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CHARACTERIZATION OF OVINE LYMPHATIC ENDOTHELIAL CELLS AND THEIR INTERACTIONS WITH LYMPHOCYTES

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ABSTRACT

The interaction of blood-borne lymphocytes with blood vascular endothelial cells is a fundamental component of lymphocyte circulation. The role of lymphatic endothelial cells is less certain. These studies describe the isolation, characterization and lymphocyte binding capacity of efferent lymphatic endothelial cells from ovine mesenteric lymphatic vessels. Lymphatic endothelial cells had anti-thrombin 3, von Willebrand Factor and MHC I on their surface. The cells also actively metabolized acetylated low density lipoprotein. The morphological appearance was indistinguishable from blood vascular endothelium but quite different from cultured smooth muscle cells. Lymphocyte binding to lymphatic endothelial cells was not significantly different from binding to carotid artery or jugular vein endothelial cells. The degree of binding in all cases could be enhanced by incubating endothelial cells in medium containing rh TNF-α (recombinant human tumor necrosis factor alpha).

Lymphocytes continuously travel from the blood through the lymph nodes and into efferent lymphatic vessels. This migration is regulated at the level of the blood vascular endothelium and also within the lymph node itself. Many antigens can promote increased lymphocyte recruitment from the blood and also retard the release of lymphocytes into the efferent lymph. Most of this lymphocyte retention occurs during the first day of an immune response. In addition, during the subsequent period when an overall increase in lymphocyte output is observed in the efferent lymph, specific antigen-reactive lymphocytes still appear to be selectively detailed until they are released into the lymph as lymphoblasts (1).

It is clear that the increase in blood flow and therefore lymphocyte delivery to the lymph node is one measurable consequence of antigenic challenge (2) and potential mediators for increasing blood flow, particularly arachidonic acid metabolites and phospholipase A_2 have been assayed in the lymph at appropriate times after antigenic challenge (3,4). In addition, several studies have shown that the blood vascular endothelium plays an active role, particularly with respect to the adhesion of lymphocytes. Molecules like TNF-α and interferon (IFN)-γ enhance lymphocyte binding to blood vascular endothelium *in vitro* (5-7). In sheep it has been shown that blood vascular endo-thelial cells from lymph nodes as well as larger blood vessels can be stimulated by such molecules *in vitro* (8).

The possible participation of lymphatic endothelial cells, perhaps within the lymph node itself has not been studied before. The data in this report characterizes lymphatic endothelium derived from sheep and examines the binding capacity of such endothelium for lymphocytes. An impetus for these studies came from the description and the cultivation of bovine lymphatic endothelial cells by Johnston and Walker (9).

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MATERIALS AND METHODS

Isolation and Culture of Ovine Endothelium

Tissues used for the isolation of carotid artery, lymphatic and jugular vein endothelium were obtained from adult ewes at necropsy. The protocol used for harvesting the endothelial cells was based on the procedure developed by Jaffe et al (10). A 10 to 20 cm length of carotid artery, jugular vein or efferent mesenteric lymphatic was cannulated at both ends with sterile polyethylene cannulae (Intramedic, Clay Adams, Parsippany, NJ). Blood or mesenteric lymph was flushed from the vessel with 20ml serum-free M199 media (Gibco, Grand Island, NY) and two, 20 cc sterile syringes (Becton Dickinson, Rutherford, NJ) connected to the cannulae via 18 gauge needles (Becton Dickinson). A 0.2% solution of (wt/vol) collagenase type 2 (Sigma Chemical Co., St. Louis, MO) was infused into the vessel. The vessel was then immersed in a beaker containing prewarmed serum-free media for 10 minutes. The digestion solution was then drawn out through one cannula with a 20 cc syringe while a similar syringe was used on the opposite cannula to inject 40ml of tissue culture media containing: 20% lamb serum (Gibco), 50IU/ml penicillin (Gibco) and 10U/ml of heparin (Organon). This media was infused gradually and taken up by the opposing cannula. The entire 60ml suspension was then poured into two, 50ml conical tubes (Sarstedt, Que, Canada) and centrifuged for 5 minutes at 558xg. The resulting pellets were resuspended in 2ml of tissue culture media each and plated in one 15x60 mm dish (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) that was precoated with 2% gelatin solution (Sigma). Cultures were then treated with endothelial cell growth supplement (ECGS, Sigma) so that primary cultures grew in media with 100μg/ml of ECGS at 37°C in 5% CO_2. Later the endothelial cells were subcultured and grown in 20x100mm dishes (Falcon) with only 20μg/ml ECGS.

Endothelial cells were used between the sixth and fifteenth passages. Just prior to confluence, cultures were treated for 15 minutes at 37°C and 5% CO_2 with a trypsin/EDTA solution (0.05%, Gibco). Media containing an added 10% dimethylsulfoxide (DMSO, BDH Chemicals, Toronto) was prepared for cells frozen down at a concentration of 1-2x10^6/ml in individual cryotubes (1.8 ml Nunc Cryotubes, Kampstrup, Denmark). The tubes were then stored in liquid nitrogen. Julie Yu, a project student in the lab, and Nevin Abernethy (unpublished) established the feasibility of growing the ovine lymphatic endothelium, prior to the re-isolation of these cells for this investigation.

Identification and Characterization of Carotid Artery, Jugular Vein and Lymphatic Endothelium

All three cell types were determined to be endothelial cells on the basis of their cobblestone morphology and their ability to metabolize acetylated low density lipoprotein labeled with 1.1'-dioctadecyl-1-3,3,3'3,-tetramethylindocarboocyanine perchlorate (Di1-Ac-LDL). The confluent 20x100 mm culture dishes were washed 3 times with serum-free media and then treated with trypsin/EDTA solution. The cell suspensions were fixed with 1% (wt/vol) solution of phosphate buffeted saline and paraformaldehyde (BDH) for 30 minutes. Suspensions were then centrifuged and the pellets resuspended in a solution of 0.1% sodium azide, 10% bovine serum albumin and M199 media. Samples were stored in 400μl volumes at 5°C in the dark until they were analyzed by flow cytometry, using a Coulter Epics V with an argon laser (Coulter Electronics Hialeah, FL). Acetylated-LDL treated cells and their untreated controls were analyzed with an excitation wavelength of 514nm. Previously isolated ovine jugular vein endothelial cell cultures were used as a positive control. Ovine smooth muscle cells isolated from collagenase

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digestion of a peripheral lymph node were used as a negative control. In all cases, both treated and untreated cell types were tested during the same experiment. Lymphatic and jugular vein endothelial cell cultures were further analyzed for their expression of certain cell surface antigens. The primary antibodies used were: rabbit polyclonal anti-sheep, anti-thrombin 3 (AT-3) a gift from Dr. Safia Wasi, Canadian Red Cross, Ottawa, rabbit polyclonal anti-sheep von Willebrand Factor (vWF), a gift from Dr. Marlene Rabinovitch, Dept. of Cardiology, Hospital for Sick Children, Toronto, mouse monoclonal antibody directed against the sheep major histocompatibility complex (MHC) class I antigen, purchased from Dr. M. Brandon at the University of Melbourne, Australia. The secondary antibodies used were goat anti-mouse IgG monoclonal antibody conjugated with R-phycoerythrin (Sigma) and goat anti-rabbit IgG monoclonal antibody with a fluorescein conjugate (Cedarlane Laboratories, Ltd., Hornby, ON). All conditions were performed in duplicate.

Lymphocyte/Endothelial Cell Binding Assay

An adhesion assay that measures the binding activity of lymphocytes to endothelium was developed to evaluate the ability of lymph plasma to alter baseline binding between allogeneic ovine lymphocytes and ovine carotid artery or lymphatic endothelium (12). Lymphocytes were obtained from efferent lymph (13) and were predominantly resting T cells. Endothelial cells were cultured on 2% gelatin coated (Sigma), flat bottomed, 96 well tissue culture plates (Nunc). Each well received $5x10^4$ cells. After plating out the endothelium in 100µl of serum-free M-199 plus antibiotics, 100µl of media containing 40% lamb serum and antibiotics was added to each well. Recombinant human TNF-α was used as a positive control at a concentration of 0.1µg/ml. Wells containing no endothelial cells were used as negative controls. All monolayers were incubated with serum, lymph plasma, or serum and rh TNF-α (Genentech, San Francisco, CA) for 17 hours prior to washing the monolayers 3 times with warm tissue culture media. Each experiment was performed in quadruplicate. When 17 hours had elapsed $1x10^6$ ^{111}indium labeled lymphocytes were added/well in 200µl of warm tissue culture media. The plate was incubated under standard conditions (37°C, 5% CO_2) for one hour. The wells were washed four times with an 8-channel micropipetter (Titertek, Flow Laboratories) and warmed media, 200µl/wash. The micropipetter was used to vigorously aspirate and dispense the media into each well 10 times/wash. After each wash, the plate was inverted to remove unbound cells. A solution of 1N NaOH was added to each well at a volume of 200µl. The plate was then left at -20°C for 20 minutes to lyse all remaining cells. Later each well was washed two times with 200µl water which was dispensed into 10µl plastic tubes for gamma counting. Lymphocyte binding activity was expressed as:

counts/min in 0.6 ml of lysate/counts/min in lysate of original lymphocyte suspension added x 100
= % of bound lymphocytes ± the standard deviation of quadruplicate cultures.

Statistical analysis was performed on data using a Macintosh computer.

RESULTS

Metabolism of Dil-Ac-LDL

Prior to this investigation, ovine lymphatic endothelia had not been characterized metabolically, immunologically, or for their ability to interact with cytokines and lymphocytes. The endothelial cells were identified for their purity in culture, using metabolic and surface antigen properties characteristic of endothelial cells. The top two panels of *Fig. 1* show that both ovine carotid

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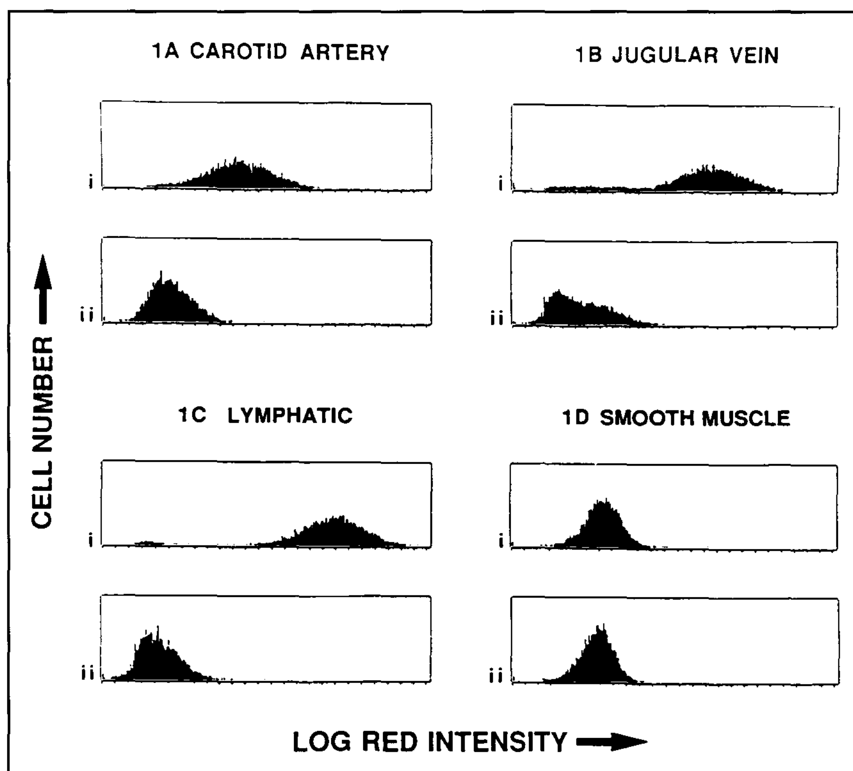

Fig. 1. The uptake and metabolism of fluorescent acetylated low density lipoprotein (DiI-Ac-LDL) by ovine endothelial cell cultures harvested from (A) carotid artery, (B) jugular vein, (C) efferent mesenteric lymphatic, and (D) by lymph node smooth muscle cells. In each case, treated cultures (i) were compared with untreated cells from the same source (ii). A minimum of 5000 cells were analyzed per condition.

artery (1Ai) and jugular vein (1Bi) endothelial cells take up and metabolize an acetylated low density lipoprotein conjugated to a fluorescent molecule. This was apparent when compared with untreated cultures from the same source (1Aii and 1Bii, respectively). The cells harvested by collagenase digestion of lymph node tissue did not demonstrate any ability to bind or metabolize the molecule (1Di) and therefore there was no observable difference in the amount of fluorescence when treated (1Di) and untreated (1Dii) smooth muscle cultures were compared.

Endothelial Cell Surface Antigens

The lymphatic endothelial cell cultures were also characterized for their ability to bind monoclonal antibodies specific for two endothelial cell surface antigens and compared to the known positive jugular vein cultures: antithrombin 3 and von Willebrand factor (Factor 8 related antigen). Subtraction analysis using Coulter software demonstrated that the lymphatic endothelial cell cultures were greater than 95% positive for the antithrombin 3 surface antigen (95.91%); similarly, the jugular vein cultures were 94.12% positive for this antigen using the same analysis *(Figs. 2Ai and Bi, respectively).* The ovine lymphatic endothelial cell cultures were also strongly positive for the von Willebrand factor surface antigen, as nearly 95% of the cells positively expressed this molecule (92.88%) *(Fig. 2Ci).* Subtraction analysis also showed that the ovine jugular vein endothelial cell cultures expressed the von Willebrand factor antigen as 86.84% of the cells were positive *(Fig. 2Di).* Both the lymphatic and jugular vein endothelial cell cultures were tested for the surface expression of the ovine MHC Class 1 molecule. *Figures 2E and 2F* (i) show the subtraction analysis of these histograms from negative control

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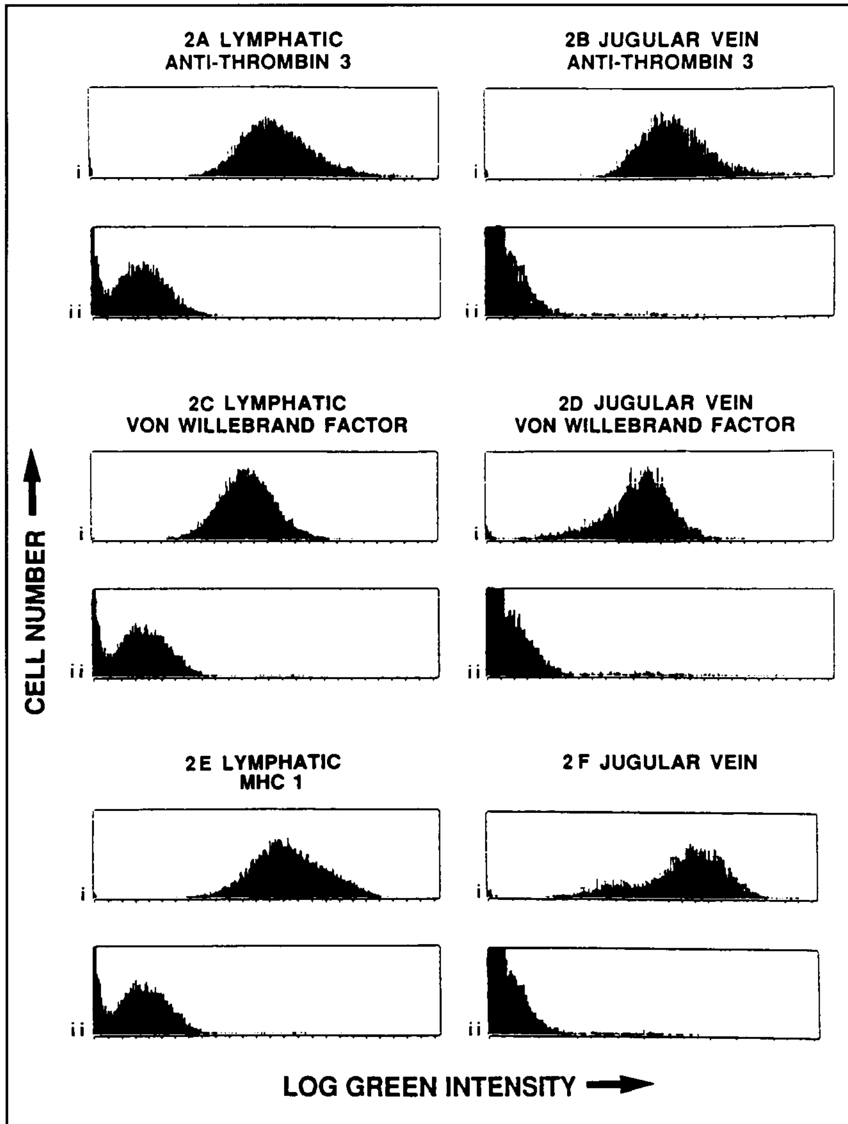

Fig. 2. A polyclonal antiserum from rabbits directed against ovine anti-thrombin 3 was added to lymphatic (2Ai) and jugular vein endothelial cells (2Bi). Similarly, staining was done with antibody to von Willebrand factor (2C,2D) and MHC 1 (2E,2F). In both cases, the top histogram shows staining with primary and secondary antibodies (i), while the lower histograms indicated staining with secondary antibody control (ii). The percent positive cells was calculated by the subtraction of background fluorescence from the experimental condition, yielding a figure for percent antigen positive cells.

cultures (ii). The subsequent calculation of the percentage of cells in culture that expressed this antigen on their surface demonstrated that of the ovine lymphatic cells, 95.3% of the cells expressed MHC I, whereas 92.3% of the ovine jugular vein cultures expressed MHC I on their surface.

Comparative Lymphocyte Binding to Venous, Arterial and Lymphatic Endothelium

The simultaneous testing of 3 different endothelial cell cultures and one smooth muscle culture for the ability to bind efferent lymphatic lymphocytes was performed on three separate occasions *(upper panel, Fig. 3).* These experiments contained a further negative control consisting of lymphocytes bound to gelatin coated plastic. These data show firstly that lymphatic endothelium had the ability to bind lymphocytes *in vitro* and

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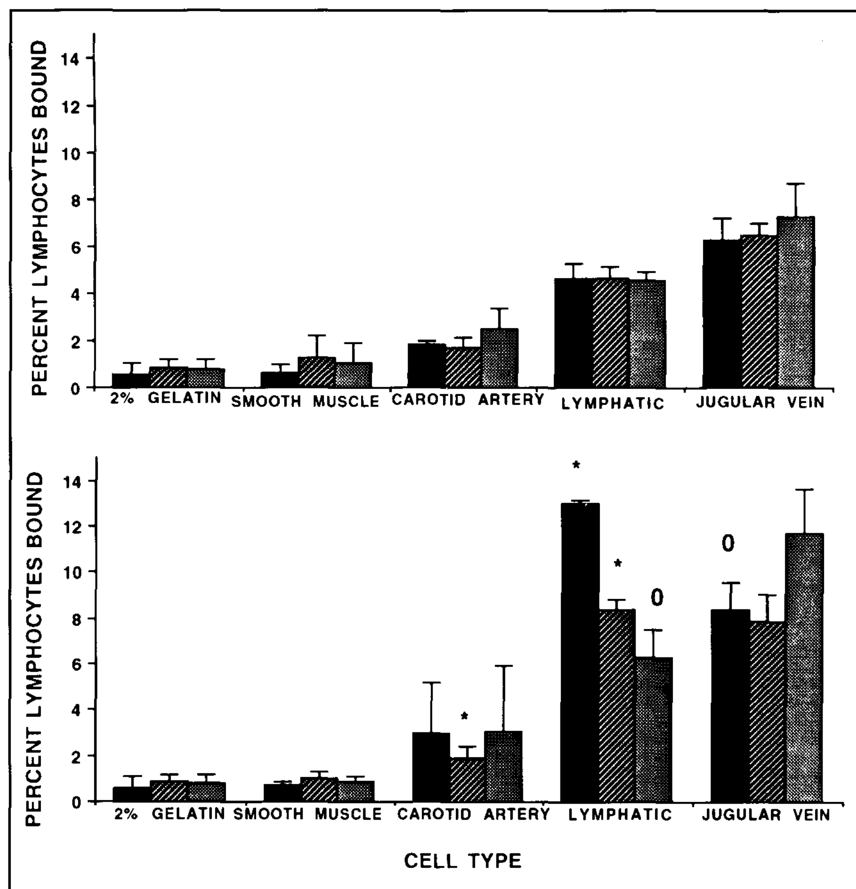
*Fig. 3. The comparison of 3 separate experiments showing the mean percentage of lymphocytes bound to: 2% gelatin coated wells, smooth muscle cells, carotid artery endothelial cells, lymphatic endothelial cells, and jugular vein endothelial cells that were preincubated in the absence (upper panel) or presence (lower panel) of rh TNF-α. Error bars represent the standard deviation of the mean. *, represents p<0.001 while o represents p<0.01.*



secondly that lymphatic endothelium had comparable binding activity to arterial and venous endothelium *in vitro*. Pretreatment of these same endothelial cell cultures with 0.1µg/ml of rh TNF-α resulted in varying degrees of enhanced lymphocyte adhesion *(lower panel Fig. 3)*. In all 3 experiments, lymphatic endothelium pretreated with rh TNF-α had significantly higher lymphocyte binding activity than untreated cultures. Jugular vein and carotid artery endothelium exposed to rh TNF-α also showed significantly increased lymphocyte binding activity. Statistical levels of significance are shown in the Figure Legend. All of these cultures were more efficient than either smooth muscle or gelatinized plastic, which consistently showed no observable difference in binding activity nor any significant increase in lymphocyte adhesion after preincubation with rh TNF-α.

DISCUSSION

These experiments are the first to characterize ovine lymphatic endothelium by means of cell surface antigens and metabolic activity. Furthermore, this characterization includes functional studies that describe the ability of lymphatic endothelium to bind lymphocytes and the increase in this lymphocyte binding activity after exposure to rh TNF-α. The strong expression of several different endothelial cell surface antigens; namely anti-thrombin 3, Factor 8 related antigen and the common surface antigen MHC I indicates that the lymphatic endothelial cells maintain their

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basic endothelial cell phenotype for long periods in culture. By simultaneously characterizing these lymphatic cells with blood vascular endothelial cell cultures, it has been observed that *in vitro* neither cell type differs significantly from the other in terms of the cell surface antigens that were tested. The lymphocyte binding properties which these cells possess appear equally stable over the time period that the experiments were performed and no obvious differences were seen between lymphatic and blood vascular endothelial cell binding patterns. Several studies report a similar range of results for the mean percentage of lymphocytes bound to endothelium after an incubation period of 1 hour with blood vascular endothelium from different sources (5,7,8,12,14). The ability of endothelium to respond to TNF-α and increase the capacity to bind lymphocytes has been demonstrated by using human umbilical vein endothelial cells (6), rat endothelial cells (12) and sheep lymph node endothelial cells (8). The experiments performed in our study demonstrate the specificity that rh TNF-α has for endothelial cells, as opposed to the smooth muscle cell cultures which do not respond to rh TNF-α treatment.

Previous *in vivo* studies by Kalaaji et al (15) using rbov TNF-α in sheep have clearly demonstrated that TNF-α is a potent mediator of lymphocyte localization in dermal tissues. This phenomenon was first shown in the rat by Issekutz et al (16).

Taken collectively, the data are ambiguous as to whether the lymphatic endothelium is different from blood vascular endothelium (17). Evidence in favor of this idea was partially derived from the observation that thoracic duct endothelium demonstrated differences in tumor cell adhesion compared with blood vascular endothelium from various tissue sources. Some suggest that Kaposi sarcoma is a neoplasm which originates in the lymphatic endothelium (18). Moreover, reports have described a variety of monoclonal antibodies directed against human endothelial cells. Some of these antibodies have been reported to react with lymphatic endothelium and others do not (19). When lymphatic vessels are severed, they reanastomose with existing lymphatics and generally not with blood vessels (20), a phenomenon that implies that lymphatic endothelium is different. If it is different, what is the nature of the transition cells between lymphatic endothelium and venous endothelium at the site of thoracic duct or right lymph duct entry into the blood? Lymphocytes are not known to cross lymphatic endothelium as they are to cross post-capillary venular endothelium. It is plausible that the same group of biologically active molecules induce the blood vascular endothelial cells to bind more lymphocytes and increase lymphocyte migration into the lymphoid tissue also simultaneously activate the lymphatic endothelium to bind lymphocytes, thereby modulating their exit from the challenged lymph node. Therefore, the significance of lymphocyte/lymphatic endothelial cell interaction remains speculative. The successful cultivation and further characterization of lymphatic endothelium may help clarify its relation to blood vascular endothelium.

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