The Influence of Afferent Lymphatic Vessel Interruption on Vascular Addressin Expression

Reina E. Mebius,* Philip R. Streeter,‡ John Brevé,* Adrian M. Duijvestijn,§ and Georg Kraal*

*Department of Cell Biology, Division Histology, Medical Faculty, Vrije Universiteit, Amsterdam; ‡SyStemix Inc., Palo Alto, California 94303; and §Department of Immunology, Medical Faculty, University of Limburg, Maastricht, The Netherlands

Abstract. Tissue-selective lymphocyte homing is directed in part by specialized vessels that define sites of lymphocyte exit from the blood. These vessels, the post capillary high endothelial venules (HEV), are found in organized lymphoid tissues, and at sites of chronic inflammation. Lymphocytes bearing specific receptors, called homing receptors, recognize and adhere to their putative ligands on high endothelial cells, the vascular addressins. After adhesion, lymphocytes enter organized lymphoid tissues by migrating through the endothelial cell wall. Cells and/or soluble factors arriving in lymph nodes by way of the afferent lymph supply have been implicated in the maintenance of HEV morphology and efficient lymphocyte homing. In the study reported here, we assessed the influence of afferent lymphatic vessel interruption on lymph node composition, organization of cellular elements; and on expression of vascular addressins. At 1 wk after occlusion of afferent lymphatic vessels, HEV became flat walled and expression of the peripheral lymph node addressin disappeared from the luminal aspect of most vessels, while being retained on the abluminal side. In addition, an HEV-specific differentiation marker, defined by mAb MECA-325, was undetectable at 7-d postocclusion. In vivo homing studies revealed that these modified vessels support minimal lymphocyte traffic from the blood. After occlusion, we observed dramatic changes in lymphocyte populations and at 7-d postsurgery, lymph nodes were populated predominantly by cells lacking the peripheral lymph node homing receptor LECAM-1. In addition, effects on nonlymphoid cells were observed: subcapsular sinus macrophages, defined by mAb MOMA-1, disappeared; and interdigitating dendritic cells, defined by mAb NLDC-145, were dramatically reduced. These data reveal that functioning afferent lymphatics are centrally involved in maintaining normal lymph node homeostasis.

LYMPHOCYTES migrate continuously between various lymphoid and extra-lymphoid tissues of the body by way of the blood and lymph vascular systems. Lymphocyte entry into peripheral lymph nodes, mucosal lymphoid tissues, and sites of chronic inflammation is directed in part by tissue-selective interactions between blood-borne lymphocytes and specialized cells lining postcapillary high endothelial venules (HEV).[1] Functional studies have revealed that lymphocyte adhesion to HEV is governed at the lymphocyte level by homing receptors (6, 12, 13, 18), and at the endothelial cell level by vascular addressins (21, 22). In the mouse system, two structurally and functionally distinct vascular addressins have been described. The peripheral lymph node addressin mediates the interaction of lymphocytes with HEV in peripheral lymphoid organs (22), and the mucosal addressin directs equivalent cellular interactions in mucosal lymphoid tissues (21). The peripheral lymph node and mucosal addressins are defined, and the adhesive interactions directed by these molecules are functionally inhibited by the anti-addressin mAb MECA-79 (22) and MECA-367 (21), respectively.

Several reports indicate the importance of afferent lymph in lymphocyte migration into lymph nodes (3, 11). Hendriks et al. described a model in which interruption of afferent lymphatic vessels of rat popliteal lymph nodes results in a flattening of high endothelial venule cells, and a decline in lymphocyte influx into modified lymph nodes (11). Within 1 d after afferent lymphatic occlusion lymphocyte influx is observed to decrease and this decrease is followed by the flattening of high endothelial cells (3). Here we have adapted this model to the mouse system, to study the influence of afferent lymphatic vessel interruption on vascular addressin expression, and the presence and distribution of lymph node leukocyte populations.

Materials and Methods

Animals

BALB/c mice were either purchased from Bomholtgard (Ry, Denmark), or were obtained from the animal colony maintained at SyStemix. All animals were kept under routine laboratory conditions.

1. *Abbreviation used in this paper*: HEV, high endothelial venules.

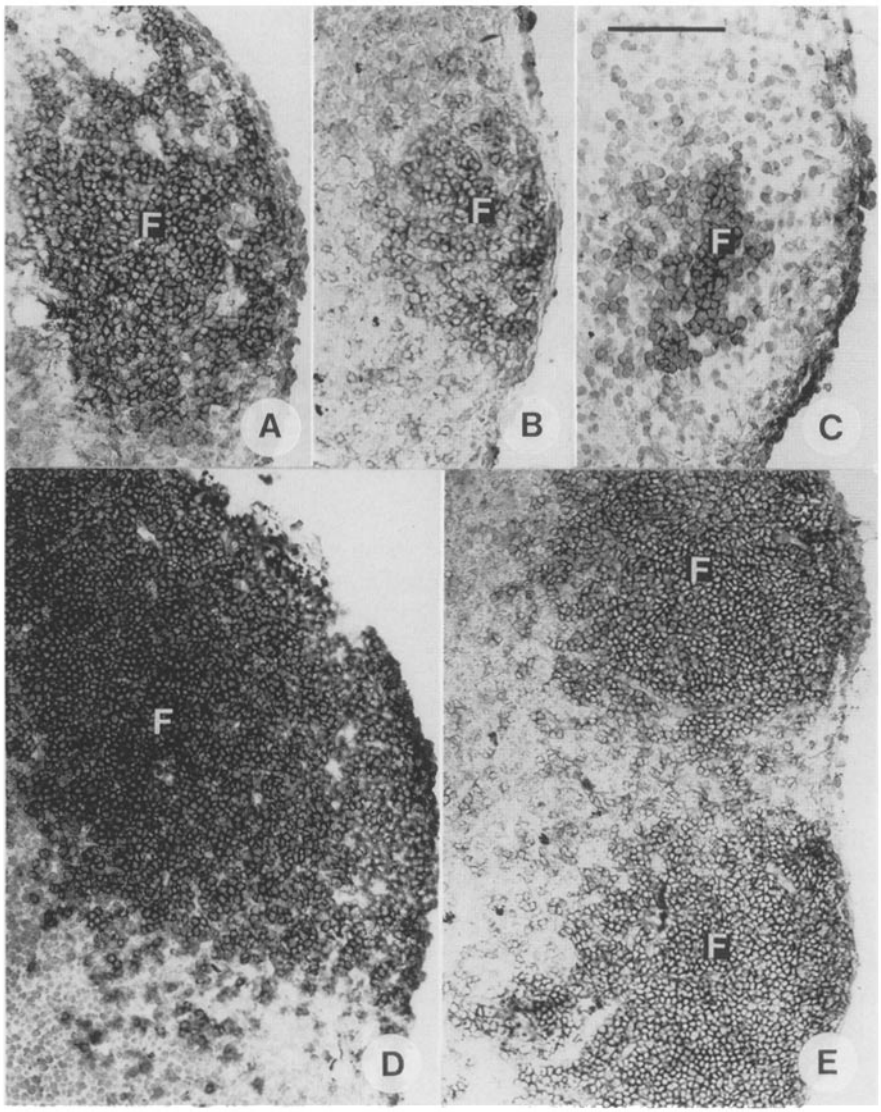


Figure 1. B cell follicles in operated and control lymph nodes. (*A–E*) are all stained with mAb 6B2, which reacts with the B200 antigen on B cells. (*A*, *B*, and *C*) are sections of different operated lymph nodes, 7 d after interruption of afferent lymph supply, and show small B cell follicles compared with B cell follicles in control lymph nodes (*D* and *E*). *F*, follicle. Bar, 80 μm.

Occlusion of Afferent Lymphatics

Mice were anaesthetized by intramuscular injection of 1 μl/g body weight of a 4:3 mixture of Aescoket (Aesculaap N. V., Gent, Belgium) and Rompun (Bayer, Leverkussen, Germany). The operative procedure was adapted from the procedure developed for the rat (9). Briefly, popliteal lymph nodes were exteriorized, and afferent lymphatic vessels were severed. Efferent lymphatic vessels and blood vessels, emerging from the hilus, were left intact. Mice were killed at different timepoints after afferent lymphatic vessel interruption. Before excision of the operated lymph node, 0.05 ml of a 10% India ink solution was injected into the ipsilateral footpad to determine the completeness of the operation. Lymph nodes with intact afferent lymphatics, as demonstrated by the uptake of ink, were discarded. The contralateral popliteal lymph node served as unoperated control tissue. Excised lymph nodes were snap-frozen in liquid nitrogen and stored at −20°C until use.

In Vitro HEV-binding Assay

Lymphocyte adherence to HEV in vitro was assayed by the method of Butcher et al. (1), a modification of the assay originally described by Stamper and Woodruff (20). Briefly, lymphocyte suspensions, containing 1×10^6 cells/100 μl were incubated at 4°C on unfixed frozen sections (8 μm) of operated and control lymph nodes. During the assay, lymph node sections were rotated for 30 min. Upon completion of the assay, unbound cells were decanted, and sections were fixed in PBS containing 1% glutaraldehyde. Cell binding to HEV was assessed microscopically under darkfield or normal illumination after staining by methyl green-pyronin, and the mean number of bound cells per HEV was determined.

Immunohistochemistry

Cryostat sections of popliteal lymph nodes (5–8 μm thick) were fixed in cold acetone for 10 min and air dried for at least 15 min. The sections were incubated for 30 min with various monoclonal rat anti-mouse antibodies at saturating concentrations in 50 mM Tris buffer (pH 7.2–7.6) containing 1% FCS (Tris/FCS). After washing thoroughly in 50 mM Tris buffer (pH 7.2–7.6), slides were incubated for 30 min with a 1:150 dilution of peroxidase-conjugated goat anti-rat IgG/IgM (Jackson Immuno Research, San Francisco, CA) in Tris/FCS containing 5% normal mouse serum. After washing in Tris buffer again, the peroxidase activity was visualized with 3,3′-diaminobenzidine-tetrahydrochloride (DAB; Sigma Chemical Co., St. Louis, MO) at a concentration of 0.5 mg/ml, in Tris buffer (pH 7.2–7.6) containing 0.01% H_2O_2 (10–15 min-incubation at room temperature). The following mAbs were used: MECA-325 (HEV-differentiation antigen; 4), MECA-79 (peripheral lymph node addressin; 22), MECA-367 (mucosal addressin; 21), NLDC-145 (interdigitating cells; 15), MOMA-1 (macrophages in the subcapsular sinus of lymph nodes; 14), 53-2.1 (Thy 1.2 antigen on T cells; 16), 6B2 (B220 antigen on B cells; 2), and MEL-14 (peripheral lymph node homing receptor LECAM-1; 6).

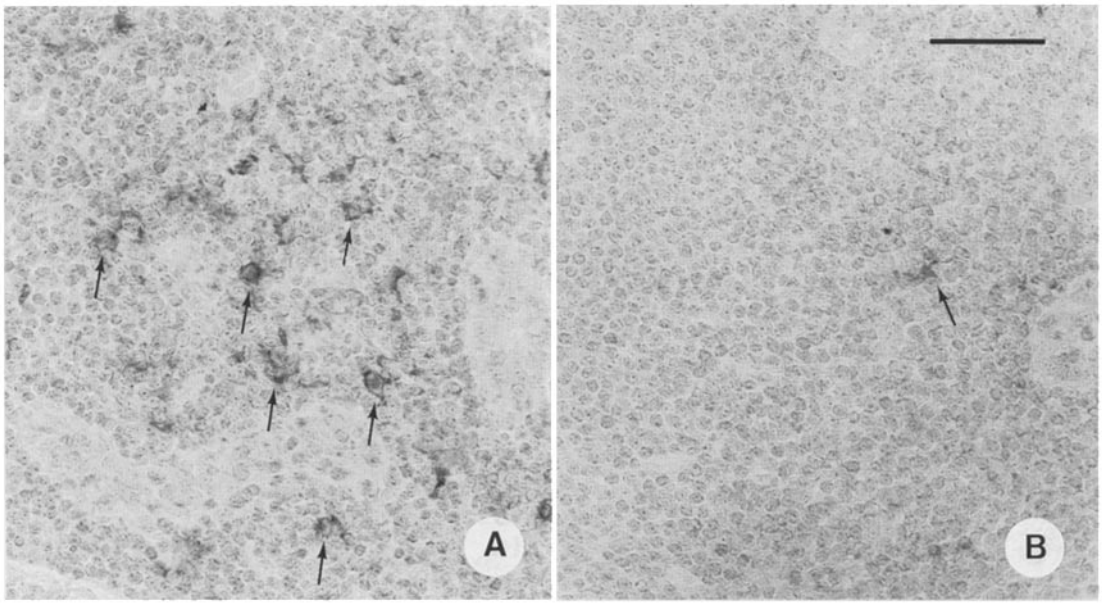


Figure 2. Interdigitating cells in the paracortical area of control and operated lymph nodes (day 4), as recognized by mAb NLDC-145. The number of interdigitating cells (*arrows*), as seen in the T cell-dependent area of control nodes (*A*), had diminished within 4 d of afferent lymphatic occlusion (*B*). Bar, 40 μm.

Flow Cytometric Analysis

Cell fluorescence was analyzed with a FACStar® (a registered trademark of Becton-Dickinson, Mountain View, CA). Single-cell suspensions were made from control and operated lymph nodes. For triple-color immunofluorescence staining, the following procedure was used. Briefly, cells were treated with the mAb MEL-14, washed in HBSS containing 2% FCS, and treated with Texas red–conjugated goat anti-rat IgG (Caltag Laboratories, San Francisco, CA) in HBSS/FCS containing 5% normal mouse serum. After washing in HBSS/FCS the cells were incubated for 5 min with an excess of rat Ig. The cells were then incubated with FITC-conjugated 53-2.1 and biotin-conjugated 6B2. After an additional wash, cells were incubated with phycoerytherin conjugated streptavidin (Biomeda, Foster City, CA). Each incubation step took 20 min and was performed at 4°C. In addition to the above three colors, forward angle scatter was measured and used to gate out debris and dead cells.

Lymphocyte Homing

In Vitro Labeling of Lymphocytes with ^{51}Cr. Lymph node cells (obtained from axillary, brachial, inguinal, and mesenteric lymph nodes) and spleen cells from BALB/c mice were pooled and labeled with ^{51}Cr as described earlier (21). Briefly, lymphocytes were labeled by incubation of 2.5×10^7 cells/ml for 1.5 h at 37°C, with mixing every 15 min, in DME supplemented with 20 mM Hepes, 5% FCS, and 100 μCi/ml sodium chromate ($Na_2{}^{51}CrO_4$; New England Nuclear, Boston, MA). After labeling, the cells were centrifuged through a layer of FCS, and washed twice with HBSS. 2.5×10^7 chromium-labeled cells were injected intravenously into each mouse, which were operated on 5 h, 1, 5, or 8 d previously. Lymphocyte localization was determined by quantitating the cpm in the operated and the contralateral control node. Results are presented as percent of contralateral control (±SD).

Results

The Influence of Afferent Lymphatic Occlusion on the Lymph Node

When afferent lymphatic vessels of popliteal lymph nodes were interrupted, and the nodes replaced in their original position (i.e., within the popliteal fossa), afferent lymphatic vessels were found to reconnect to the operated lymph nodes within 7 d. To prevent this reassociation, we attached the operated lymph nodes, with intact efferent lymphatics and

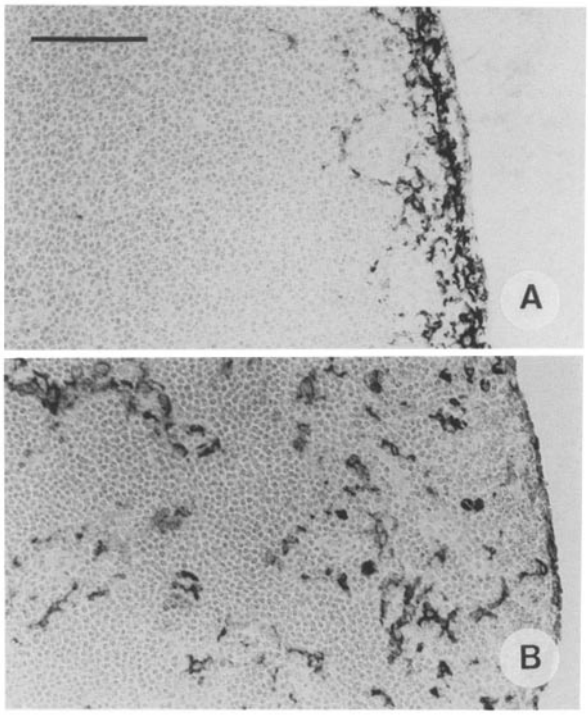


Figure 3. Macrophages in the subcapsular sinus and the paracortical area of control and operated lymph nodes (day 4), as recognized by mAb MOMA-1. The subpopulation of MOMA-1-positive macrophages was restricted to the subcapsular sinus in normal lymph nodes (*A*), whereas they could be observed in the T cell-dependent area at 4 d of afferent lymphatic occlusion (*B*). Bar, 80 μm.

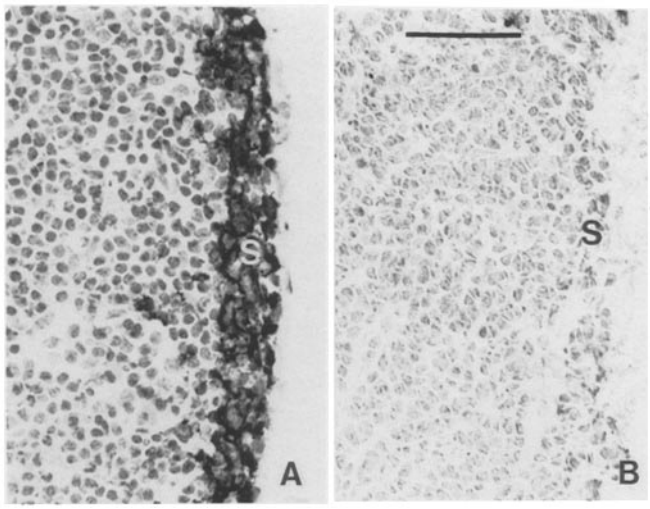


Figure 4. Macrophages in the subcapsular sinus in normal and operated lymph nodes (day 7). (*A* and *B*) are both stained for MOMA-1. The subpopulation of macrophages in the subcapsular sinus of normal lymph nodes (*A*) had disappeared completely, within 7 d of afferent lymphatic vessel interruption (*B*). Bar, 40 μm.

blood supply, onto the fascia directly under the skin. In this subcutaneous position afferent lymphatic vessels draining the footpad were not able to reassociate with the node. The original blood supply remained intact during these manipulations. Reconnection of afferent lymphatic vessels was assessed by injection of India ink into footpads 20 min before lymph nodes excision.

Removal of afferent lymphatics had an effect on lymph node size, and within 1 wk of interruption, the number of lymphocytes present within manipulated lymph nodes had diminished considerably. At 7 d after lymphatic vessel interruption, T cell zones were markedly depleted of T cells, and the follicles within the lymph nodes had decreased in size (Fig. 1). In contrast to normal lymph node compartmentalization, increased numbers of B cells were observed in T cell zones in manipulated lymph nodes. The number of interdigitating cells in the T-cell dependent areas, stained by mAb NLDC-145, had diminished by day 4 after occlusion (Fig. 2). At 7 d after surgery, weakly NLDC-145-positive cells were present in the paracortical area, while strong positive cells, characteristically found in the cortical area, were not detectable. MOMA-1-positive subcapsular sinus macrophages were observed in the paracortical area at 4 d after surgery (Fig. 3), and were not detectable by day 7 after lymphatic vessel occlusion (Fig. 4).

The Influence of Afferent Lymphatic Occlusion on Lymph Node High Endothelial Venules

Hendriks et al. have reported that interruption of lymph node afferent lymphatic vessels has a negative effect on HEV function and morphology (11). Their study was done in rats, however, where mAb defining vascular addressins or specific markers of HEV differentiation are not available. In the present investigation, involving mice rather than rats, the effects of afferent lymphatic vessel occlusion on HEV was assessed. Expression of both an HEV specific differentiation marker, defined by mAb MECA-325, and the peripheral lymph node addressin as recognized by mAb MECA-79 was evaluated at

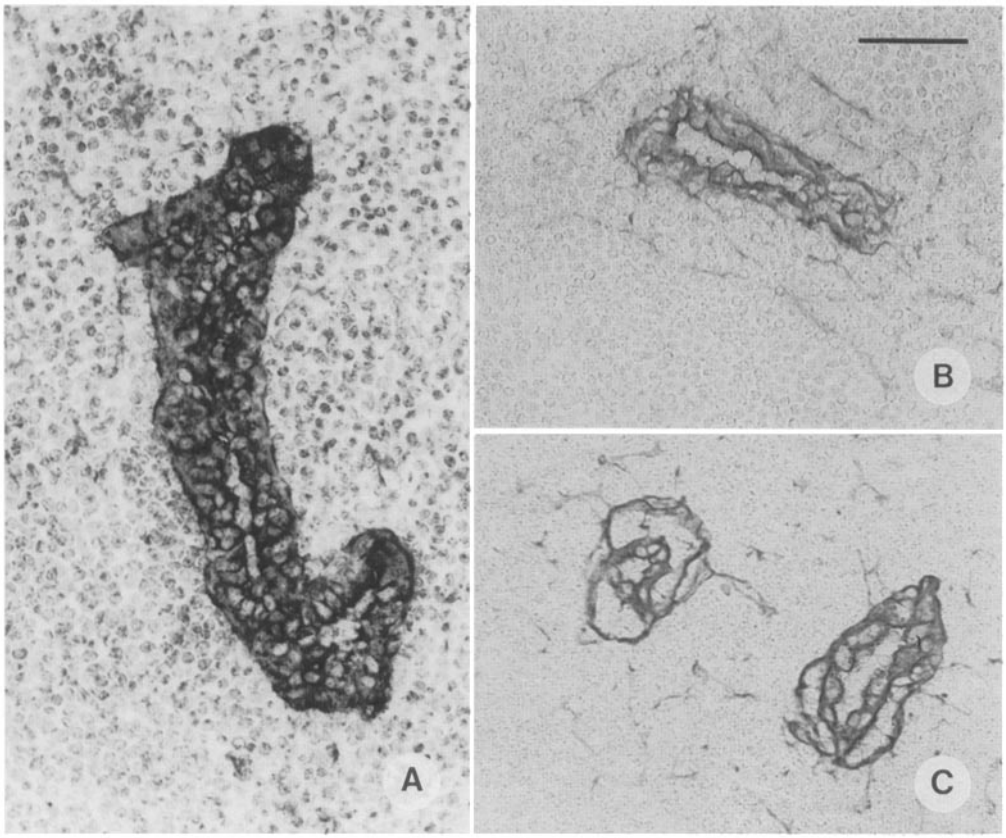


Figure 5. Expression of the peripheral lymph node addressin in control and operated lymph nodes at day 4 after afferent lymphatic interruption. *A* shows the pronounced addressin expression on high walled endothelial cells in the control nodes. At 4 d after occlusion of afferent lymphatics more flat walled vessels could be observed (*B*). Some vessels showed a loss of the peripheral lymph node addressin (*C*). In most cases the addressin was still expressed on the luminal side of the venules. Bar, 40 μm.

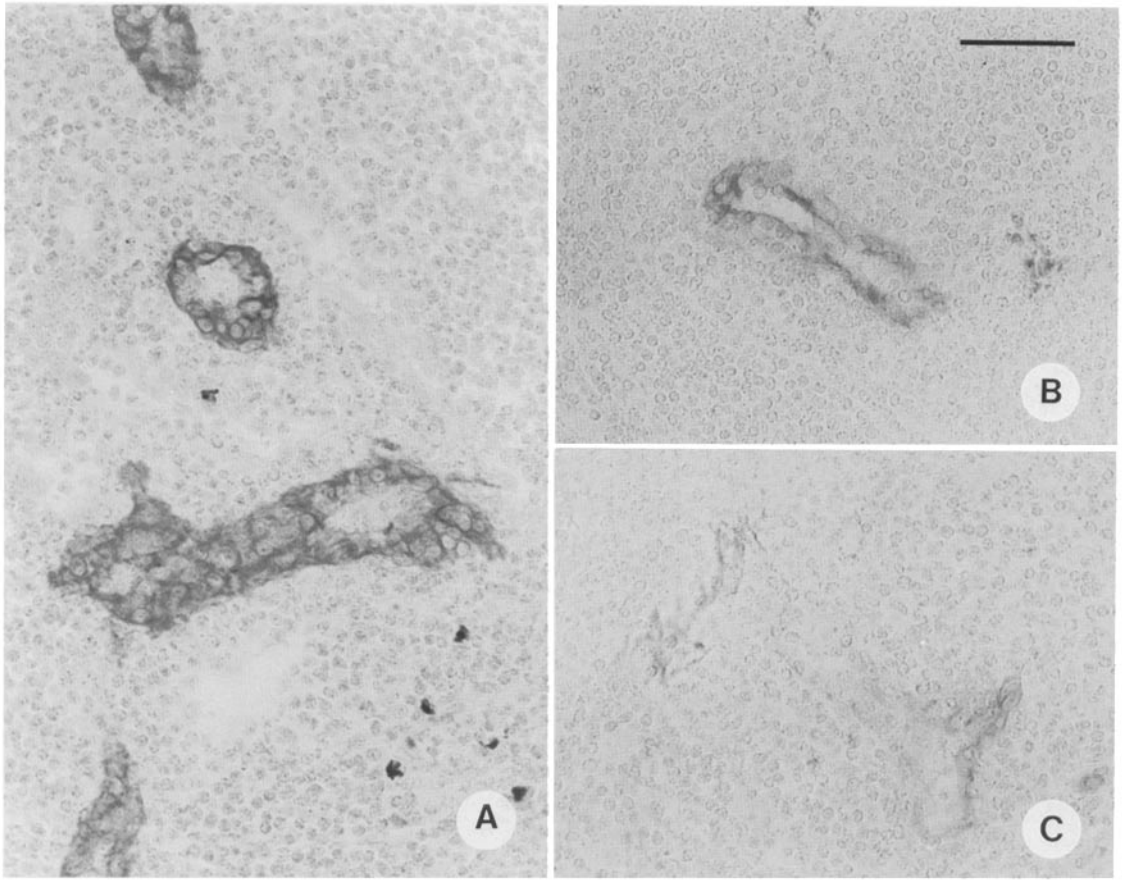


Figure 6. Expression of the MECA-325 antigen on vessels in control and operated lymph nodes (day 4). The high expression of the MECA-325 antigen, as seen on HEV in control nodes (*A*), was weak in operated lymph nodes at 4 d after afferent lymphatic occlusion (*B* and *C*). Sometimes MECA-325 was restricted to the luminal side of the vessels. Bar, 40 μm.

different timepoints after occlusion. This phenotype analysis was performed in combination with functional in vivo and in vitro tests.

4 d after afferent lymphatic vessel interruption, expression of the peripheral lymph node addressin was observed on both high- and flatwalled vessels (Fig. 5). These vessels were also weakly positive for the HEV differentiation antigen MECA-325, with expression sometimes restricted to the luminal side of the endothelium (Fig. 6). It was striking that some high endothelial cells of several individual HEV expressed the mucosal addressin (Fig. 7). While normal peripheral lymph node HEV can express low levels of the mucosal addressin (21), increased numbers of mucosal addressin expressing high endothelial cells were observed in lymph nodes 4 d after afferent lymphatic occlusion.

7 d after occlusion of afferent lymphatic vessels, low levels of the MECA-325 antigen were observed on rare HEV; peripheral lymph node addressin expression appeared to be restricted, in most instances, to the abluminal aspect of relatively flat-walled endothelium (Fig. 8). The abluminal expression was confirmed by the intravenous injection of MECA-79, which failed to localize at high levels in operated lymph nodes, but did localize in the contralateral control nodes of the same animal (Fig. 9). Restriction of the peripheral lymph node addressin to the abluminal side of the vessels, with no expression of the mucosal addressin, suggested that these vessels would not support high levels of lymphocyte adhesion or lymphocyte trafficking from the blood into operated lymph nodes. Indeed, in vivo homing studies revealed that homing into operated lymph nodes at 8 d after operation was decreased by >80% (Fig. 10). Decreased entrance of lymphocytes started at around day 5. At this timepoint, trafficking of labeled cells into operated lymph nodes was comparable to the contralateral control nodes, but varied greatly between animals. The binding of lymphocytes to these vessels at day 4, measured in an in vitro binding assay, was decreased by >30% (Fig. 11). At 7 d after lymphatic occlusion HEV failed to support lymphocyte adhesion.

The Effect of Afferent Lymphatic Occlusion on Peripheral Lymph Node Homing Receptor Expression

The rapid disappearance of lymphocytes from afferent lymphatic vessel occluded lymph nodes prompted us to study the phenotype of residual lymph node cell populations by FACS analysis. The ratio of T and B cells and the proportion of LECAM-1-positive vs. -negative cells was determined by triple staining for 53-2.1, which recognizes the Thy-1 antigen on T cells, 6B2, which recognizes the B220 antigen on B cells, and MEL-14, which recognizes the peripheral lymph node homing receptor LECAM-1. On day 4 after in-

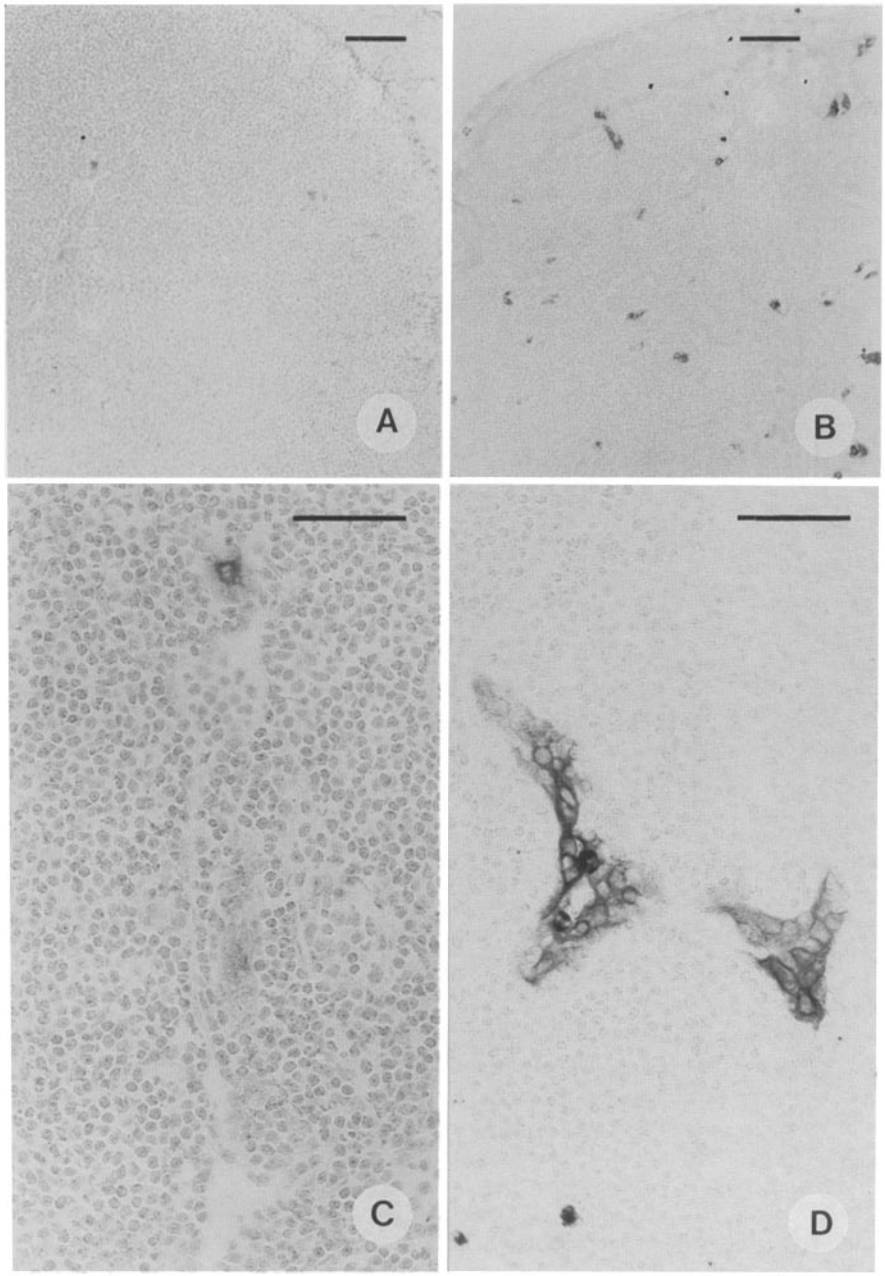


Figure 7. Expression of the mucosal addressin on vessels of control and operated lymph nodes (day 4). Peripheral lymph nodes can express low levels of the mucosal addressin (*A* and *C*). Interestingly, occlusion of afferent lymphatics resulted in a transient increase in expression of this addressin on high endothelial cells at day 4 (*B* and *D*). Bar, 40 μm.

terruption of afferent lymphatic vessels, the percentage of LECAM-1 positive B cells had decreased, while the relative percentage of LECAM-1 positive T cells remained constant (Figs. 12 and 13). On day 7 postoperation, the percentage of LECAM-1 positive B cells and T cells had decreased. At this timepoint, we observed a high percentage of cells negative for 6B2, Thy 1.2, and LECAM-1 (>55%). The identity of these cells is still uncertain.

Reversal of the Effects of Afferent Lymphatic Occlusion

To investigate the reversibility of the effects of afferent lymphatic vessel interruption, we occluded the popliteal lymph node afferent lymphatics of eight animals, and attached the nodes subcutaneously on top of the fascia. After 1 wk the fascia of four animals was opened, and the nodes were replaced in the popliteal fossa, the original popliteal lymph node environment. During this manipulation the efferent lymphatics and the blood supply were unaffected. 2 wk after replacing the nodes into the popliteal fossa, we examined the nodes of the two groups by immunohistochemistry. 20 min before excision of the nodes, India ink was injected into the ipsilateral footpad to detect reconnected afferent lymphatic vessels. In the group that had their lymph nodes replaced in the popliteal fossa, three out of four lymph nodes contained a small amount of India ink, demonstrating partial restoration of afferent lymphatic vessels. Although these nodes were small in size, staining with mAb specific for T and B cells showed a well-compartmentalized T cell zone, and a B

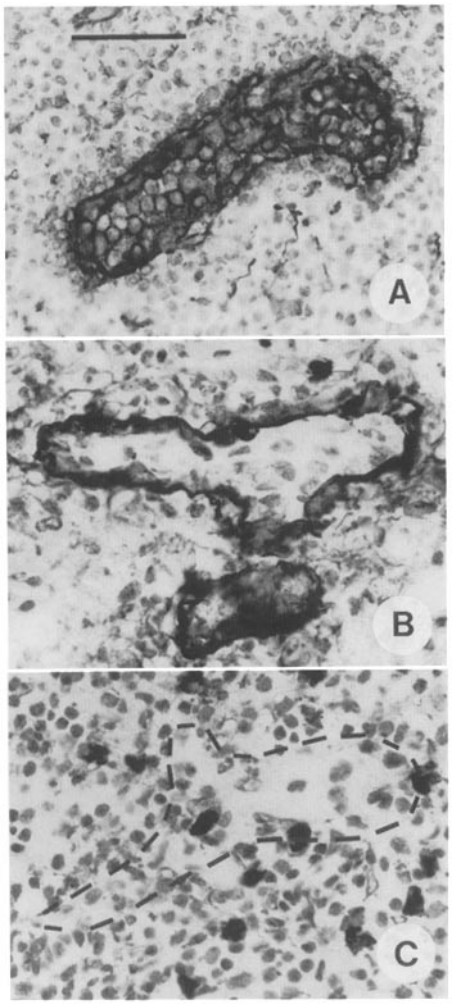


Figure 8. Expression of the peripheral lymph node addressin and MECA-325 antigen by vessels in control and operated lymph nodes (day 7). The morphologically distinct HEV of control lymph nodes, which express high levels of the peripheral lymph node addressin (*A*), are quite distinct from their counterparts in operated lymph nodes. *B* illustrates the modified expression of the peripheral lymph node addressin in lymph nodes 7 d after interruption of afferent lymphatics. The vessels became flat walled, and expression of the peripheral lymph node addressin appeared restricted to the abluminal aspect of the vessels. At 7 d after occlusion of afferent lymphatics the vessels did not show any expression of the MECA-325 antigen (*C*). Bar, 40 μm.

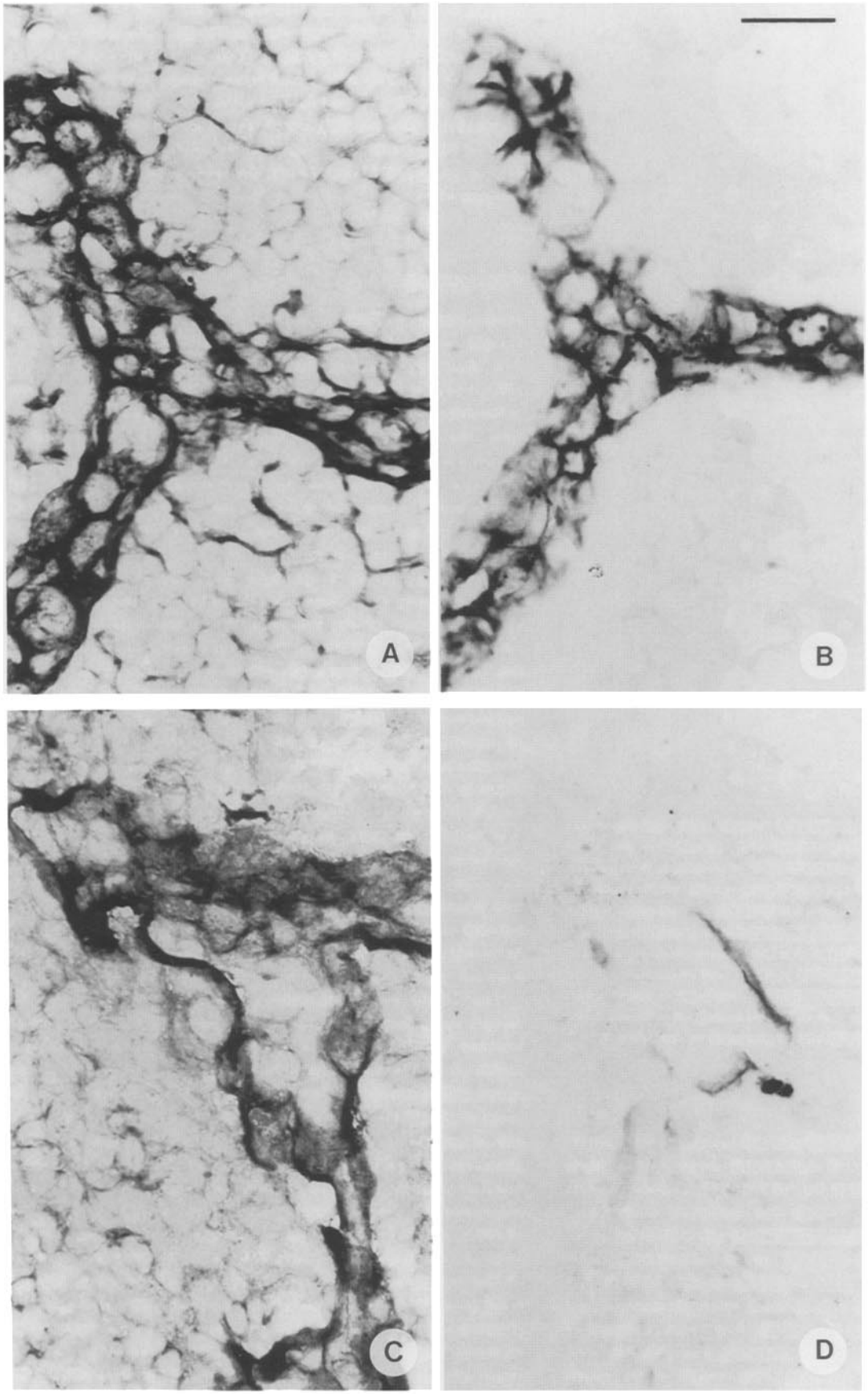
cell area comparable to normal control lymph nodes. Interdigitating cells in the T cell dependent areas within these nodes were strongly positive for NLDC-145, and these cells were abundant (Fig. 14). Within this relatively short time-period, we also observed a restoration of the MOMA-1-positive subpopulation of macrophages in the subcapsular sinus (Fig. 14). Staining with the HEV-specific marker MECA-325 showed high walled HEV, which were strongly positive for the peripheral lymph node addressin. The node replaced in the popliteal fossa that had not reconnected its afferent lymphatics was too small for analysis. The lymph nodes which were left on top of the fascia for 3 wk, rather than being replaced in the popliteal fossa, showed small B cell compartments, with B cells present in the T cell zones. No MOMA-1-positive subcapsular sinus macrophages were seen in these nodes and NLDC-145-positive cells were not as well represented as in nodes which had been replaced in the popliteal fossa. Interestingly, while India ink did not drain from the footpad into these nodes, we observed an intermediate phenotype of HEV, with both high and flat walled vessels expressing the peripheral lymph node addressin and the MECA-325 antigen (Fig. 15).

Discussion

Interruption of afferent lymphatic vessels to mouse popliteal lymph nodes affected the function and phenotype of HEV. 7 d after occlusion of the lymph supply to lymph nodes, HEV became flat walled and expression of the HEV-specific differentiation marker MECA-325 had disappeared. At this time, the peripheral lymph node addressin was restricted in most cases to the abluminal side of the vessels, a site where it would not be available to circulating lymphocytes, and functional in vitro and in vivo assays confirmed that these vessels did not support normal levels of lymphocyte adhesions and homing, respectively.

Removing afferent lymphatic vessels had a traumatic effect on lymphocyte entry into lymph nodes: 5 h after lymphatic vessel interruption the entrance of labeled cells had diminished by 70% compared to the contralateral control node in the same animal. This early effect of surgery, likely related to trauma to operated nodes, was short lived, with recovery occurring 4–5 d after surgery. After 5 d, however, the lack of incoming lymph appeared to begin to have an effect on lymph node function. At this timepoint the entrance of lymphocytes was still comparable to the contralateral control nodes. However, lymphocyte homing in different animals was highly variable, suggesting that changes occur around this time. Immunohistologic assessment of lymph nodes at 4 d after surgery also showed significant variation: both high and flatwalled vessels were observed, and some venules exhibited a partial loss of the peripheral lymph node addressin. In most cases, however, peripheral addressin was still expressed on the luminal side of the venules.

The observed abluminal expression of the peripheral lymph node addressin, the flat appearance of the HEV, and the low trafficking of lymphocytes into operated lymph nodes at 8 d after afferent lymphatic occlusion are all in agreement with each other. Absence of the peripheral lymph node addressin on the luminal aspect of the vessels prevents the adhesion of lymphocytes to HEV. As a consequence lymphocytes cannot enter the nodes, which is shown in in vivo homing studies. Previous studies have shown that decreased migration of lymphocytes across HEV can cause flattening of HEV, which become high walled again after reconstitution with cells that migrate across HEV (7, 23). Our observation of flat walled venules in lymph nodes not receiving afferent lymph could therefore be a consequence of impaired lymphocyte migration across the venular wall. Our experiments however showed that the effect of afferent lymphatic vessel interruption on HEV morphology could be reversed when the nodes were left on top of the fascia for an additional 2 wk. These data suggest that, if lymph-borne factors or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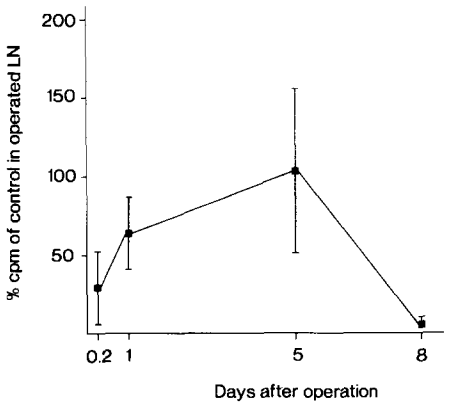

Figure 10. In vivo lymphocyte homing to operated lymph nodes. Afferent lymphatics to popliteal lymph nodes were interrupted and ^{51}Cr-labeled lymphocytes were injected intravenously at 5 h, 1, 5, or 8 d after operation. 1 h after injection of labeled lymphocytes recipients were killed and cell localization in operated and contralateral control nodes was determined by quantitating the cpm in these lymph nodes. Relative lymphocyte localization in operated lymph nodes is expressed as a percent of lymphocytes localizing in the contralateral control nodes. Three to six animals were included in each group. Occlusion of afferent lymphatic vessels resulted in an early reduction in lymphocyte homing into operated lymph nodes. This reduction was reversed during the days following occlusion. A pronounced effect of lymphatic occlusion on lymphocyte homing into operated lymph nodes was observed at day 8 after afferent lymphatic vessel occlusion.

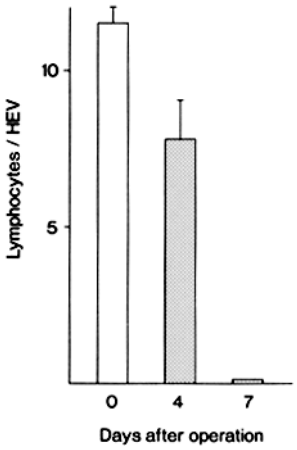

Figure 11. In vitro binding of lymphocytes to HEV in operated and control lymph nodes. Interruption of afferent lymphatics induced a 30% decrease (±SD) in binding of lymphocytes to HEV at day 4 compared to control nodes (day 0), measured in an in vitro binding assay. At 7 d after afferent lymphatic occlusion no specific lymphocyte binding to vessels in the nodes was observed.

cells are responsible for HEV function, they may be arriving by way of newly connected lymphatics.

Alternatively, HEV morphology and function could be regulated by resident cells within lymph nodes, or factors

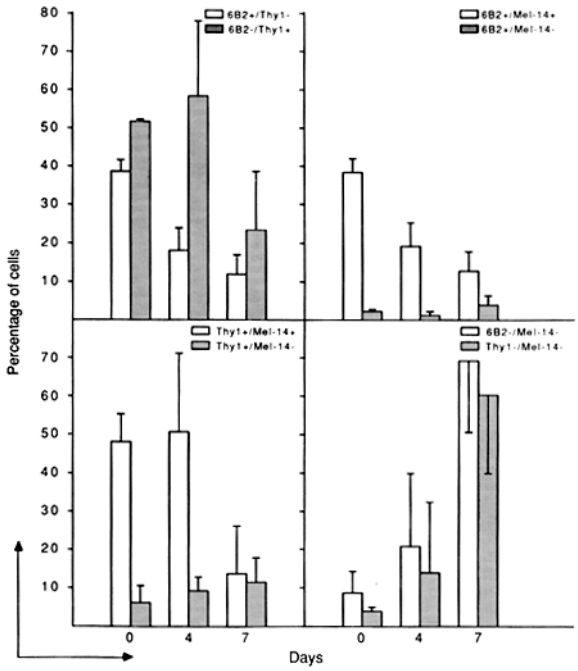

Figure 12. Phenotypic characterization of cell populations in control and operated lymph nodes on days 4 and 7 after afferent lymphatic vessel interruption. Cells were stained for Thy-1.2, B220, and MEL-14, which recognizes the peripheral lymph node homing receptor LECAM-1, and 5×10^3 cells were analyzed by FACS. 5–10 separate lymph nodes were analyzed at each time point. Data are expressed as the mean percentage of cells positive or negative for each mAb (±SD).

produced by such cells. The effects of afferent lymph on lymph node macrophage populations have been described previously in rats (10) and it was suggested that macrophages are directly or indirectly responsible for HEV function. Previously we reported in the rat system that subcapsular ED3 macrophages disappeared upon interruption of afferent lymphatic vessels (17). In the present study, this finding was confirmed for MOMA-1 positive subcapsular macrophages in the mouse. However, when HEV regained their characteristic morphology and phenotype in lymph nodes in the subcutaneous site at 3 wk after surgery, this population of macrophages was not replaced, revealing that the presence of this lymph node macrophage population is not required for HEV differentiation. The MOMA-1-positive subcapsular macrophage population was restored rapidly however, when lymph nodes were replaced in the popliteal fossa, and afferent lymphatics allowed to connect. These data suggest that MOMA-1-positive cells in the lymph node turn over rapidly, and that they can be replaced by cells entering via afferent lymphatics draining the footpad.

Figure 9. Analysis of the presence of the peripheral lymph node addressin on the luminal aspect of HEV in control nodes (*A* and *B*) and operated nodes (*C* and *D*) at day 7 after afferent lymphatic occlusion. Briefly, 100 μg of MECA-79 was administered intravenously into mice. Recipients were killed 15 min after injection and gently perfused with HBSS to remove unbound antibody from the vasculature. Membrane-bound MECA-79 was visualized on sections by immunoperoxidase staining with anti-rat IgM (*B* and *D*). HEV were shown by incubation of serial sections with mAb MECA-79 followed by an immunoperoxidase staining with anti-rat IgM (*A* and *C*). The luminal expression of the peripheral lymph node addressin, as seen in the control node (*B*) was confirmed to be absent in the operated node at day 7 (*D*). Thus, the peripheral lymph node addressin expression observed in this node (*C*) is associated with the abluminal aspect of the HEV. Bar, 16 μm.

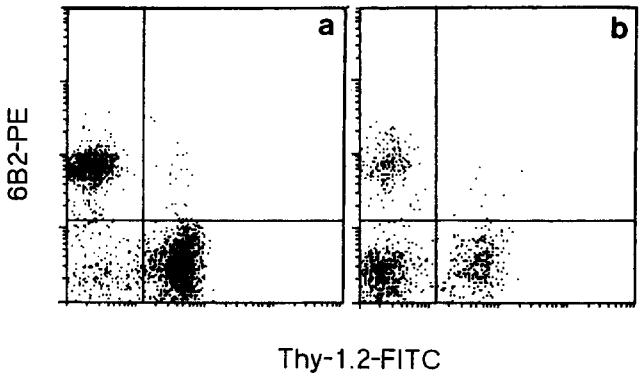

Figure 13. Phenotypic characterization of different cell populations in control and operated lymph nodes. Per lymph node 5×10^3 cells were analyzed and cells other than small, viable cells were gated out. (*a*) In control lymph nodes, B cells, as defined by mAb 6B2, and T cells, as defined by Thy-1.2, represented 37 and 51% of the recovered cells, respectively. Double negative cells represented roughly 10% of the cells obtained from these lymph nodes. (*b*) In operated lymph nodes (7 d after occlusion), the distribution was markedly different, with B cells and T cells representing 20 and 27% of the recovered cells, respectively. Roughly 50% of the cells was negative for both Thy-1.2 and 6B2.

While the physiological processes controlling HEV induction and vascular addressin expression are unclear, a role for interdigitating cells in the HEV induction process has been suggested (3). Our experiments, in which lymph nodes were left on top of the fascia for longer periods support these findings. 7 d after disconnection of afferent lymphatics, the NLDC-145 antigen was weakly expressed on interdigitating cells and HEV had become flat walled. Interdigitating cells might either have left the lymph node or lost their ability to express the NLDC-145 antigen as a consequence of afferent lymphatic vessel occlusion. At 3 wk after surgery increased levels, as compared to 1 wk after occlusion, of the NLDC-145 antigen was observed in lymph nodes with both high and flat walled vessels expressing the MECA-325 antigen and the peripheral lymph node addressin. The correlation between NLDC-145 antigen expression and HEV induction, including peripheral lymph node addressin expression, suggests a role for interdigitating cells in this inductive process.

The importance of soluble factors in HEV differentiation and function should also be considered. Experiments in which we diminished the entrance of soluble factors and/or cells to a greatest extent by surrounding the nodes with parafilm, revealed lymph nodes with HEV which became flat walled more rapidly, and had fewer LECAM-1 expressing cells than lymph nodes without parafilm (results not shown).

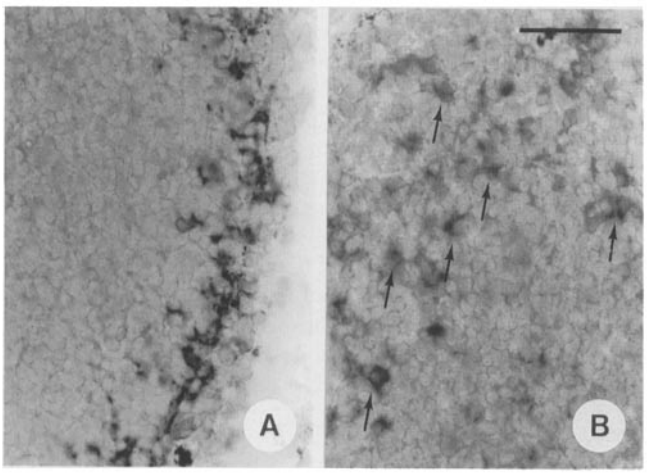

Figure 14. Macrophages in the subcapsular sinus and interdigitating cells in the paracortical area of an operated lymph node that was replaced in the popliteal fossa 1 wk after operation and examined 2 wk after replacement of the node in the popliteal fossa. The MOMA-1-positive subpopulation of macrophages in the subcapsular sinus was restored within 2 wk (*A*) and numerous interdigitating cells (*arrows*), strongly positive for mAb NLDC-145, could be observed in the T cell-dependent area (*B*). Bar, 40 μm.

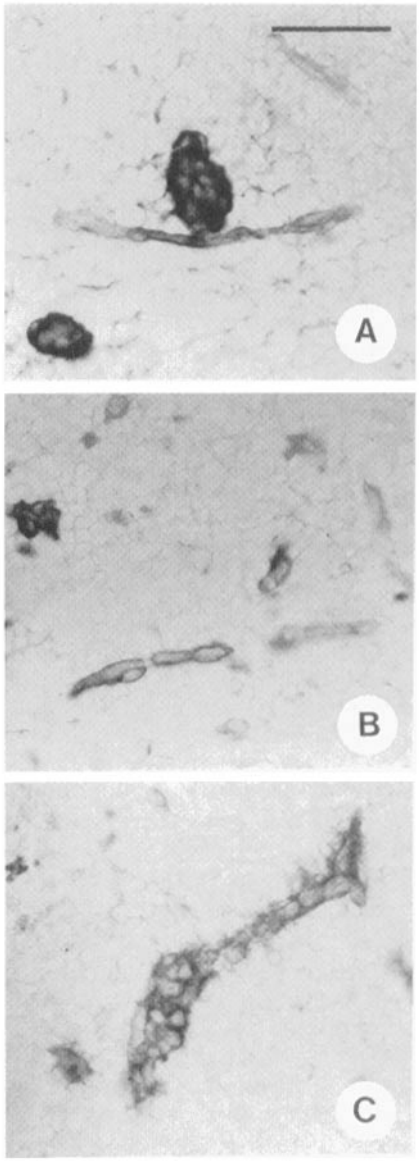

Figure 15. Expression of the peripheral lymph node addressin (*A* and *B*) and the MECA-325 antigen (*C*) on vessels in operated lymph nodes (day 21). Both flat and high walled vessels expressing the peripheral lymph node addressin and the MECA-325 antigen could be observed. Bar, 40 μm.

4 d after interruption of the afferent lymph supply to lymph nodes, a decrease in the percentage of LECAM-1 expressing B cells was observed. By contrast, a decrease in the percentage of LECAM-1 expressing T cells was not observed until day 7 after occlusion. It should be mentioned that operated lymph nodes as a whole had decreased in size by day 4 postsurgery. The observed early decrease in the percentage of LECAM-1 expressing B cells in operated lymph nodes could be explained by a direct effect of lymph flow on lymphocyte migration to B cell follicles. B cells home to follicles in a highly selective manner, migrating against lymph flow (8), and having a variable transit time (5). Interruption of the lymph flow may disrupt lymph node compartmentalization by preventing the directed migration of B cells to follicular regions. Those B cells not localizing in follicles may leave the node more rapidly. Our observation of increased numbers of B cells within the T cell regions is consistent with an inability of B cells to localize in follicular regions. Alternatively, interruption of lymph flow may directly induce a loss of follicular integrity, a scenario which would also explain both the presence of B cells in the T cell zones, and the rapid depletion of B cells from the lymph node. In contrast to our studies, investigations of prolonged thoracic duct cannulation indicated that T cells leave lymph nodes more rapidly than B cells (19). These studies differ from our own, since the afferent lymph supply was intact.

In conclusion, our experiments demonstrate the crucial role of lymph-borne cells and/or factors in the presence, and in some cases, the phenotype of cell populations within lymph nodes. Upon afferent lymphatic occlusion, venules that normally support lymphocyte migration lose their high walled phenotype, they show decreased expression of the peripheral lymph node addressin on the luminal side, and they do not support high levels of lymphocyte homing. Lymph node leukocyte populations are also influenced, with dramatic losses of B cells, T cells, subcapsular sinus macrophages, and interdigitating cells. While these leukocyte population shifts are due, at least in part, to reduced cellular trafficking into lymph nodes via the blood, a direct effect of lymph components on cellular compartmentalization and cellular retention within lymph nodes is also possible. These observations reveal that lymph node homeostasis is maintained by the complex cellular and molecular interactions that occur in normal lymph nodes; interactions that are dramatically altered by afferent lymphatic occlusion.

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