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## High Endothelial Venules of the Lymph Nodes Express Fas Ligand

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**SUMMARY** Fas (CD95, APO-1) is widely expressed on lymphatic cells, and by interacting with its natural ligand (Fas-L), Fas induces apoptosis through a complex caspase cascade. In this study we sought to survey Fas-L expression in vascular and sinusoidal structures of human reactive lymph nodes. Immunohistochemical Fas-L expression was present in all paracortical high endothelial venules (HEVs), in cells lining the marginal sinus wall, and in a few lymphocytes, but only occasionally in non-HEV vascular endothelium. In the paracortical zone over 60% of all vessels and all paracortical HEVs showed Fas-L expression, whereas in the medullary zone less than 10% of the blood vessels were stained with Fas-L. Normal vessels outside lymph nodes mostly showed no Fas-L expression. We show that in human reactive lymph nodes Fas-L expression is predominantly present in HEVs. Because the circulating lymphocytes gain entry to nodal parenchyma by transendothelial migration through HEVs, the suggested physiological importance of Fas-L expression in these vessels lies in the regulation of lymphocyte access to lymph node parenchyma by possibly inducing Fas/Fas-L mediated apoptosis of activated Fas-expressing lymphoid cells. The Fas-L expressing cells in the marginal sinus might have a similar function for cells accessing the node in afferent lymph. (*J Histochem Cytochem* 52:693–699, 2004)

**KEY WORDS**

Fas-L  
CD95-L  
HEV  
lymph node  
immune privilege

Fas (CD95, APO-1) is a type I transmembrane glycoprotein that is a member of the tumor necrosis factor (TNF) superfamily. It is widely expressed on various types of cells, being found on lymphatic cells such as activated B- and T-cells, NK-cells, and myeloid cells (Itoh et al. 1991; Iwai et al. 1994). Only a minority of resting peripheral blood lymphocytes express Fas (Yoshino et al. 1994). Interacting with its natural ligand (Fas-L), Fas induces apoptosis through a complex caspase cascade (Pinkoski et al. 2000; Wajant 2002; Lee and Ferguson 2003). Fas-L is a TNF-related type II transmembrane molecule (Krammer 1999), which is expressed not only by activated T-cells and NK-cells (Nagata and Golstein 1995) but also by activated macrophages (Bradley et al. 1996), neutrophils (Suda et al. 1993; Liles et al. 1996), and some epithelial cells (Pinkoski et al. 2000).

Fas-mediated apoptosis has a crucial role in the downregulation of immunological reactions. The process of downregulation starts when lymphocytes are activated. When activated lymphocytes begin to express both Fas and Fas-L, the process is called activation-induced cell death (AICD), meaning that AICD cells are predetermined to go through apoptosis sometime after activation (Lynch et al. 1995). Fas-L expression is not restricted to lymphocytes only; it is also expressed by Sertoli cells (Bellgrau et al. 1995) in testis and by epithelial cells of the eye (Griffith et al. 1995). These sites are said to be “immune privileged” sites because of their ability to kill activated lymphocytes via Fas-mediated apoptosis. By eliminating lymphocytes, the eye and testis avoid immune reaction, which could damage the tissue (Abbas 1996; Lee and Ferguson 2003). In the endothelium of blood vessels, Fas-L expression regulates the extravasation of leukocytes (Sata and Walsh 1998). Endothelial overexpression may be a factor that confers protection from atherosclerosis in transplanted vessels (Wang and Adegboyega 2002).

Lymphocyte homing to peripheral lymph nodes is a

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Received for publication September 3, 2003; accepted January 14, 2004 (3A6163).

highly regulated process that occurs exclusively in specialized high endothelial venules (HEVs) in the nodal paracortex (von Andrian and M'Rini 1998). HEVs are lined by tall cuboidal rather than the usual flat endothelial cells. On their surface HEVs also express specific lymphocyte-binding molecules known as addressins, which enable lymphocytes to bind to the endothelium as the first step of migrating across into the tissue (Mebius et al. 1991).

In this study we sought to survey the Fas-L expression in vascular structures of human lymph nodes with different types of reactive alterations. We were able to demonstrate that Fas-L expression is predominantly present in the HEVs, thus providing these vessels a potential means for a selective exclusion of Fas-expressing lymphocytes entering the lymph node parenchyma. Moreover, we showed Fas-L to be expressed in the mononuclear phagocytic cells lining the parenchymal site of the subcortical sinus, suggesting their role in a similar protective activity against Fas-expressing cells in the afferent lymph.

## Patients and Methods

### Patients

Lymph node samples from 12 patients (aged 1–73 years, mean 38.0 years; eight males, four females) representing different types of reactive alterations were selected from the files of the Department of Pathology, University of Oulu. Lymph nodes had all been enlarged and removed for diagnostic purposes. Their anatomic distribution was varied, including neck, axilla, abdominal cavity, and inguinal region. Three of the patients were under 18 years, five were 19–55 years, and four were over 55 years. Material included six paracortical hyperplasias, one sinus histiosytosis, three follicular hyperplasias, and two cases with a mixed reaction.

### Immunohistochemistry

Immunohistochemical (IHC) staining for Fas-L was performed on 4- $\mu$ m sections of formalin-fixed, paraffin-embedded tissue section using a modified DAKO EnVision (DAKO; Glostrup, Denmark) detection system kit with 3,3'-diaminobenzidine (DAB) as a chromogen. Sections were pretreated by microwaves (150 W) for 15 min in Tris-EDTA (pH 9.00). After cooling at RT for 20 min, the sections were washed in PBS-Tween (PBST) for 10 min, treated with peroxidase blocking solution for 15 min, and washed again in PBST for 10 min. Primary Fas-L antibody (mouse monoclo-

nal, clone G247-4; Pharmingen, San Diego, CA) was diluted 1:1500 in PBS and incubated overnight at 4C. For demonstration of factor VIII (von Willebrand factor, rabbit antihuman, code No. A 0082; DAKO) immunoreactivity, the sections were treated as for Fas-L staining but the antibody was diluted 1:5000 and incubated for 60 min at 37C. After a 10-min washing procedure, visualization was performed using EnVision solution for 30 min and the sections were washed in PBST for 15 min with three changes. DAB was incubated for 5 min and slides were washed again for 10 min. For CD68 IHC, a LabVision Autostainer (Labvision; Fremont, CA) was used with CD68 (clone KP-1, mouse monoclonal, code No. M0814; DAKO) diluted 1:600 and pretreated with 0.4% pepsin for 1/2 hour at 37C. For Fas-L and FVIII IHC stainings counterstaining was performed with Methyl Green and in CD68 with Meyers HTX. For negative control stainings, primary antibody was omitted or an irrelevant antibody was used. Specimens from testis were used as positive controls.

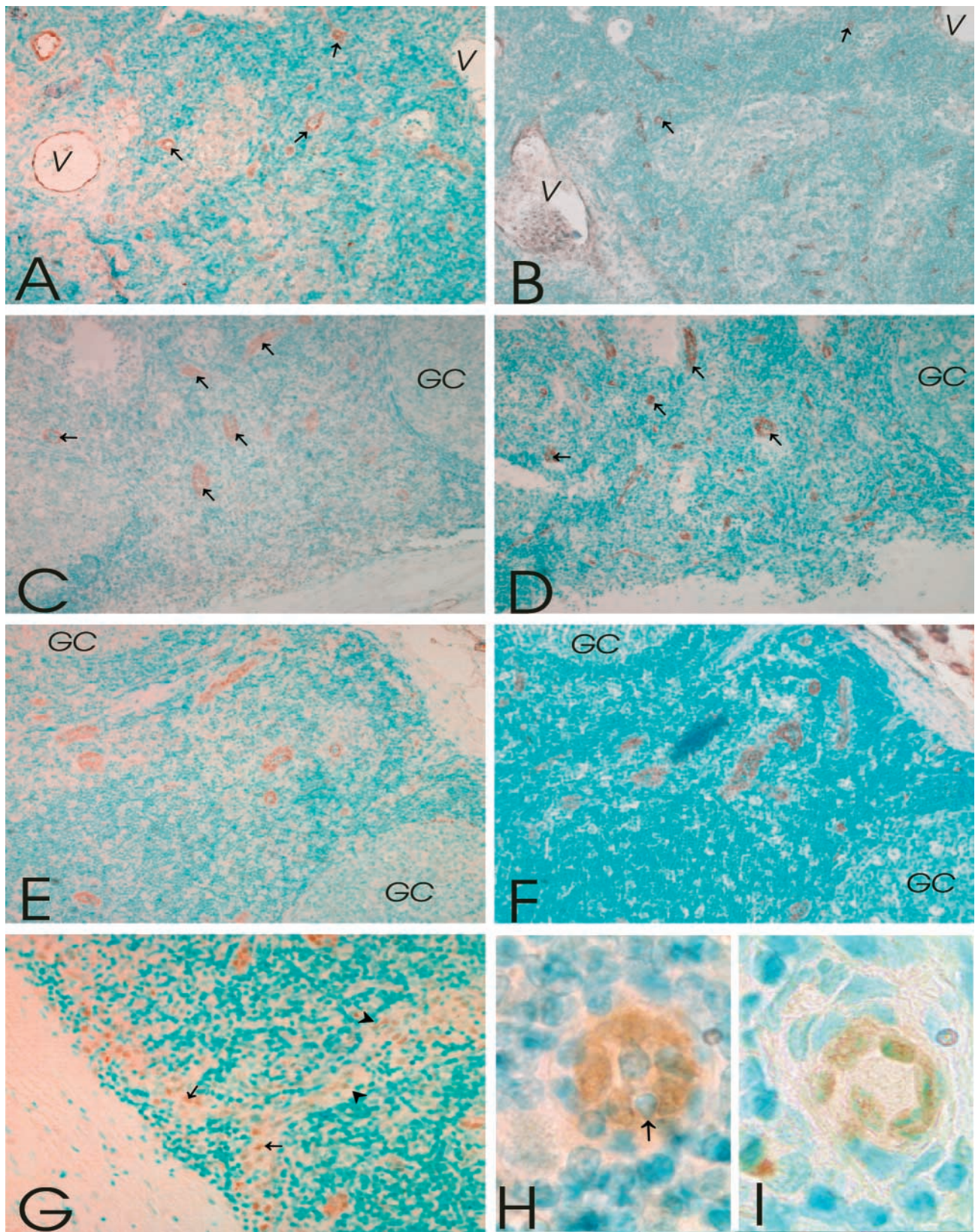
### Evaluation of the Stainings

For evaluation of the proportion of blood vessels expressing Fas-L in different regions and reaction types of lymph nodes, the area density of blood vessels showing expression was counted by using an ocular graticule with a  $10 \times 10$  grid and  $\times 40$  magnification, the counting area being 0.0625 mm<sup>2</sup>. At least three fields were counted in each case and in four regions: paracortex, medulla, germinal centers, and perifollicular area in the cortex. The total density of blood vessels was counted similarly by using FVIII-stained sections, and the proportion of the vessels with Fas-L expression was calculated for each area. To see whether there was a difference in Fas-L expression among different types of blood vessels, we classified the vessels as HEV or non-HEV (including capillaries, arteries, and veins in the lymph node). HEVs were identified by the cuboidal shape of their endothelial cells (Figure 1H). We also estimated the staining intensity in each vessel type by using a scale of negative (-), weak (+), moderate (++), and strong intensity of staining (+++). Strong intensity was the same as the most intensive staining found in positive lymphocytes and it also served as a positive control in each section. For evaluation of reproducibility of vessel counts, five cases were assessed twice. The correlation between two HEV counts was 0.74, indicating a reasonable reproducibility.

### Statistical Evaluation

The staining patterns in the different reaction types of lymph node were compared with the nonparametric Kruskal-Wallis test, and the staining patterns among the different ana-

**Figure 1** IHC stainings of reactive lymph nodes showing immunoreactivity for Fas-Ligand (A,C,E,H,I) or Factor VIII (B,D,F). A–B, C–D, and E–F each form a pair representing the same patient and the same area. A and B show the medullary zone. There are more FVIII<sup>+</sup> vessels (B) than Fas-L<sup>+</sup> ones (A). V marks two non-HEV vessels to indicate a reference point in two serial sections, and some HEV vessels are marked with arrows. C and D show the paracortical area, arrows indicating HEVs. In this area the density of Fas<sup>+</sup> vessels is similar to that of FVIII<sup>+</sup> vessels. Germinal centers (GC) show no positive vessels in either staining. E and F show another paracortical area in the cortex demonstrating a similar extent of expression of Fas-L<sup>+</sup> and FVIII<sup>+</sup> vessels. In G, Fas-L expression is seen in the cells lining the inner wall of a marginal sinus (arrows) and extending to the cortical sinus (arrowheads). H and I show the difference in intensity of expression of Fas-L in HEVs (H) and a



vessel with low endothelium (I), the latter showing less expression than the HEV. In the wall of HEV there is also a lymphocyte (arrow) showing no expression of Fas-L. Magnifications: A-F  $\times 10$ ; G  $\times 20$ ; H,I  $\times 100$ .

tomic regions of lymph nodes were compared with Wilcoxon's rank sum test by using SPSS v. 10.1 (SPSS; Chicago, IL). Nonparametric tests were used due to skewness of the distributions.

## Results

In the reactive lymph nodes occasional lymphocytes or sinusoidal macrophages expressing Fas-L were found in 12/15 samples. Three samples showed no positive structures for any Fas-L, even in repeated IHC stainings, and they were excluded from further analysis. Absence of any immunoreactivity for Fas-L in these three cases was not related to the age of the patients, anatomic location, or any reaction type.

In all cases showing expression of Fas-L in lymphoid cells, the antigen was also expressed in a selection of endothelial cells (Figure 1). The intensity of overall Fas-L staining in different types of blood vessels in lymph nodes and their vicinity is summarized in Table 1. There were no Fas-L-expressing vessels in the germinal centers or in the perifollicular areas, and only a minority of extracapsular vessels showed expression. Most Fas-L<sup>+</sup> vessels appeared in the paracortical region and had the morphology of high endothelial venules; the difference of HEVs and other vessels is shown in Figures 1H and 1I. HEVs showed most constant expression and the intensity in these vessels was higher than in the other vessels, ranging from weak to moderate in the HEVs and negative to weak in vessels with low endothelium (Table 1). The staining intensity in the HEVs was similar to that in Sertoli cells in testicular tissue, which was used as a positive control (not shown). In the endothelial cells, staining was diffuse in the cytoplasm, with accentuation on luminal plasma membrane in some cells. In addition, Fas-L expression was occasionally present in cells lining the inner wall of the marginal sinus (Figure 1G). These cells did not show any FVIII positivity but their distribution corresponded to that of CD68<sup>+</sup> cells seen in serial sections, suggesting that these cells are of mononuclear phagocyte lineage.

**Table 1** The intensity of the overall Fas-L staining in different types of vessels in the lymph nodes and their vicinity on the scale negative (-), weak (+), moderate (++), and strong (+++). Strong intensity was the same as the maximal lymphocyte staining intensity. HEV were not present (NP) in some areas

	HEV	non-HEV
Lymph node		
Germinal center	NP	-
Perifollicular zone	NP	-
Paracortex	- to ++	- to +
Medulla	- to +	- to +
Extranodal vessels	NP	- to +

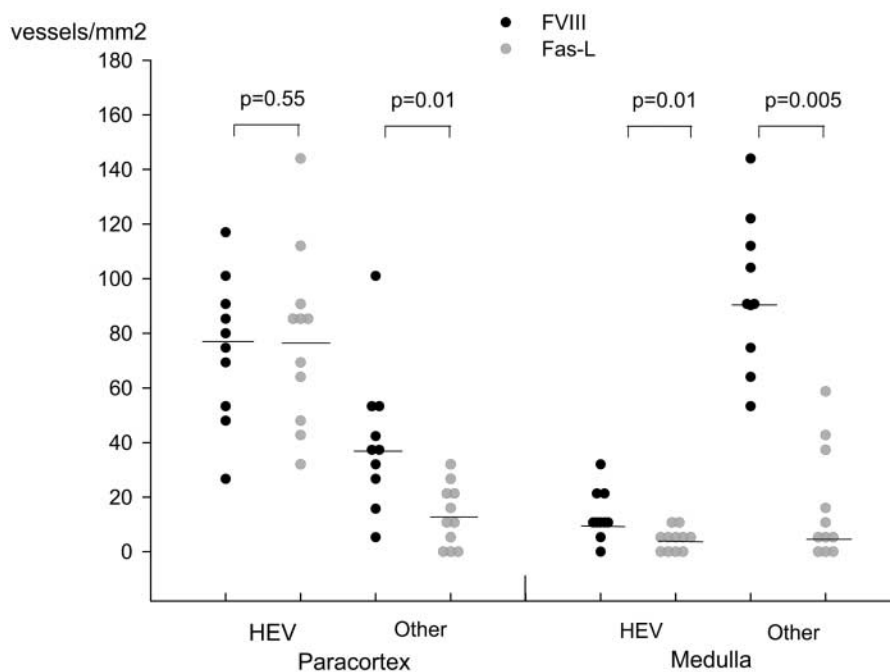
To get a more precise view of preferential expression of Fas-L in blood vessels in terms of functional region of lymph nodes and vessel type, we first counted the area density of all blood vessels as indicated by FVIII positivity in the paracortex and medulla. Vascular density in the germinal centers and perifollicular cortex was not counted because these areas were devoid of Fas-L<sup>+</sup> vessels. Vessels with high and low endothelium were counted separately to see what proportions of HEV and non-HEV vessels were positive. The density of all blood vessels (FVIII<sup>+</sup> vessels) was similar in the paracortex (median 106.7 vessels/mm<sup>2</sup>; mean 115.1/mm<sup>2</sup>; min 75; max 219) and medulla (median 102.5 vessels/mm<sup>2</sup>; mean 107.9/mm<sup>2</sup>; min 64; max 176;  $p=0.721$ ; Figure 2). As expected, the density of FVIII<sup>+</sup> HEVs was higher in the paracortex (median 77.3 vessels/mm<sup>2</sup>; mean 74.7/mm<sup>2</sup>; min 27; max 117) than in the medulla (median 10.7 vessels/mm<sup>2</sup>; mean 13.3/mm<sup>2</sup>; min 0; max 32), but the density of FVIII<sup>+</sup> non-HEVs was lower in the paracortex (median 37.3 vessels/mm<sup>2</sup>; mean 40.4/mm<sup>2</sup>; min 5; max 101) than in the medulla (median 90.7 vessels/mm<sup>2</sup>; mean 94.6/mm<sup>2</sup>; min 53; max 144), indicating that HEVs can be found in both medulla and cortex, but their proportion of all vessels is much higher in the paracortex.

The area density of Fas-L<sup>+</sup> vessels was then counted similarly. The density of vessels was significantly higher in the paracortex (median 82.6 vessels/mm<sup>2</sup>; mean 90.7/mm<sup>2</sup>; min 53; max 176) than in the medulla (median 16.0 vessels/mm<sup>2</sup>; mean 20.9/mm<sup>2</sup>; min 0; max 69; Figure 2). Focusing on the specific vessel types, the density of Fas-L<sup>+</sup> HEVs was much higher in the paracortical zone (median 77.3 vessels/mm<sup>2</sup>; mean 77.3/mm<sup>2</sup>; min 32; max 144) than in the medullary zone (median 5.3 vessels/mm<sup>2</sup>; mean 4.4/mm<sup>2</sup>; min 0; max 11;  $p=0.003$ ; Figure 2). The density of non-HEV vessels expressing Fas-L was equally low in the paracortex (median 13.3 vessels/mm<sup>2</sup>; mean 13.3/mm<sup>2</sup>; min 0; max 32) and the medulla (median 5.3 vessels/mm<sup>2</sup>; mean 16.5/mm<sup>2</sup>; min 0; max 59).

The densities of Fas-L<sup>+</sup> and FVIII<sup>+</sup> vessels were compared to gain an insight about proportion of each vessel type that expresses Fas-L in paracortex and medulla. In the paracortex, the counts of FVIII- and Fas-L-expressing HEVs did not differ ( $p=0.553$ ), indicating that most paracortical HEVs express Fas-L (Figures 1E and 1F). In the medulla, the density of Fas-L<sup>+</sup> HEVs was only ~25% of FVIII<sup>+</sup> HEVs, indicating that only a minority of medullary HEVs express Fas-L. Of the paracortical non-HEV vessels, only 0.3% were Fas-L<sup>+</sup>, the corresponding figure in the medulla being 11%.

Finally, the densities of Fas-L<sup>+</sup> and FVIII<sup>+</sup> vessels were compared in the different patterns of lymph node reaction, including paracortical hyperplasia ( $n=6$ ), fol-

**Figure 2** Scatter plot showing area densities of blood vessels expressing FVIII or Fas-L in lymph node paracortex and medulla.



licular hyperplasia ( $n=3$ ), and mixed reaction ( $n=2$ ). No significant differences were seen among the different reaction types.

## Discussion

A major finding emerging from this study is that human lymph nodes with different activation states express Fas-L in most of the HEVs in the paracortical region and in the cells lining the parenchymal side of the subcortical sinus, whereas in the other nodal vessels the expression is weak or absent. This specific expression may have an important role in the regulation of immune responses, similar to the way in which eye and testis gain an immune-privileged status via Fas-L expression and Fas/Fas-L-mediated apoptosis of the lymphoid cells.

Previous studies addressing Fas-L expression in lymph nodes have mainly focused on expression in lymphoid cells and are conflicting about endothelial cell expression. Sträter et al. (1999) noted inconsistent Fas-L mRNA transcripts and protein in epitheloid endothelial cells in reactive lymph nodes, and strong staining in all endothelial cells in lymph nodes affected by Hodgkin's disease was reported by Verbeke et al (2001). Wang et al. (2002) did not demonstrate any Fas-L protein expression in endothelial cells in reactive lymph nodes (Wang and Adegboyega 2002). These discrepancies may be due to the antibody properties or differences in the pretreatment methods used, or both. In evaluation of different anti-Fas-L antibod-

ies, Sträter et al. (2001) showed that several of the commercial antibodies, including one used by Verbeke et al.(2001) were not specific enough and that only one monoclonal antibody (clone G247-4), used in the present study and that of Sträter et al. (1999), showed high specificity for detection of Fas-L. We used a polymer-based EnVision staining method, which has a much higher sensitivity than the avidin-biotin- and streptavidin-based methods (Sabattini et al. 1998). Moreover, we used pretreatment with Tris-EDTA (pH 9.0) instead of the commonly used citrate buffer (pH 6.0), which may relieve more epitopes for the antibodies to attach to and may explain our more constant and extensive HEV expression compared with the results of Sträter et al. (1999) using the same antibodies. As all of our control stainings were negative there is no doubt about nonspecific false-positive staining. In our material, three lymph node specimens showed no immunoreaction, positive staining being absent from lymphocytes as well. Because no evidence for association with any lymph node reaction type and absence of immunoreaction was seen, we consider this a false-negative staining, possibly related to fixation of the specimens.

The functional significance of the observed Fas-L expression in the HEVs of a lymph node was not addressed in this study. However, evidence from previous studies about the functional role of these vessels and expression of Fas in lymphocytes supports the idea that Fas-L expression in HEVs has an important role in the regulation of lymph node function. Lymphocyte migration into the parenchyma through

HEVs is a complicated process, involving a multistep adhesion cascade involving selectins, integrins, and HEV glycoproteins. The adhesion molecules of the immunoglobulin superfamily are followed by a chemokine-dependent arrest and migration. Only naïve T-lymphocytes are able to migrate through HEVs (McKay 1992). In the lymph node parenchyma they get into a contact with an antigen presenting cells and become activated in cases where they meet their specific target antigen. In contrast, activated lymphocytes are directed into peripheral target tissues where they meet their antigens and become further activated. It has been shown that T-lymphocytes that are stimulated by binding of the antigen into their receptors are later killed by Fas-L-mediated apoptosis (Lenardo et al. 1999). Similarly, resting B-lymphocytes do not express Fas, but B-cells activated by CD40 ligand express Fas and become susceptible to Fas/Fas-L-mediated apoptosis (Garrone et al. 1995; Schattner et al. 1995). We speculate that HEVs with Fas-L expression form a selective border that restricts the entrance of activated lymphocytes. The physiological advantage of the exclusion of activated T-cells is evident, because contact between antigen and activated T-lymphocyte in the lymph node parenchyma would lead to an activation cascade and eventually to destruction of the lymph node structures.

The phagocytosis of apoptotic leukocytes is known to take place in HEVs, supporting the significance of this process in protecting the parenchyma of a lymph node and further suggesting that HEVs limit the entrance of apoptotic lymphocytes into the peripheral lymphoid tissues (Hess et al. 1997). Additional evidence for importance of Fas/Fas-L-mediated apoptosis in the regulation of paracortical function comes from observations in an autoimmune lymphoproliferative syndrome caused by mutation in the Fas gene. In this syndrome, generalized lymphadenopathy is associated with an expansion of paracortical zones and a decreased rate of T-lymphocyte apoptosis in the paracortical region (Lim et al. 1998).

Although most paracortical HEVs expressed Fas-L, only a minority of the rare medullary HEVs showed any expression. This difference in the expression pattern indicates functional heterogeneity of HEVs related to their location in lymph node parenchyma. HEVs are dynamic structures. It has been shown that HEV convert from high- to flat-walled endothelial morphology and lose their ability to support lymphocyte traffic when lymph nodes are deprived of afferent lymph (Hendriks and Eestermans 1983), indicating that antigen load or some other factor in the afferent lymph might be regulating their formation. Whether such exogenous factors or local environment in the lymph node parenchyma regulate Fas-L expression in lymph node vasculature is currently unknown.

In addition to being expressed by HEVs, Fas-L expression was seen in cells at the parenchymal side of the marginal sinus, this kind of expression not being reported previously. The distribution of Fas-L expressing cells was similar to that of occasional sinus lining mononuclear phagocytes expressing CD68. Although the exact role of Fas-L expression in these cells remains speculative, a similar protective function as suggested for vascular endothelial expression is plausible. It has been shown that macrophages express Fas-L, the expression is upregulated in activated cells (Bradley et al. 1996), Fas-L may be released during activation (Kiener et al. 1997) and, finally, that macrophages may induce Fas-L-mediated apoptosis (Boyle et al. 2001). The marginal sinus is the first compartment in the lymph node at which the incoming lymph arrives with immunologically active cells, and it may be necessary to protect the cortical structures from their activity. It is of interest that malignant cells traveling with lymph first lodge in this compartment, where neoplastic metastatic growth usually starts. Therefore, Fas-L expression in the lining cells might be one factor that modifies the settlement of tumor cells during the formation of lymph node metastases.

In summary, this study shows that Fas-L is preferentially expressed in paracortical HEVs of the lymph nodes and in cells lining the parenchyma of the subcortical sinus. The suggested physiological importance lies in the formation of an immune-privileged area in the lymph node via Fas/Fas-L-mediated apoptosis of activated lymphoid cells, leaving only naïve T-lymphocytes to access the node.

#### Acknowledgments

We thank Ms Riitta Vuento and Mr Manu Tuovinen for technical advice.

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