Novel chondroitin sulfate-modified ligands for L-selectin on lymph node high endothelial venules

Catherine J. Derry, Christelle Faveeuw, Kelly R. Mordsley and Ann Ager

Division of Cellular Immunology, National Institute for Medical Research, London, GB

The migration of lymphocytes into lymph nodes via high endothelial venules (HEV) is dependent on the expression of L-selectin on the lymphocyte cell surface. HEV express several L-selectin ligands including CD34, GlyCAM-1, MAdCAM-1 and two sulfated glycoproteins (Sgp) of 200 kDa and 170 kDa which remain to be identified. In this investigation, labeling with sodium [^{35}S]sulfate, which is incorporated into and forms part of the functional carbohydrate ligand, has been used to isolate and characterize macromolecular L-selectin ligands. High endothelial cells (HEC) cultured from rat lymph node HEV were shown to express ligands for L-selectin. HEC synthesized two groups of sulfated glycoproteins of 150 kDa and $>$200 kDa, which were present in conditioned medium. These coeluted on anion exchange chromatography at 1.0–1.2 M NaCl and supported calcium-dependent L-selectin-mediated cell adhesion. In common with known L-selectin ligands, Sgp 150/$>$200 were shown to be O-sialoglycoproteins; however, in contrast to other ligands, Sgp 150/$>$200 contained chondroitin sulfate glycosaminoglycan modifications which were required for L-selectin recognition. Chondroitin sulfate-modified ligands for L-selectin were expressed at the HEC surface and by HEV in lymph nodes, suggesting that they may participate in lymphocyte interactions with HEV *in vivo*.

Received	11/5/98
Revised	9/9/98
Accepted	4/11/98

Key words: L-selectin ligand / High endothelial venule / Lymphocyte adhesion

1 Introduction

The cells of the immune system are unique in their ability to migrate around the body, ensuring continuous immune surveillance. In particular, naive lymphocytes constitutively migrate from the blood into lymph nodes (LN), where they encounter and respond to antigen. Initial identification of L-selectin as the peripheral LN homing receptor [1] has been confirmed in L-selectin-deficient mice, which have severely reduced levels of lymphocyte migration to peripheral LN and an inability to mount immune responses in these organs [2, 3]. L-selectin is also required for migration of lymphocytes to mesenteric lymph nodes and, to a lesser extent, Peyer's patches [4]. The predominant function of the three members of the selectin family of adhesion molecules L-, E- and P-selectin, is to mediate the tethering and rolling step of leukocyte binding to endothelium. This is the first of a series of interactions that constitute the adhesion cascade, allowing leukocytes to bind and transmigrate across endothelium into the underlying tissue [5].

L-selectin, in common with E- and P-selectin, contains an amino terminal C-type lectin domain, that binds carbohydrate ligands in a calcium-dependent manner [5]. In LN, this interaction occurs between L-selectin-positive naive lymphocytes and ligands expressed on the luminal surface of specialized high endothelial venules (HEV). HEV have been shown to synthesize several ligands for L-selectin: a specific glycoform of CD34 [6], glycosylation-dependent cell adhesion molecule (GlyCAM)-1 [7] and a specific glycoform of MAdCAM-1 [8]. In addition, several other sulfated glycoproteins (Sgp) have been shown to bind to soluble forms of L-selectin: in the mouse glycoproteins of 200 kDa and 170 kDa [9] and in the rat glycoproteins of 55, 65, 120, 190, $>$250 kDa and $>$500 kDa [10]. However, none of these molecules have been fully characterized to date.

CD34, GlyCAM-1 and Sgp 200 all contain sulfate-modified carbohydrate groups and sulfation is required for L-selectin recognition [9, 11]. In this study, we have

[I 18465]

Abbreviations: GAG: Glycosaminoglycan **GlyCAM-1:** Glycosylation-dependent cell adhesion molecule-1 **HEC:** High endothelial cells **HEV:** High endothelial venules **OSGP:** O-sialoglycoprotein endopeptidase **Sgp:** Sulfated glycoprotein

0014-2980/99/0202-419$17.50+.50/0

used metabolic labeling with $Na_2{}^{35}SO_4$ to isolate sulfated L-selectin ligands. High endothelial cells (HEC) cultured from rat LN HEV were used as a source of L-selectin ligands. We demonstrate here that HEC express ligands for L-selectin. Specifically, sulfated glycoproteins of 150 kDa and >200 kDa have been co-purified from HEC-conditioned media and shown to support calcium-dependent, L-selectin-mediated cell adhesion. Sgp 150/>200 contain chondroitin sulfate glycosaminoglycan (GAG) modifications which are required for L-selectin recognition and L-selectin ligands with these modifications are expressed on the surface of HEC and by HEV in LN.

2 Results

2.1 Expression of L-selectin ligands by cultured HEC

To determine whether cultured HEC express ligands for L-selectin, binding of a soluble L-selectin fusion protein (LEC-Ig) to confluent monolayers of HEC was measured by ELISA (Fig. 1). VCAM-Ig was used as a control since HEC do not express α4 integrins [12], and therefore should not bind VCAM-Ig. LEC-Ig bound to HEC at 8–14-fold higher levels than VCAM-Ig. Absorbance values were low (<0.03) for VCAM-Ig and were not affected by the various treatments used to detect L-selectin-dependent binding. The absorbance values, of identically treated HEC, for VCAM-Ig were subtracted from those for LEC-Ig to give specific LEC-Ig binding. LEC-Ig bound to HEC in an L-selectin-dependent manner, inhibitable by a blocking antibody to mouse L-selectin, MEL-14 (69 % inhibition). The interaction was inhibited by the L-selectin ligand mimics, fucoidan (74 %) and PPME (65 %), and by the Ca^{2+} chelator EGTA (99 %). LAM101, a non-blocking antibody [13], had no effect on binding of LEC-Ig and the results were pooled with those of untreated HEC. Similar results were obtained in ELISA on three different primary cultures of HEC, and at passage numbers up to 25, and therefore the data were pooled (Fig. 1). HEC were lightly fixed to prevent loss of HEC during the multiple incubation and washing steps for the ELISA. Viable, unfixed HEC gave similar levels of specific LEC-Ig binding (data not shown), indicating that the fixation step did not affect detection of ligand.

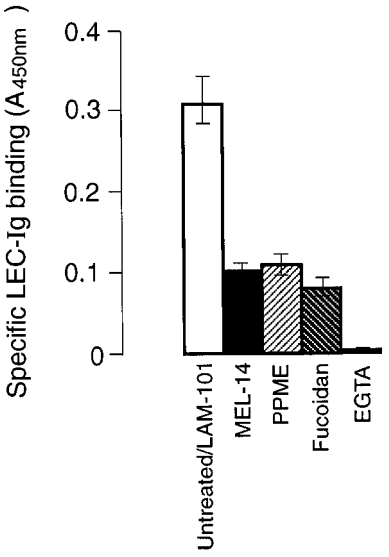

Figure 1. Binding of soluble L-selectin to cultured HEC. Confluent primary cultures of HEC were fixed in 0.04 % glutaraldehyde and incubated with an L-selectin fusion protein (LEC-Ig) or a control fusion protein (VCAM-Ig) at 50 μg/ml, in the presence or absence of LAM1–101 (200 μg/ml), MEL 14 (200 μg/ml), fucoidan (500 μg/ml), PPME (500 μg/ml) or EGTA (10 mM). Absorbance values for VCAM-Ig were subtracted from those for LEC-Ig to give specific LEC-Ig binding. Assays were performed two to three times and pooled results were expressed as absorbance at 450 nm ± SEM (n = 8–12). MEL-14, fucoidan, PPME and EGTA all significantly ($p<0.001$) reduced LEC-Ig binding in comparison with untreated/LAM-101 samples.

2.2 Metabolic labeling with $Na_2{}^{35}SO_4$ and isolation sulfated glycoproteins

Since sulfation is required to form a functional carbohydrate ligand, we metabolically labeled HEC with $Na_2{}^{35}SO_4$, isolated labeled metabolites and determined whether they supported L-selectin-dependent cell adhesion. SDS-PAGE and PhosphorImager analysis of supernatants harvested from $Na_2{}^{35}SO_4$-labeled HEC revealed the presence of several $Na_2{}^{35}SO_4$-labeled macromolecules (Fig. 2 A). These separated into two major groups on SDS PAGE: one of approximately 150 kDa and the other with >200 kDa.

Anion exchange chromatography of HEC supernatant demonstrated one peak of $Na_2{}^{35}SO_4$-labeled molecules (Fig. 2 B). This eluted in the range 1.0–1.2 M NaCl and SDS-PAGE analysis revealed that it contained several $Na_2{}^{35}SO_4$-labeled macromolecules. The major species were approximately 150 kDa and >200 kDa, which corresponded to the high molecular mass species seen prior to anion exchange chromatography (Fig. 2 A). Analysis of individual fractions by SDS-PAGE showed the presence of both 150 kDa and >200 kDa molecules in each fraction. Silver staining of SDS-PAGE gels revealed the absence of detectable protein following anion exchange chromatography. Since it was likely that these molecules were heavily glycosylated and therefore diffi-

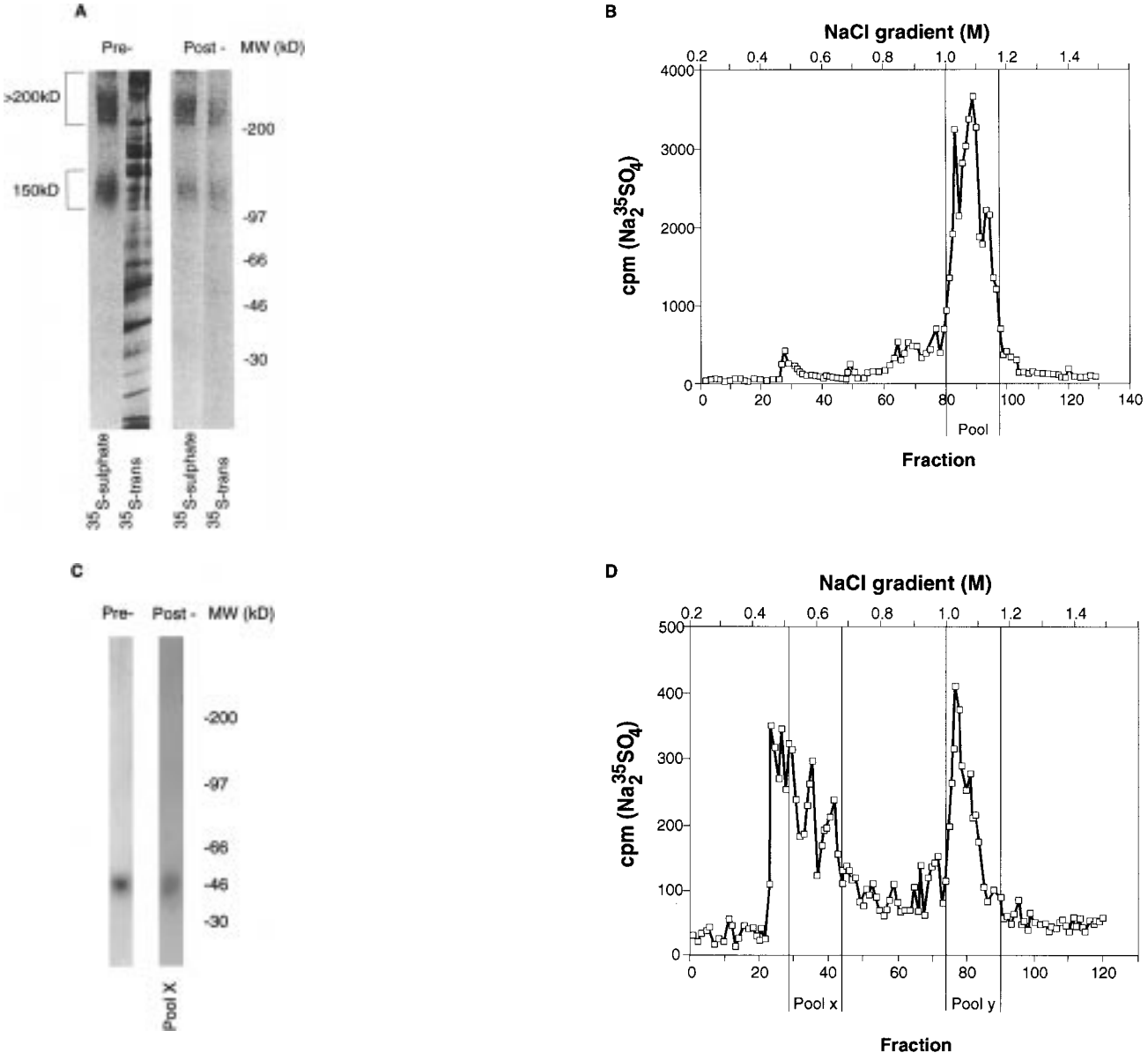

Figure 2. Analysis of HEC and LN extracts following metabolic labeling. (A) Cultured HEC were labeled for 16 h with 50 μCi/ml $Na_2{}^{35}SO_4$ or TRAN^{35}S-LABEL™ and media collected. Labeled components (20 000 cpm/lane) were analyzed by non-reducing 5–15 % gradient SDS-PAGE before separation (Pre-) and following separation (Post-) by anion exchange chromatography. (B) $Na_2{}^{35}SO_4$-labeled supernatants were separated on a Hi Trap Q anion exchange cartridge (Pharmacia). Bound material was eluted with a continuous salt gradient (0.2–1.5 M NaCl). Fractions were analyzed for incorporation of $Na_2{}^{35}SO_4$ (cpm) and marked fractions pooled for SDS-PAGE analysis. (C) LN slices were labeled for 16 h with 50 μCi/ml $Na_2{}^{35}SO_4$ and media analyzed by SDS-PAGE before separation (Pre-). Pool x was separated by anion exchange chromatography (Post-). (D) $Na_2{}^{35}SO_4$-labeled supernatants were separated by anion exchange chromatography as described in (B) and marked fractions pooled for SDS-PAGE analysis.

cult to detect by silver staining, we used TRAN^{35}S LABEL™ to label the protein backbone in order to assess purity. As shown in Fig. 2 A, two groups of proteins eluted at 1.0 – 1.2 M NaCl. These corresponded to the $^{35}SO_4$-labeled species of 150 kDa and $>$200 kDa. By analogy with the nomenclature used in the mouse [9], the high molecular mass molecules will be referred to as sulfated glycoproteins (Sgp) 150/$>$200. On Western blotting, purified Sgp150/$>$200 was not detected by anti-CAMO2, an antibody to a GlyCAM-1 peptide (data not shown).

2.3 Isolation of GlyCAM-1 from peripheral lymph nodes

GlyCAM-1, a soluble ligand for L-selectin, was isolated for direct comparison with Sgp 150/>200. Since GlyCAM-1 is anionic, we used anion exchange chromatography as a first step for isolation. LN synthesized one major component of 46 kDa (Fig. 2 C). This corresponds to the molecular mass of the rat L-selectin ligand, GlyCAM-1 [14].

Two peaks of $Na_2{}^{35}SO_4$-labeled molecules were separated by anion exchange chromatography of LN supernatants (Fig. 2 D). One eluted in the range 0.5–0.7 M NaCl (Pool x) and the other in the range 1.0–1.2 M NaCl (Pool y). Pool x contained a $Na_2{}^{35}SO_4$-labeled molecule of 46 kDa which corresponded to the major metabolite seen prior to anion exchange chromatography (Fig. 2 C). Pool y contained several $Na_2{}^{35}SO_4$-labeled macromolecules which ran as a smear on SDS-PAGE and were >150 kDa. The latter molecules were present in trace amounts in unpurified supernatants (data not shown). Because the high molecular mass species did not give a clear banding pattern on SDS-PAGE they were not analyzed further.

The identity of the 46 kDa molecule in Pool x was confirmed as rat GlyCAM-1 by Western blotting with an antibody raised to a C-terminal peptide of GlyCAM-1 [14] (data not shown). The quantity of GlyCAM-1 in Pool x, as measured by $Na_2{}^{35}SO_4$-labeling, was enriched approximately tenfold by purification on a wheat germ agglutinin affinity column, which has been shown to bind GlyCAM-1 [7]. This step was used to enrich GlyCAM-1 following anion exchange chromatography.

2.4 Adhesion of L-selectin positive cells to immobilized ligands

To determine whether Sgp150/>200 contain ligands for L-selectin, an adhesion assay was established using GlyCAM-1 as a positive control. Binding of the mouse EL4 lymphoma which had been stably transfected with cDNA encoding full length rat L-selectin (RM1 cells) [10] was compared with that of untransfected EL4 cells. At least 85 % of RM1 cells expressed cell surfce rat L-selectin, as detected by flow cytometry. EL4 cells did not express endogenous L-selectin (data not shown). The adhesion of RM1 cells to immobilized GlyCAM-1 was four- to sixfold higher than that of EL4 cells (Fig. 3). RM1 cell binding to GlyCAM-1 was reduced to the background level of EL4 cells in the presence of an antibody to rat L-selectin, HRL-1, which blocks L-selectin ligand interactions [10]. In contrast, HRL-2, a non-blocking antibody to rat L-selectin, did not inhibit binding of L-selectin-positive cells. The interaction of L-selectin-positive cells with GlyCAM-1 was completely inhibited in the presence of the Ca^{2+} chelator, EGTA. Similarly, inclusion of the L-selectin ligand mimic fucoidan prevented cell adhesion to GlyCAM-1 (Fig. 3). The binding of EL4 cells to GlyCAM-1 represented nonspecific adhesion, since EL4 cells showed similar levels of binding to an irrelevant protein (BSA: 66 ± 8 cells/mm^2; GlyCAM-1: 70 ± 8 cells/mm^2).

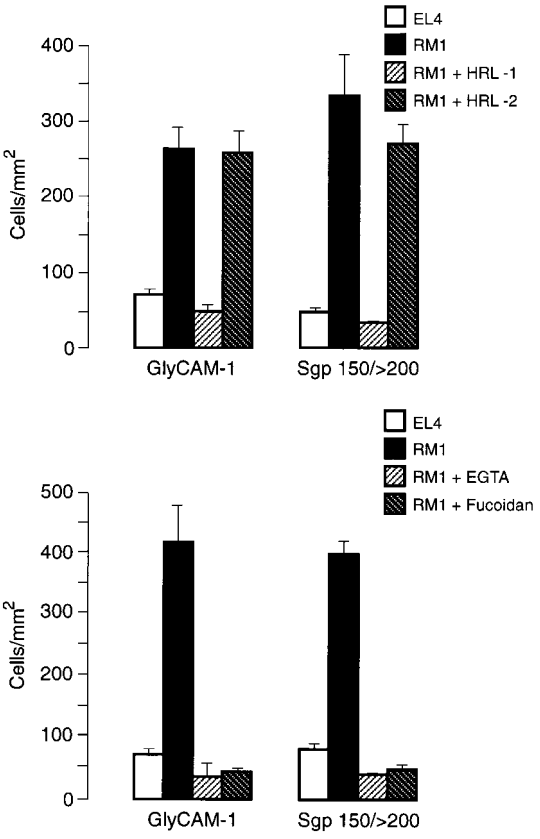

Figure 3. Adhesion of lymphoid cells to Sgp 150/>200. EL4 (L-selectin$^-$) or RM1 cells (L-selectin$^+$) were allowed to adhere to immobilized Sgp 150/>200 for 30 min at 4°C with rotation. Cells were preincubated for 30 min at 4 °C in the presence or absence of blocking (HRL-1) or non-blocking (HRL-2) antibodies to rat L-selectin at 50 μg/ml, fucoidan (500 μg/ml) or EGTA (10 mM). Adherent cells were counted by light microscopy and pooled results are expressed as cells bound/mm^2 ± SEM (n = 9). HRL-1, EGTA and fucoidan all significantly ($p < 0.001$) inhibited binding of RM1 cells to GlyCAM-1 and Sgp150/>200. HRL-2 had no significant effect.

The adhesion of L-selectin-positive RM1 cells to Sgp150/>200 was six- to sevenfold higher than that of EL4 cells (Fig. 3). As found for RM1 cell binding to

GlyCAM-1, this interaction was inhibited by fucoidan, EGTA and the antibody HRL1, but not HRL-2. There was no change in morphology, or clumping, of adherent EL4 compared with RM1 cells, or when RM1 cells were preincubated with antibodies to rat L-selectin. L-selectin-negative EL4 cells showed similar levels of binding to BSA and purified Sgp150/>200, indicating nonspecific adhesion (BSA: 66 ± 8 cells/mm^2; Sgp150/>200: 76 ± 10 cells/mm^2). Since Sgp150/>200 was purified from HEC conditioned medium, we checked whether FCS contained similar L-selectin ligands. An equivalent volume of FCS containing media was fractionated by anion exchange chromatography and the fractions eluting at 1.0–1.2 M NaCl pooled, dialyzed and tested in an adhesion assay. There was no difference in the binding of RM1 and EL4 cells (data not shown), indicating that the source of Sgp150/>200 was indeed cultured HEC.

2.5 Characterization of Sgp150/>200 as chondroitin sulfate modified O-sialoglycoproteins

Since carbohydrate modifications, including sulfate incorporation, are important for L-selectin/ligand interactions, we compared Sgp150/>200 with GlyCAM-1 for sensitivity to various proteases and glycosidases (Fig. 4). As found previously, GlyCAM-1 was digested by trypsin and O-sialoglycoprotein endopeptidase (OSGP), an enzyme that selectively degrades O-sialoglycoproteins. GlyCAM-1 was not affected by the GAG degrading enzymes chondroitinases ABC or ACI or by heparinases I or III (Fig. 4 a) [15].

Like GlyCAM-1, Sgp150/>200 were sensitive to trypsin and OSGP (Fig. 4 b). Increases in the mobility of digested components on SDS-PAGE were observed following both trypsin and OSGP treatment. Trypsin-digested material separated on SDS-PAGE was detectable by silver staining and even after 3 h digestion of Sgp150/>200 there were still large molecules (>50 kDa) present (data not shown) that might support binding of L-selectin. In contrast to GlyCAM-1, Sgp150/>200 was degraded by chondroitinase ABC (Fig. 4 b). On SDS-PAGE analysis, this was seen as complete removal of incorporated $Na_2{}^{35}SO_4$. Chondroitinase ACI, which is more selective for chondroitin sulfate, also completely removed incorporated $Na_2{}^{35}SO_4$. However, treatment with heparinase I or III had no effect on the migration pattern of Sgp150/>200 on SDS-PAGE (Fig. 4 b). Activity of

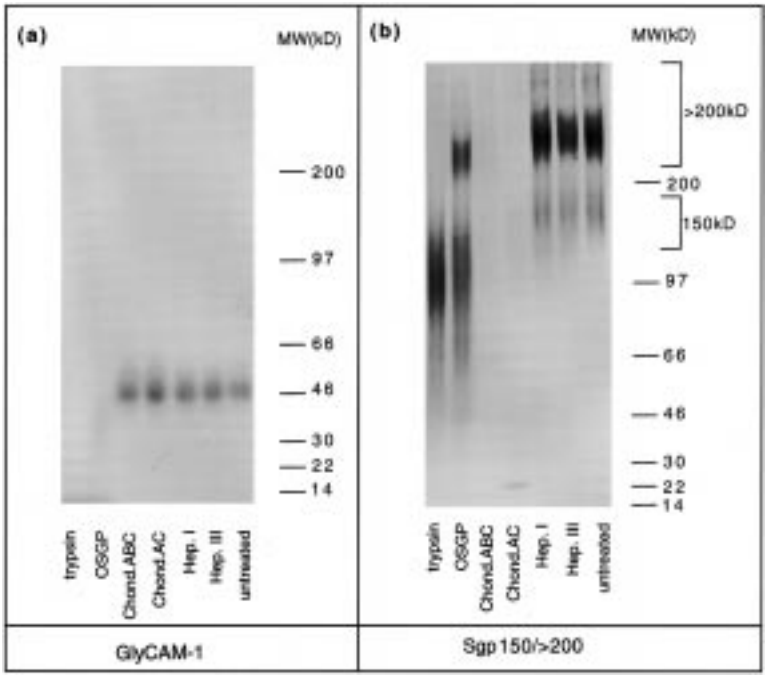

Figure 4. SDS-PAGE analysis of isolated L-selectin ligands digested with proteolytic or glycosaminoglycan degrading enzymes. $Na_2{}^{35}SO_4$-labeled L-selectin ligands, (a) GlyCAM-1 and (**b**) Sgp150/>200, were treated with the following enzymes at 37 °C for 3 h: trypsin (50 μg/ml), OSGP (500 μg/ml), chondroitinase ABC (Chond. ABC) (0.05 U/ml) and chondroitinase ACI (Chond. AC) (0.05 U/ml), heparinase I (Hep. I) (5 U/ml) and heparinase III (Hep. III (2.5 U/ml). Digested samples were analyzed by 5–15 % gradient SDS-PAGE, and gels dried for PhosphorImager analysis.

the heparinases was confirmed by test digestions of heparan sulfate for heparinase I and heparin for heparinase III, according to the manufacturer's protocol (Sigma, Poole, Dorset, GB), and was measured as 200 U/mg for heparinase I and 300 U/mg for heparinase III. The lack of effect of chondroitinases and heparinases on GlyCAM-1 demonstrates the lack of proteolytic activity in the glycosidases used. Having identified Sgp 150/>200 as chondroitin sulfate-modified proteins, the BlySCAN dye-binding assay was used to determine the yield of Sgp150/>200 from HEC at 5 $\mu g/10^7$ cells/16 h.

To determine the importance of chondroitin sulfate modifications for Sgp150/>200 function, the effect of chondroitinase digestion of immobilized Sgp150/>200 on the adhesion of L-selectin-positive cells was determined (Fig. 5). Chondroitinase ABC inhibited RM1 cell binding by 84 % and chondroitinase ACI inhibited binding by 64 %. The chondroitin sulfate-modified proteoglycan biglycan was tested for L-selectin ligand activity. As shown in Fig. 5 there was no specific binding of L-selectin transfectants to biglycan.

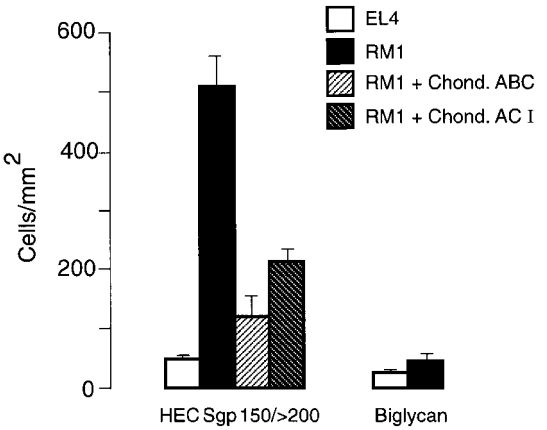

Figure 5. Adhesion of lymphoid cells to chondroitinase digested L-selectin ligands. EL4 and RM1 cells were allowed to adhere to immobilized Sgp150/>200 or Biglycan with rotation. Sgp150/>200 were pretreated for 3 h at 37 °C with chondroitinase ABC (Chond. ABC) or chondroitinase ACI (Chond. ACI) at 0.05 U/ml. Results are as given in legend to Fig. 3. Chondroitinase treatment of Sgp150/>200 significantly ($p < 0.001$) reduced binding of RM1 cells. The binding of EL4 and RM1 cells to biglycan were not significantly different.

2.6 Cell-associated chondroitin sulfate-modified L-selectin ligands are expressed by cultured HEC and in lymph node HEV

Chondroitin sulfate-modified L-selectin ligands were isolated from HEC-conditioned media. To determine whether chondroitinase sensitive ligands for L-selectin are expressed at the cell surface, HEC were treated with chondroitinases ABC or ACI for 3 h prior to measurement of specific LEC-Ig binding. As shown in Fig. 6 A, chondroitinase ABC reduced LEC-Ig binding by 54 % and chondroitinase ACI by 50 %. Trypsin pretreatment reduced LEC-Ig binding by 66 %.

To determine whether chondroitin sulfate-modified L-selectin ligands are expressed by HEV in lymph nodes, the Stamper Woodruff frozen section assay was performed. The blocking antibody to rat L-selectin HRL-1 abolished the binding of lymphocytes to HEV, whereas the non-blocking antibody HRL-2 had no effect as found previously [10] (data not shown). Sections were pretreated with chrondroitinases for the shorter period of 1 h to ensure maintenance of tissue architecture for subsequent image analysis. Chondroitinase ABC or ACI reduced the binding of lymphocytes to HEV, relative to untreated sections, as demonstrated by confocal microscopy (Fig. 6 C). The efficacy of chondroitinase ABC digestion was demonstrated by staining with 2B6 (data not shown), a monoclonal antibody that recognizes the 4,5 unsaturated saccharides formed after chondroitinase ABC digestion [16], and which has been shown to bind to chondroitinase ABC-treated rat LN HEC [17]. Quantitation of lymphocyte binding revealed a decrease in the number of HEV with 6 or more lymphocytes bound and an increase in the number with 0–5 lymphocytes bound. The effects of chondroitinase ABC and ACI were similar giving increases of 59 and 68 %, respectively, in the number of HEV with 0–5 lymphocytes bound, decreases of 34 and 40 %, respectively, in the number of HEV with 6–15 lymphocytes bound and 50 or 55 %, respectively, in the number of HEV with more that 15 lymphocytes bound (Fig. 6 B).

3 Discussion

In this study we have used GAG degrading enzymes to demonstrate novel ligands for L-selectin containing functional chondroitin sulfate modifications. Three separate assays have been used: lymphocyte binding to HEV, binding of soluble L-selectin to cultured HEC and lymphocyte binding to isolated ligands. Two groups of chondroitin sulfate-modified glycoproteins of 150 kDa and >200 kDa were co-purified from HEC conditioned media at 1.0–1.2 M NaCl and shown to support L-selectin-dependent adhesion. Identical analysis of conditioned media from LN organ cultures demonstrated sulfated macromolecules of >150 kDa which eluted at 1.0–1.2 M NaCl, but these species did not give a clear banding pattern on SDS-PAGE. Sulfated macromole-

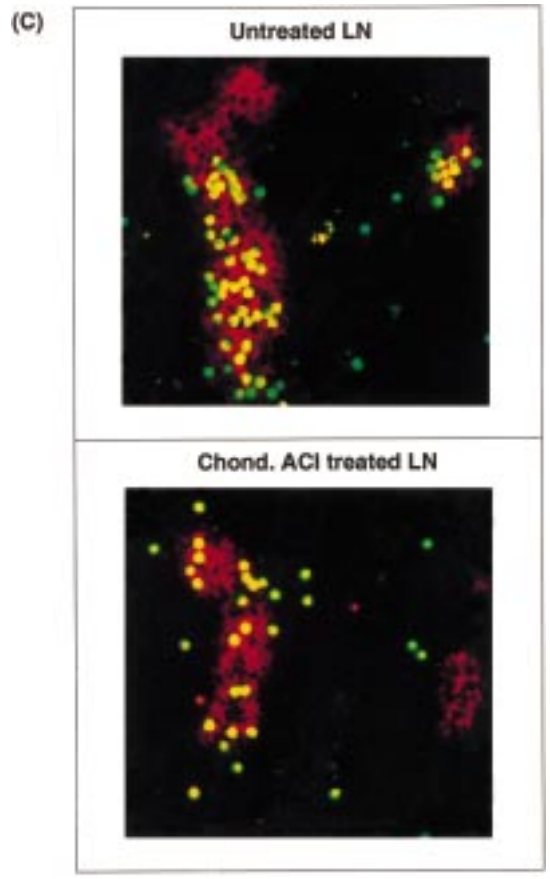

Figure 6. Cell associated chondroitin sulfate-modified ligands for L-selectin. (A) Cultured HEC were fixed and pretreated with chondroitinase ABC (Chond. ABC) or ACI (Chond. ACI) at 0.05 U/ml or trypsin at 50 μg/ml for 3 h at 37 °C prior to measuring specific LEC-Ig binding as described in legend to Fig. 1. All enzyme treatments significantly (***$p < 0.001$) reduced LEC-Ig binding. (B) LN sections were incubated at 37 °C for 1 h in the presence or absence of Chond. ABC or Chond. ACI at 0.25 U/ml prior to the adhesion assay. Lymphocytes bound to HEV were counted by light microscopy. Pooled results from three experiments were expressed as the percentage of HEV with 0–5, 6–15 and > 15 cells bound ± SEM. ***$p < 0.001$ and *$p < 0.05$ relative to untreated sections. (C) Confocal microscope images of CFSE-labeled lymphocytes adhering to untreated or Chond. ACI-treated LN sections. HEV were stained with a GlyCAM-1 antisera, anti-CAMO2. Magnification x200.

cules of > 150 kDa, which ran as a smear on SDS-PAGE, were also isolated from detergent lysates of cultured HEC (C. Derry, K. Mordsley and A. Ager, unpublished). Therefore, further studies are required to determine whether the chondroitin sulfate-modified ligands expressed by HEV and at the HEC surface contain Sgp150/> 200. Identification of the protein backbone(s) of Sgp150/> 200 will assist in this analysis and also resolve the issue of whether Sgp150 and Sgp> 200 represent one or more proteins with chondroitin sulfate modifications.

Thus far, the best characterized ligand for L-selectin is GlyCAM-1 and a major carbohydrate capping group which demonstrates L-selectin ligand function is 6-sulfated sialyl Lewis x [18]. The insensitivity of GlyCAM-1 to chondroitinases demonstrates clearly that the carbohydrate ligands identified in this study are distinct from those presented by GlyCAM-1. L-selectin ligand function in all assays was equally sensitive to degradation by chondroitinases ABC and ACI suggesting that the functional GAG modifications are unlikely to be dermatan sulfate (chondroitin sulfate B). The relative contribution of

chondroitin-4-sulfate (A) and chondroitin-6-sulfate (C) to L-selectin ligand function remain to be determined. Early studies failed to identify a role for chondroitin sulfate in L-selectin-dependent assays. Chondroitin sulfate from bovine trachea did not block lymphocyte adhesion to HEV in the frozen section assay [19], binding of anti-L-selectin antibodies to lymphocytes [20] or migration of lymphocytes into LN [21]. In this study, biglycan from bovine articular cartilage, a 200–350 kDa proteoglycan containing chondroitin sulfate side chains did not support L-selectin-dependent adhesion. Together, these results suggest that chondroitin sulfate *per se* does not necessarily have L-selectin ligand function. The precise composition of the GAG side chains (*e.g.* chondroitin A *vs.* C), the protein backbone on which it is presented and the degree of clustering of the GAG side chains on individual proteins may all contribute to generating a functional ligand. Sulfation is just one of several requirements for L-selectin ligand function. The others are sialylation and fucosylation, as demonstrated by the lack of L-selectin ligand in HEV of the fucosyltransferase VII knockout mouse [22]. All three are contained within the carbohydrate (6-sulfated sialyl Lewis x) modifications on GlyCAM-1 [18]. But, by analogy with PSGL-1 where the protein backbone rather than the oligosaccharide is sulfated [23], sulfation, sialylation and/or fucosylation of Sgp150/>200 could occur in different areas of the molecule. Chondroitin sulfate, although clearly an important constituent, may therefore constitute just a part of the functional carbohydrate ligand.

The relationship between Sgp150/>200 and other L-selectin ligands expressed by HEV, such as mouse Sgp 170 and Sgp 200, remain to be determined. Like Sgp150/>200, mouse Sgp 170 and 200 exist in soluble forms [24]. MECA 79, a rat anti-mouse mAb that recognizes sulfate modifications on L-selectin ligands, binds to mouse Sgp 200 and blocks L-selectin-dependent recognition [9], but the sugar modifications in Sgp 200 have not yet been characterized. MECA 79 does not detect sulfated ligands in the rat and therefore cannot be used to study Sgp150/>200. Non-lymphoid endothelial cells have been shown to express GAG-modified ligands for L-selectin. Norgard-Sumnicht et al. [15, 25] demonstrated heparinase, but not chondroitinase sensitive, proteoglycans synthesized by cultured calf pulmonary aortic endothelial (CPAE) cells and human umbilical vein endothelial cells (HUVEC), which are also secreted into the medium. These high molecular mass (>200 kDa) heparan sulfate-modified proteoglycans bind LEC-Ig in a calcium-dependent manner. CPAE ligands migrated on SDS-PAGE as a broad smear between 50–100 kDa with a second band at >200 kDa, whereas HUVEC showed broadly smeared material of >200 kDa. Interestingly, the interaction between CPAE-derived ligands and L-selectin was dependent on the presence of the heparan sulfate GAG chains [15, 25]. Similarly, Giuffrè et al. [26] demonstrated heparinase, but not chondroitinase sensitive, ligands for L-selectin that supported monocyte adhesion to activated bovine aortic endothelium under flow. Thus, HEC-derived Sgp150/>200 is clearly distinct from heparan sulfate modified L-selectin ligands synthesized by non-lymphoid endothelial cells.

Miyasaka et al. [10] have shown that an L-selectin fusion protein will bind to HEC-derived sulfated molecules of similar molecular mass to those identified in this study. In addition to confirming that soluble L-selectin detects Sgp150/>200, it would be interesting to determine whether Sgp150/>200 contains podocalyxin. The human homologue, podocalyxin – like protein, has recently been identified as a 160 kDa L-selectin ligand [27]. Although we did not identify Sgp150/>200 expressed by cultured HEC, it was clear that these cells to not synthesize GlyCAM-1. GlyCAM-1 expression by LN HEV is down-regulated by ligation of afferent lymphatics [28] and also 3–4 days following antigen activation [24]. In contrast to HEV in LN, cultured HEC are maintained in isolation from an intact LN microenvironment and the cells are rapidly proliferating [32], a response that is only seen in HEV after antigen activation [29]. The fact that cultured HEC do express macromolecular ligands for L-selectin ([10] and this study) indicates that the expression of individual L-selectin ligands is independently regulated, as previously shown for GlyCAM-1 and Sgp 200 [24].

It is clear from this and previous studies that chondroitin sulfate-modified ligands represent just one of several L-selectin ligands expressed by HEV in LN. Interestingly, L-selectin ligands are either soluble (GlyCAM-1 [7]), cell associated (CD34 [6]) or both (Sgp200 [24]). GlyCAM-1 up-regulates integrin function on naive lymphocytes [30] and thus could activate these cells, perhaps in combination with chemokines on the surface of HEV, to convert rolling to stable adhesion from flow. Cell associated chondroitin sulfate-modified L-selectin ligands could play a key role in presenting chemokines to rolling lymphocytes [31].

4 Materials and methods

4.1 Cell culture

Individual primary cultures of HEC were isolated from cervical LN of single AO(RT1^u) rats and cultured as previously described [32]. Three different HEC primary cultures were used at between passage number 5 and 25. EL4, a mouse T

cell lymphoma and RM1, the parent EL4 cell line transfected with rat L-selectin [10] were kindly provided by Dr. Masa Miyasaka (Osaka University, Japan).

4.2 Reagents

The following Ab to L-selectin were used: MEL-14 (rat IgG2a) from ATCC; LAM101 (rat IgG2a) from Dr. T. Tedder [13] (Duke University Medical Center, Durham, NC); HRL-1 and HRL-2 from Pharmingen (San Diego, CA). GlyCAM-1 was detected using anti-CAM02 from Steve Rosen [7]. An antibody, 2B6, that detects chondroitin-4-sulfate "stubs" after digestion with Chondroitinase ABC [16], was from ICN (Thame, GB). Fucoidan and biglycan (B8041) were from Sigma (Poole, Dorset, GB) and polyphosphomannan (PPME) was a kind gift of Dr. Chris Parish (University of Canberra, Australia). The following enzymes were used: Chondroitinases ABC (EC. 4.2.2.4) and ACI (EC. 4.2.2.5) from ICN (Thame, GB); Heparinases I (EC. 4.2.2.7) and III (EC. 4.2.2.8) and trypsin (EC. 3.4.21.4) from Sigma (Poole, Dorset, GB); O-sialoglycoprotein endopeptidase (OSGP) (EC. 3.4.24.57) from Cedarlane Labs (Hornby, Ontario, Canada). The L-selectin fusion protein [33] was kindly provided by Dr. Larry Lasky (Genentech, CA) and VCAM-Ig by Dr. Roy Lobb (Biogen, Cambridge, MA) [34]. D-PBS is defined as 0.9 mM Ca^{2+} and 0.49 mM Mg^{2+} containing PBS. All other reagents were purchased from standard laboratory suppliers.

4.3 $Na_2{}^{35}SO_4$ and TRAN^{35}S-LABEL^{TM} metabolic labeling and isolation of ^{35}S-labeled material

Confluent HEC monolayers (approximately 7×10^6 cells in a 175-mm^2 tissue culture flask) or 2–3 mm slices of pooled axillary, brachial and cervical LN from five 6–8-week-old LOU ($RT1^u$) rats (1.5–2.0 g in a 75-mm^2 tissue culture flask), were each incubated overnight in 20 ml RPMI containing 2 % FCS and 50 μCi/ml $Na_2{}^{35}SO_4$ or 50 μCi/ml TRAN^{35}S-LABEL^{TM} (ICN, Thame, GB). Labeled supernatants were harvested and analyzed by SDS-PAGE or dialyzed against 75 mM Tris, pH 7.7 for anion exchange chromatography.

4.4 Anion exchange chromatography

Labeled supernatants, containing 0.2 M NaCl, were recirculated for 2 h through HiTrap Q columns (Pharmacia Biotech, St. Albans), which had been preequilibrated in 75 mM Tris, pH 7.7 containing 0.2 M NaCl, before washing off unbound material. Bound material was eluted with a linear gradient of 0.2–1.5 M NaCl and ^{35}S-label in 1.0 ml fractions measured on a β-counter (Wallac 1205, Milton Keynes, GB). Collected fractions were dialyzed against PBS for SDS-PAGE and for adhesion assays.

4.5 Wheat germ agglutinin affinity chromatography

Wheat germ agglutinin conjugated to agarose beads (Vector, Burlington, CA) was used to enrich further LN anion exchange purified material for GlyCAM-1 [7]. Bound material was eluted with 0.2 M N-acetylglucosamine (Sigma, Poole, Dorset, GB) in PBS. Collected fractions were analyzed for ^{35}S-label by β-counting and by SDS-PAGE. The presence of GlyCAM-1 in LN preparations was confirmed by Western blotting with an antibody raised to a C-terminal GlyCAM-1 peptide, anti-CAMO2 [7].

4.6 SDS-PAGE

^{35}S-labeled components were separated by non-reducing SDS-PAGE on a Hoefer SE400 gel apparatus (Hoefer, San Francisco, CA) using a gradient of 5–15 % polyacrylamide. Gels were dried and exposed on a PhosphorImager (Molecular Dynamics, Chesham, GB) overnight to reveal the presence of ^{35}S-labeled components. Densitometry analysis was carried out using ImageQuant software (Molecular Dynamics, Chesham, GB). In some experiments, equal aliquots of ^{35}S-labeled material were treated with one of the following enzymes at 37 °C for 3 h: trypsin (50 μg/ml), OSGP (500 μg/ml), chondroitinase ABC (0.05 U/ml), chondroitinase ACI (0.05 U/ml), heparinase I (5 U/ml) or heparinase III (2.5 U/ml). Reactions were stopped by acetone precipitation, and precipitates redissolved in 50 μl of Laemlli buffer for SDS-PAGE analysis.

4.7 BlySCAN assays

The BlySCAN assay is a quantitative dye-binding method for the *in vitro* analysis of proteoglycans and sulfated GAG. The method is based on the specific binding of the cationic dye 1,9-dimethylmethylene blue to sulfated GAG and was used here to determine the concentration of isolated Sgp150/$>$200. Assays were performed according to manufacturer's instruction (Biocolor Ltd., Belfast, GB). A standard curve, from 0–100 μg/ml, was constructed using the chondroitin sulfate standard provided.

4.8 Lymphocyte adhesion to immobilized ligands

Triplicate wells of PTFE coated multispot slides (Hendley, Loughton, Essex, GB) were coated with 50 μl/well of purified Sgp150/$>$200 at 9 μg/ml overnight at +4 °C. GlyCAM-1, 50 μl/well, was coated at a concentration of approximately 100-fold that of original supernatant. Sgp150/$>$200 was digested for 3 h using 0.05 U/ml chondroitinases at 37 °C, and slides were washed three times with PBS. Unbound sites were blocked by treatment with 1 % BSA in D-PBS, for 1 h at 22 °C. Slides were cooled to +4 °C, and 100 μl of EL4 and RM1 cells (10^6 cells) in 1 % FCS/RPMI pipetted onto each well. Cells treated with antibodies, fucoidan or EGTA

were preincubated with the appropriate concentration of inhibitor, for 0.5 h at +4 °C, prior to inclusion in the assay. Slides were rotated at 65 rpm for 30 min at 4°C and unbound cells removed by two to three washes in PBS. The number of cells bound was counted under phase contrast microscopy (Olympus CK2 microscope with a x20 objective) using a graticule with an area of 0.137 mm^2. Each assay was performed three times. Pooled results are expressed as cells bound/mm^2 ± SEM (n = 9). Percentage inhibition of adhesion was calculated following subtraction of nonspecific binding to BSA blocked wells.

4.9 Binding of LEC-Ig to cultured HEC by ELISA

HEC were grown to confluence in Nunc 96-well plates (Life Technologies, Glasgow, GB), fixed in 0.04 % glutaraldehyde in D-PBS for 10 min, and stored for up to 7 days at 4 °C. Nonspecific binding sites were blocked with 1 % BSA in D-PBS for 1 h at 22 °C (300 μl/well). HEC were incubated in quadruplicate with 50 μg/ml LEC-Ig or VCAM-Ig in D-PBS containing 1 % BSA (50 μl/well), in the presence or absence of L-selectin inhibitors for 1 h at 22 °C. Plates were washed three times with D-PBS, and incubated for 1 h in HRPO-conjugated $F(ab')_2$ fragments of rabbit anti-human IgG at 1 in 500 (Dako, Glostrup, Denmark) (50 μl/well). After washing, bound enzyme was detected using 0.1 mg/ml 3,3',5,5'-tetramethylbenzidine (TMB) in 0.05 M phosphate-citrate buffer, pH 5.0 (50 μl/well), the reaction stopped using 50 μl 2 M H_2SO_4/well and absorbance measured at 450 nm. Calcium-dependent binding of LEC-Ig to cultured HEC was detected by co-incubation with 10 mM EGTA. Lectin-dependent binding was determined by preincubation of LEC-Ig with 500 μg/ml fucoidan or PPME, or with 50 μg/ml MEL-14 or LAM101 for 30 min at room temperature, prior to inclusion in the assay. For enzyme treatments, fixed HEC were pretreated with chondroitinase ABC (0.05 U/ml), chondroitinase ACI (0.05 U/ml) or trypsin (50 μg/ml) for 3 h at 37 °C prior to the blocking step. Absorbance values for identically treated HEC using VCAM-Ig were subtracted from those for LEC-Ig to give specific LEC-Ig binding. Each assay was performed two to three times, and pooled results were expressed as absorbance at 450 nm ± SEM (n = 8–12).

4.10 Lymphocyte-HEV adhesion assay

The Stamper Woodruff frozen section assay was performed as described previously [35] with some modifications. Three to six 8 μm cryostat sections of rat cervical LN were air dried onto each well of PTFE-coated multispot slides (Hendley, Loughton, Essex, GB) and stored for up to 2 weeks at – 70 °C. Triplicate wells were digested at 37 °C for 1 h using 0.25 U/ml chondroitinase ABC or ACI. To check for chondroitinase ABC digestion, treated sections were stained with 200 μg/ml 2B6, and bound antibody detected with Texas Red-conjugated anti-mouse IgG at 20 μg/ml (Southern Biotechnology, Birmingham, AL).

LN sections were washed once in D- PBS, cooled to +4 °C and 100 μl of rat LN lymphocytes (3×10^6 cells) in 1 % FCS/RPMI were allowed to adhere at 4 °C for 30 min with rotation at 65 rpm. Nonadherent cells were gently tipped off and bound cells fixed in 2 % formalin in D-PBS. Sections were counterstained with 1 % methyl green/2 % pyronin and cells bound to HEV counted by light microscopy (Olympus BH2 microscope with a x20 objective). Pooled results from three experiments were expressed as the percentage of HEV with 0–5, 6–15 and >15 lymphocytes (± SEM) bound, thus taking into account the different sizes of HEV present in LN cryostat sections. At least 100 HEV were counted per experiment, with a total count of 364 HEV for untreated sections and 350 or 364 HEV for chondroitinase ACI or chondroitinase ABC treated sections respectively.

For confocal microscopy, rat lymphocytes were labeled at 5×10^7/ml with the green fluorescent dye CFSE [5(6)-carboxyfluorescein diacetate, succinimidyl ester] (Molecular Probes, Leiden, The Netherlands) at 2 μM in Ca^{2+}- and Mg^{2+}-free PBS, for 15 min at 37 °C. After fixing in 2 % formalin, HEV were stained with the GlyCAM-1 antisera, anti-CAMO2 at 250 μg/ml, for 1 h at 22 °C and bound antibody was detected with Texas Red-conjugated anti-rabbit IgG at 20 μg/ml (Southern Biotechnology, Birmingham, AL). Slides were viewed on the MRC 600 confocal microscope (Bio-Rad, Hemel Hempstead, GB).

4.11 Statistical analysis

Data are presented as mean ± SEM. A Student's t-test was used for multiple comparisons.

Acknowledgements: We are grateful to the following people for poviding reagents: Larry Lasky, Roy Lobb, Masa Miyasaka, Chris Parish, Steve Rosen and Tom Tedder. We would like to acknowledge the expertise of the Photo-Graphics department at the NIMR and the technical assistance of Graham Preece, Cellular Immunology, NIMR and Liz Hirst, Membrane Biology, NIMR. Our thanks also go to Charles Pusey, Helen Yarwood and Rose Zamosyka for helpful comments on this manuscript. This work was funded by the Medical Research Council (GB).

5 References

1 **Gallatin, W. M., Weissman, I. L. and Butcher E. C.,** A cell-surface molecule involved in organ-specific homing of lymphocytes. *Nature* 1983. **304:** 30–34.

2 **Arbones, M. L., Ord, D. C., Ley, K., Ratech, H., Maynard, C. C., Otten, G., Capon, D. J. and Tedder, T. F.,** Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin-deficient mice. *Immunity* 1994. **1:** 247–260.

3 **Xu, J., Grewal, I. S., Geba, G. P. and Flavell, R. A.,** Impaired primary T cell responses in L-selectin-deficient mice. *J. Exp. Med.* 1996. **183:** 589–598.

4 **Hamann, A., Jablonski-Westrich, D. and Thiele, H. G.,** Homing receptors re-examined: mouse LECAM-1 (MEL-14 antigen) is involved in lymphocyte migration into gut-associated lymphoid tissue. *Eur. J. Immunol.* 1991. **21:** 2925–2929.

5 **Kansas, G. S.,** Selectins and their ligands: Current concepts and controversies. *Blood* 1996. **88:** 3259–3287.

6 **Baumhueter, S., Singer, M. S., Henzel, W., Hemmerich, S., Renz, M., Rosen, S. D. and Lasky, L. A.,** Binding of L-selectin to the vascular sialomucin CD34. *Science* 1993. **262:** 436–438.

7 **Lasky, L. A., Singer, M. S., Dowbenko, D., Imai, Y., Henzel, W. J., Grimley, C., Fennie, C., Gillett, N., Watson, S. R. and Rosen, S. D.,** An endothelial ligand for L-selectin is a novel mucin-like molecule. *Cell* 1992. **69:** 927–938.

8 **Berg, E. L., McEvoy, L. M., Berlin, C., Bargatze, R. F. and Butcher, E. C.,** L-selectin-mediated lymphocyte rolling on MAdCAM-1. *Nature* 1993. **366:** 695–698.

9 **Hemmerich, S., Butcher, E. C. and Rosen, S. D.,** Sulfation-dependent recognition of high endothelial venules (HEV) – ligands by L-selectin and MECA 79, an adhesion – blocking monoclonal antibody. *J. Exp. Med.* 1994. **180:** 2219–2226.

10 **Tamatani, T., Kuida, K., Watanabe, T., Koike, S. and Miyasaka, M.,** Molecular mechanisms underlying lymphocyte recirculation: III. Characterization of the LECAM-1 (L-selectin) – dependent adhesion pathway in rats. *J. Immunol.* 1993. **150:** 1735–1745.

11 **Imai, Y., Lasky, L. A. and Rosen, S. D.,** Sulphation requirement for GlyCAM-1, an endothelial ligand for L-selectin. *Nature* 1993. **361:** 555–557.

12 **Hourihan, H., Allen, T. D. and Ager, A.,** Lymphocyte migration across high endothelium is associated with increases in alpha4beta1 integrin (VLA-4) affinity. *J. Cell Sci.* 1993. **104:** 1049–1059.

13 **Steeber, D. A., Engel, P., Miller, A. S., Sheetz, M. P. and Tedder, T.,** Ligation of L-selectin through conserved regions within the lectin domain activate signal transduction pathways and integrin function in human, mouse and rat lymphocytes. *J. Immunol.* 1997. **159:** 952–963.

14 **Dowbenko, D., Watson, S. R. and Lasky, L. A.,** Cloning of a rat homologue of mouse GlyCAM-1 reveals conservation of structural domains. *J. Biol. Chem.* 1993. **268:** 14399–14403.

15 **Norgard-Sumnicht, K. E., Varki, N. M. and Varki, A.,** Calcium-dependent heparin-like ligands for L-selectin in nonlymphoid endothelial cells. *Science* 1993. **261:** 480–483.

16 **Couchman, J. R., Caterson, B., Christner, J. E. and Baker, J. R.,** Mapping by monoclonal antibody detection of glycosaminoglycans in connective tissues. *Nature* 1984. **307:** 650–652.

17 **Aruffo, A., Stamenkovic, I., Melnick, M., Underhill, C. B. and Seed, B.,** CD44 is the principal cell surface receptor for hyaluronate. *Cell* 1990. **61:** 1303–1313.

18 **Hemmerich, S. and Rosen, S. D.,** 6'-Sulfated sialyl Lewis x is a major capping group of GlyCAM-1. *Biochemistry* 1994. **33:** 4830–4835.

19 **Yednock, T. A., Stoolman, L. M. and Rosen, S. D.,** Phosphomannosyl-derivatized beads detect a receptor involved in lymphocyte homing. *J. Cell Biol.* 1987. **104:** 713–723.

20 **Yednock, T. A, Butcher, E. C., Stoolman, L. M. and Rosen, S. D.,** Receptors involved in lymphocyte homing: relationship between a carbohydrate-binding receptor and the MEL-14 antigen. *J. Cell Biol.* 1987. **104:** 725–731.

21 **Brenan, M. and Parish, C. R.,** Modification of lymphocyte migration by sulphated polysaccharides. *Eur. J. Immunol.* 1986. **16:** 423–430.

22 **Maly, P., Thall, A. D., Petryniak, B., Rogers, C. E., Smith, P. L., Marks, R. M., Kelly, R. J., Gersten, K. M., Cheng, G., Saunders, T. L., Camper, S. A., Camphausen, R. T., Sullivan, F. X., Isogai, Y., Hindsgaul, O., Von-Andrian, U. H. and Lowe, J. B.,** The alpha(1,3)fucosyltransferase Fuc-TVII controls leukocyte trafficking through an essential role in L-, E-, and P-selectin ligand biosynthesis. *Cell* 1996. **86:** 643–653.

23 **Pouyani, T. and Seed, B.,** PSGL-1 recognition of P-selectin is controlled by a tyrosine sulphation consensus at the PSGL-1 amino terminus. *Cell* 1995. **83:** 333–343.

24 **Hoke, D., Mebius, R. E., Dybdal, N., Dowbenko, D., Gribling, P., Kyle, C., Baumhueter, S. and Watson, S. R.,** Selective modulation of the expression of L-selectin ligands by an immune response. *Curr. Biol.* 1995. **5:** 670–678.

25 **Norgard-Sumnicht, K. and Varki, A.,** Endothelial heparan sulfate proteoglycans that bind to L-selectin have glucosamine residues with unsubstituted amino groups. *J. Biol. Chem.* 1995. **270:** 12012–12024.

26 **Giuffrè, L., Cordey, A. S., Monai, N., Tardy, Y., Schapira, M. and Spertini, O.,** Monocyte adhesion to activated aortic endothelium: Role of L-selectin and heparan sulfate proteoglycans. *J. Cell Biol.* 1997. **136:** 945–956.

27 **Sassetti, C., Tangemann, K., Singer, M. S., Kershaw, D. B. and Rosen, S. D.,** Identification of podocalyxin-like protein as a high endothelial venule ligand for L-selectin: parallels to CD34. *J. Exp. Med.* 1998. **187:** 1965–1975.

28 **Mebius, R. E., Dowbenko, D., Williams, A., Fennie, C., Lasky, L. A. and Watson, S. R.,** Expression of GlyCAM-1, an endothelial ligand for L-selectin, is affected by afferent lymph flow. *J. Immunol.* 1993. **151:** 6769–6776.

29 **Anderson, N. D., Anderson, A. O. and Wyllie, R. G.,** Microvascular changes in lymph nodes draining skin allografts. *Am. J. Path.* 1975. **81:** 131–153.

30 **Hwang, S. T., Singer, M. S., Giblin, P. A., Yednock, T. A., Bacon, K. B., Simon, S. I. and Rosen, S. D.,** GlyCAM-1, a physiologic ligand for L-selectin, activates beta2 integrins on naive peripheral lymphocytes. *J. Exp. Med.* 1996. **184:** 1343–1348.

31 **Tanaka, T., Adams, D. H., Hubscher, S., Hirano, H., Siebenlist, U. and Shaw, S.,** T-cell adhesion induced by proteoglycan-immobilized cytokine MIP-1β. *Nature* 1993. **61:** 79–82.

32 **Ager, A.,** Isolation and culture of high endothelial cells from rat lymph nodes. *J. Cell Sci.* 1987. **87:** 133–144.

33 **Watson, S. R., Imai, Y., Fennie, C., Geoffroy, J. S., Rosen, S. D. and Lasky, L. A.,** A homing receptor – IgG chimera as a probe for adhesive ligands of lymph node high endothelial venules. *J. Cell Biol.* 1990. **110:** 2221–2229.

34 **Jakubowski, A., Rosa, M. D., Bixler, S., Lobb, R. and Burkly, L. C.,** Vascular Cell Adhesion Molecule (VCAM)-Ig fusion protein defines distinct affinity states of the Very Late Antigen-4 (VLA-4) receptor. *Cell Adh. Commun.* 1995. **3:** 131–142.

35 **Ager, A. and Humphries, M. J.,** Use of synthetic peptides to probe lymphocyte-high endothelial cell interactions. Lymphocytes recognise a ligand on the endothelial surface which contains the CS1 adhesion motif. *Int. Immunol.* 1990. **2:** 921–928.

Correspondence: Ann Ager, Division of Cellular Immunology, National Institue for Medical Research, The Ridgeway, Mill Hill, London, NW7 1AA, GB
Fax: 0181 913 8529
E-mail: a-ager@nimr.mrc.ac.uk
C. J. Derry's present address: Infection and Immunity Research Group, Division of Life Sciences, Kings College, Campden Hill Rd, Kensington, London, W8 7AH. E-mail: catherine.derry@kcl.ac.uk