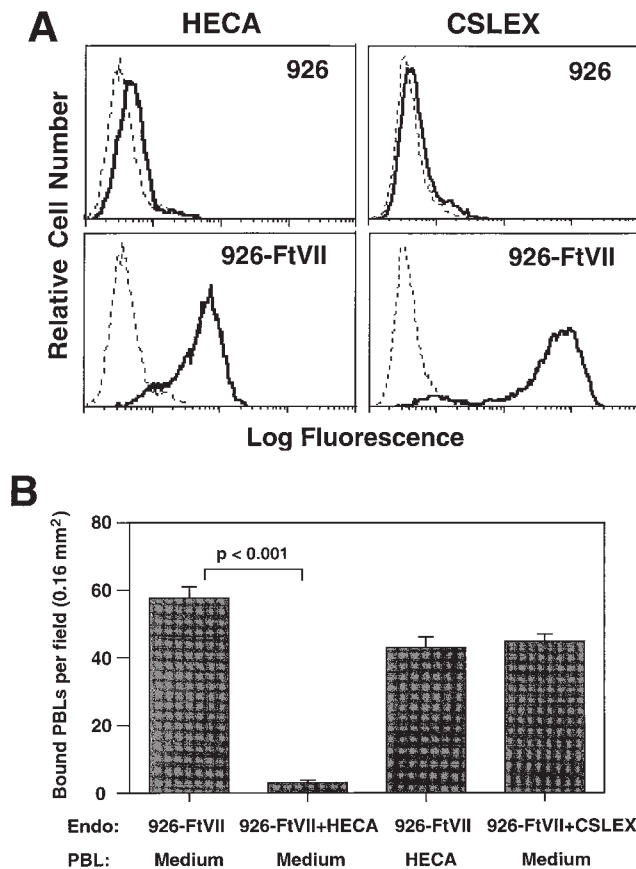


Figure 4. CLA mediates lymphocyte binding to 926-FtVII cells. (A) Staining of 926 and 926-FtVII cells with the HECA-452 (10 $\mu\text{g/ml}$) or CSLEX-1 (20 $\mu\text{g/ml}$) mAbs (bold lines) in indirect immunofluorescence assays with flow cytometry analysis. Dashed lines represent cell staining with equivalent amounts of unreactive isotype-matched control antibodies or with secondary antibody alone. (B) Lymphocyte (PBL) binding to 926-FtVII cells assessed in a nonstatic binding assay. 926-FtVII cells were preincubated with medium alone or with medium containing the HECA-452 (10 $\mu\text{g/ml}$) or CSLEX-1 (50 $\mu\text{g/ml}$) mAbs for 20 min. Lymphocytes were also incubated in medium or medium containing the HECA-452 mAb (10 $\mu\text{g/ml}$) for 20 min, and then overlaid onto $\sim 50\%$ confluent monolayers of 926-FtVII cells at 4°C with rotation. Values represent the mean (\pm SEM) number of lymphocytes bound in at least 30 0.16-mm^2 fields. Results represent those obtained in at least two experiments.

hibited (74–84%) lymphocyte attachment in nonstatic binding assays (Fig. 5 A). Although L-selectin binds to several mucin-like proteins that are sensitive to endoproteolytic cleavage by OSGE, treatment of 926-FtVII cells with OSGE did not significantly affect lymphocyte attachment in the nonstatic binding assays (Fig. 5 A) or under physiologic flow conditions (Fig. 5 B). In parallel control assays with HL-60 cells, OSGE treatment completely removed the PL-1 mAb–defined epitope from PSGL-1 (data not shown). Thus, sialic acid and sulfate are essential components of the L-selectin ligands expressed on vascular endothelium even though the ligands on vascular endothelium are OSGE-resistant.

HUVEC Activation Induces L-selectin Ligands. Whether L-selectin ligands expressed by HUVEC were CLA-dependent was assessed using TNF-activated HUVEC. The

L'IgM fusion protein reacted weakly with resting HUVEC but was bound at fourfold higher levels after TNF- α activation (Fig. 6 A). L'IgM binding peaked between 4 and 6 h of HUVEC activation and was sustained at high levels for at least 24 h. At each time point, L'IgM reactivity was reduced to background staining levels in the absence of Ca^{2+} . Similar results were obtained when HUVEC were stained with the HECA-452 mAb; low levels of CLA expression by resting HUVEC with expression significantly increased (100–200%) 6 h after TNF- α activation (Fig. 6 B). In addition, resting HUVEC expressed Fuc-TVII transcripts and relative Fuc-TVII mRNA levels increased 1.8–2.0-fold ($n = 2$) after TNF- α activation for 6 h (Fig. 6 C). 926 cells also expressed Fuc-TVII mRNA, but at levels significantly below those of 926-FtVII cells (data not shown). Therefore, the time course of increased L'IgM and HECA-452 staining correlated with the previously described induction of functional L-selectin ligands after HUVEC activation (26).

Increased CLA expression by TNF- α -activated HUVEC correlated with lymphocyte binding. In nonstatic binding assays, TNF- α activation of HUVEC induced significant lymphocyte binding, whereas resting HUVEC supported minimal binding (Fig. 6 D). The LAM1-3 mAb inhibited lymphocyte binding to activated HUVEC by 65–76%, while the non-function-blocking LAM1-14 mAb did not affect lymphocyte binding (Fig. 6 D). Treatment of activated HUVEC with the HECA-452 mAb reduced lymphocyte binding by 53–71%. Pretreatment of activated HUVEC with neuraminidase reduced lymphocyte binding by 46–55%. HUVEC cultured in the presence of 10 mM NaClO_3 for 24 h, with TNF- α added during the last 6 h of culture, also significantly inhibited (31–40%) lymphocyte binding. By contrast, OSGE treatment of cytokine-activated HUVEC did not significantly affect lymphocyte binding as previously reported (30). Thus, lymphocyte binding to activated HUVEC through L-selectin required interactions with a sulfated, CLA-bearing ligand.

CLA Expression Alone Is Insufficient for L-selectin Binding. Whether CLA expression alone was sufficient to generate L-selectin ligands was assessed using COS cells stably transfected with Fuc-TVII cDNA. COS cells did not express the HECA-452 antigen, although COS-FtVII cells expressed CLA at levels comparable to those observed on 926-FtVII cells (Fig. 4 A and Fig. 7 A). In sharp contrast with 926-FtVII cells, COS-FtVII cells did not bind lymphocytes or 300.19-L' cells in nonstatic binding assays (Fig. 7, B and C). To assess whether a specific protein backbone was required for CLA presentation, COS-FtVII cells were transiently transfected with cDNAs encoding either PSGL-1 or CD34. COS-FtVII cells expressed these cell surface antigens at high levels (Fig. 7 A). COS-FtVII cells expressing PSGL-1 bound both lymphocytes and 300.19-L' cells at high levels (Fig. 7, B and C). By contrast, COS-FtVII cells expressing CD34 did not bind either lymphocytes or 300.19-L' cells above background levels under these assay conditions. These results demonstrate that CLA may require a specific protein scaffold to serve as an L-selectin ligand, in addition to a requirement for appropriate sulfation.

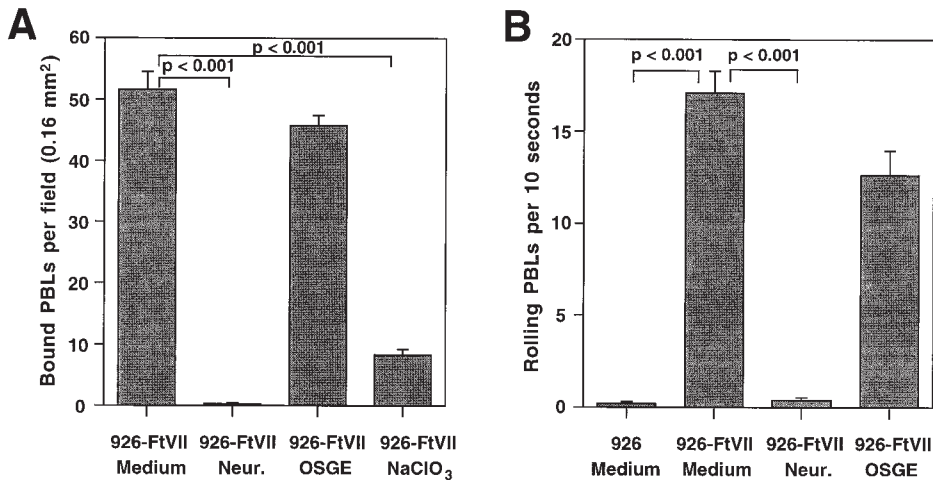


Figure 5. L-selectin ligands on vascular endothelium require sialylation and sulfation. (A) Lymphocyte (PBL) binding to ~50% confluent 926-FtVII cell monolayers was assessed in a nonstatic binding assay. 926-FtVII cells grown on glass coverslips were pretreated with medium alone, neuraminidase (Neur.), OSGE, or NaClO₃ as indicated. Values represent the mean (\pm SEM) number of lymphocytes bound in at least 30 microscopic fields. (B) Lymphocyte (PBL) rolling on confluent monolayers of 926 or 926-FtVII cells was assessed in a parallel-plate flow chamber at a shear force of 1.85 dyn/cm². 926-FtVII cells were pretreated with medium, neuraminidase (Neur.), or OSGE as indicated. Values represent the mean (\pm SEM) number of rolling lymphocytes that entered a 400- μ m-wide field over a 10-s time period

in 10 randomly chosen fields near the centerline of the flow chamber after a 10-min time period. Results represent those obtained in at least three independent experiments, except for OSGE treatment under shear flow, which was carried out twice.

Discussion

In this study, it was found that (a) transfection of a vascular endothelial cell line with Fuc-VII cDNA induced the expression of functional L-selectin ligands (Figs. 1–3); (b) vascular endothelial cells with increased Fuc-TVII expression showed significant sLe^x staining as defined by the HECA-452 (CLA) and CSLEX-1 mAbs (Fig. 4), but not MECA-79 mAb staining (data not shown); (c) HUVEC activation upregulated functional L-selectin ligand expression, Fuc-TVII transcription, and CLA expression (Fig. 6); (d) pretreatment of endothelial monolayers with the HECA-452 mAb, but not the CSLEX-1 mAb, blocked L-selectin-dependent lymphocyte attachment and adhesion to vascular endothelium (Figs. 3, 4, and 6); (e) the L-selectin ligands on vascular endothelium were a novel class of ligands that required sulfation and sialylation, but were OSGE-resistant, which distinguishes them from CD34 and PSGL-1 (Fig. 5); and (f) CLA requires appropriate presentation on the cell surface to serve as an L-selectin ligand (Fig. 7). These findings demonstrate that the HECA-452 subset of sLe^x antigens defines an essential component of the inducible vascular L-selectin ligand.

CLA, as defined by the HECA-452 mAb, is a carbohydrate determinant specifically expressed by HEV within lymphoid organs, by monocyte subsets and small numbers of leukocytes in tonsils and lymph nodes, and by endothelium at sites of long-standing chronic inflammation (50). However, most noted is that the HECA-452 antigen identifies a population of skin-homing memory T cells which bind E-selectin displayed by dermal endothelium (23, 24, 51, 52). Although the induction of CLA expression within vessels of inflamed tissues also closely correlates with intense local infiltration of lymphocytes, a role for CLA as an L-selectin ligand was initially discounted since saturating HEV with the HECA-452 mAb did not inhibit lymphocyte binding (50). However, our finding that preincubation of endothelial cells with the HECA-452 mAb blocked L-selectin-

mediated binding of leukocytes to non-HEV vascular endothelium (Figs. 3, 4, and 6) clearly demonstrates that CLA is a major component of the vascular L-selectin ligand. In addition, recent studies have shown that the HECA-452 mAb can bind sulfo sLe^x determinant(s) which may serve as L-selectin ligands on HEV (12, 20) and rat endothelium at sites of inflammation (21). Since HEV-like vessels that support lymphocyte migration are observed in chronically inflamed nonlymphoid tissues, the current studies suggest that a subset of CLA determinants induced on vascular endothelial cells functions as L-selectin ligands.

Although CLA was an essential component of the vascular L-selectin ligand, CLA expression alone was not sufficient for L-selectin binding. Sulfation was also required since NaClO₃ treatment of 926-FtVII cells (Fig. 5 A) and activated HUVEC (Fig. 6 D) abrogated L-selectin-dependent attachment. In addition, vascular endothelial cells may be required to display CLA on specialized glycoprotein scaffolds (Fig. 7). This tripartite requirement for L-selectin ligand components may explain why 926-FtVII cells appeared to express CLA at high levels, but bound lower levels of L'IgM fusion protein (Figs. 1 and 4). Thus, L-selectin may only bind a subset of CLA that is appropriately sulfated or displayed by appropriate sulfated scaffolds. Similarly, activated HUVEC bound L'IgM well, yet HECA-452 staining was not dramatically increased after activation (Fig. 6, A and B). Thus, CLA expression on HUVEC may be restricted to a subset of cell surface structures that efficiently function as L-selectin ligands. Additionally, L-selectin may bind different vascular ligands with differing affinities. It is possible that the L'IgM fusion protein binds low-affinity L-selectin ligands which are unable to support cell rolling but are visualized during immunofluorescence staining experiments with the pentameric L'IgM fusion protein. There may also be a sharp threshold for ligand expression levels required for L-selectin-mediated attachment or roll-

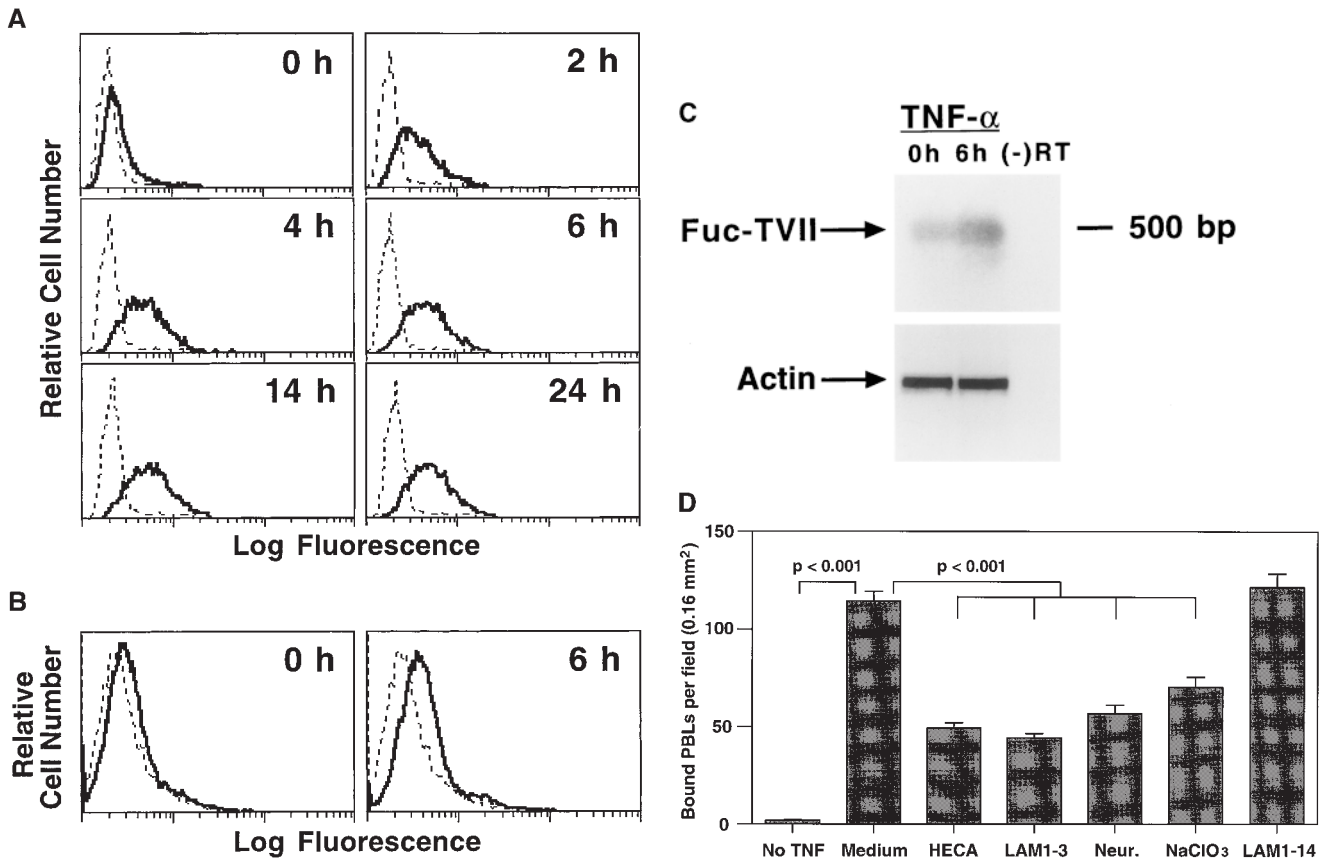


Figure 6. Functional L-selectin ligand expression by cytokine-activated endothelial cells. HUVEC monolayers cultured with medium (0 h) or TNF- α (100 U/ml) for the times indicated were stained in indirect immunofluorescence assays with (A) the L/IgM fusion protein (10 μ g/ml) or (B) the HECA-452 mAb (10 μ g/ml), and subsequently analyzed by flow cytometry. Dashed lines represent background staining with an unreactive mouse IgM (HB13b, 10 μ g/ml) or rat IgM (anti-mouse CD25, 20 μ g/ml) antibody. Identical background staining was obtained when L/IgM staining was carried out in the presence of 5 mM EDTA, with the CSLEX-1 mAb (20 μ g/ml), or with secondary antibody alone. Results represent those obtained in three experiments. (C) Fuc-TVII mRNA expression in resting and 6-h TNF- α -activated HUVEC. cDNA generated from $\sim 4 \times 10^5$ resting or TNF- α -activated (6 h) HUVEC was amplified in semiquantitative PCR assays. An autoradiograph of the Southern blot probed with a 32 P-labeled Fuc-TVII cDNA probe is shown. The (-)RT lane represents PCR amplification of control samples without cDNA added. PCR amplification of HUVEC mRNA samples in which the reverse transcriptase was omitted did not generate detectable bands (data not shown). Actin cDNA from $\sim 2 \times 10^5$ HUVEC was also PCR amplified as a control. Results represent those obtained in two experiments. (D) CLA mediates lymphocyte binding to TNF- α -activated HUVEC. HUVEC monolayers at $\sim 50\%$ confluence were cultured in medium alone or containing TNF- α (100 U/ml) for 6 h before pretreatment with medium, the HECA-452 mAb (10 μ g/ml), or neuraminidase (Neur.). Alternatively the cells were cultured previously with NaClO₃ for 24 h. Lymphocytes (PBL) were preincubated with either the LAM1-3 (50 μ g/ml) or the LAM1-14 (50 μ g/ml) mAbs for 20 min before being overlaid onto the HUVEC monolayers with lymphocyte binding assessed in a nonstatic binding assay at 4°C. Each mAb remained during the binding assay but was diluted by half. Values represent the mean (\pm SEM) of pooled numbers of bound lymphocytes in at least 60 randomly chosen microscopic fields obtained from at least three independent experiments.

ing that could make large differences in leukocyte binding to activated endothelium. Just two- to fivefold differences in L-selectin expression levels dramatically affect L-selectin binding activity for PSGL-1 *in vitro* and HEV *in vivo* (8, 53). Thus, upregulated expression of fucosyltransferases, sulfotransferases, sialyltransferases, or protein scaffolds could each contribute to the generation of functional L-selectin ligands *in vivo*.

The protein backbone of the vascular L-selectin ligand may be unknown. CLA displayed on PSGL-1 functions as the dominant L-selectin ligand on leukocytes (Tu, L., P.G. Murphy, and T.F. Tedder, manuscript in preparation), but PSGL-1 is not expressed by endothelial cells (31). CLA on endothelial cells is also unlikely to be presented by GlyCAM-1,

sgp200, or CD34. GlyCAM-1 lacks a transmembrane domain and is likely to be a secretory product of HEV found primarily in serum (54). The sgp200 HEV ligand may also be a secreted protein like GlyCAM-1 (6, 55). CD34 is only expressed by a small subset of HUVEC, is not upregulated by cytokines, and is OSGE-sensitive, whereas the vascular L-selectin ligand is OSGE-insensitive (Fig. 5, and reference 30). In addition, mice lacking CD34 are generally normal (33). Therefore, it is tempting to speculate that L-selectin will require or prefer CLA presentation by a specialized glycoprotein. Endothelial L-selectin ligands may also vary in differing vascular beds. For example, TNF induces L-selectin ligands on cultured bovine aortic endothelial cells that are not affected by neuraminidase treatment and appear to

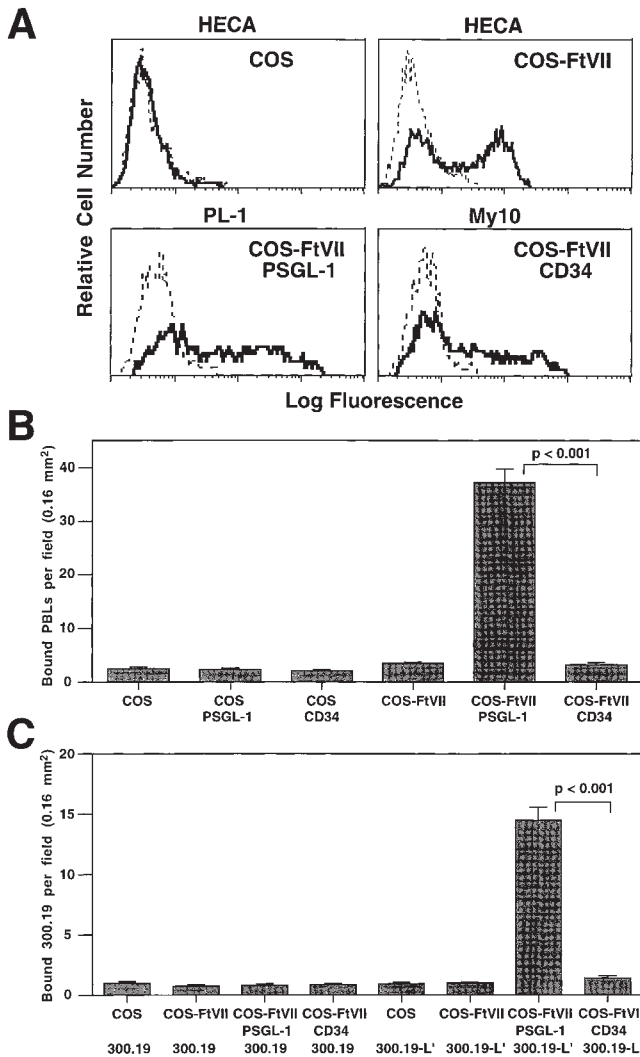


Figure 7. CLA expression alone is insufficient for L-selectin binding. (A) Staining of COS and COS-FtVII cells with the HECA-452 (10 $\mu\text{g}/\text{ml}$), PL-1 (anti-PSGL-1, 10 $\mu\text{g}/\text{ml}$), and My10 (anti-CD34, 10 $\mu\text{g}/\text{ml}$) mAbs in indirect immunofluorescence assays with flow cytometry analysis. Dashed lines represent cell staining with equivalent amounts of unreactive, isotype-matched control antibodies or with secondary antibody alone. (B) Binding of lymphocytes (PBLs) to COS or COS-FtVII cells transiently transfected with cDNAs encoding PSGL-1 or CD34 in a nonstatic binding assay. (C) Binding of 300.19 or 300.19-L' cells to COS or COS-FtVII cells transiently transfected with cDNAs encoding PSGL-1 or CD34 as indicated in a nonstatic binding assay. Values in B and C represent the mean (\pm SEM) number of lymphocytes bound to COS cells in at least 30 randomly chosen microscopic fields as previously described (8). All results represent those obtained in three independent experiments.

be related to heparan sulfate (29). Similarly, L-selectin ligands identified on human cardiac microvascular endothelial cells do not require sialic acid, but do require sulfation (56). Thus, the current studies will need to be extended to other vascular beds and the complete structure of the L-selectin ligand will need to be further characterized.

L-selectin ligands on HEV of lymphoid tissues historically have been identified by the MECA-79 mAb, although this antigen was not a component of the L-selectin ligand on human vascular endothelium. The MECA-79 mAb identifies a specific sulfation-dependent epitope on some carbohydrate side chains expressed by HEV (6, 15, 16, 19). These carbohydrates are also expressed on human venular endothelium at cutaneous lesions and sites of chronic inflammation (57, 58). The MECA-79 mAb blocks lymphocyte binding to murine peripheral lymph node HEV by 95% (15), and it inhibits lymphocyte migration into lymph nodes *in vivo* by $\sim 80\%$ (15, 59). However, in humans, the MECA-79 mAb only partially inhibits (30–50%) lymphocyte binding to HEV of peripheral lymph nodes (58, 60). In addition, OSGE-resistant L-selectin ligands distinct from the MECA-79-subset of sLe^x antigens are expressed by human HEV (60), a finding consistent with the observation that HEV of Fuc-TVII-deficient mice are unable to support L-selectin-mediated lymphocyte binding despite expressing the MECA-79 antigen at normal levels (18). Therefore, it is possible that the CLA-bearing vascular L-selectin ligand identified in this study may share functional and structural characteristics with the OSGE-resistant L-selectin ligands of HEV.

In summary, the current findings demonstrate that CLA provides a key component in the expression of L-selectin ligands on vascular endothelium that initiate leukocyte tethering and rolling. The association of CLA with the recruitment of lymphocytes to sites of inflammation in the skin (50) is consistent with a demonstrated role for L-selectin in mediating leukocyte migration to the skin during contact hypersensitivity responses and after allogeneic skin transplantation (61, 62). Moreover, CLA is expressed by vessels with the morphologic appearance of HEV in areas of extensive lymphoid infiltration in autoimmune thyroiditis, Graves and Hashimoto's disease, and in the gut of patients with Crohn's disease (50). Since the generation of CLA correlates with Fuc-TVII enzymatic activity and requires sulfation, this offers two potential sites for therapeutic regulation of L-selectin ligand expression during vascular inflammation.

We thank Ms. A. Miller for her considerable help and input into these studies, and Drs. C.-J. Edgell, B. Weston, L. Picker, K. Moore, R. McEver, S. Jalkanen, L. Robinson, B. Furie, X. Li, and D. Steeber for their help and reagents.

This work was supported by National Institutes of Health grants AI-26872, CA-54464, HL-50985, HL-53993, and HL-36028.

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Received for publication 29 May 1998 and in revised form 16 November 1998.

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