Changes in high endothelial venules in lymph nodes after vascularized and non-vascularized lymph node transfer in a murine autograft model

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Synopsis

This is the first experimental study to compare changes in the quantitative histology of transferred lymph nodes with or without vascularization in an animal model of vascularized lymph node transfer. We found that vascularized lymph node transfer preserved more functional high endothelial venules within the transferred lymph node than did non-vascularized lymph node transfer when spontaneous afferent lymphatic reconnection was obtained. Both vascularization and afferent lymphatic reconnection were needed for functional regeneration of the transferred lymph node.

Abstract

Background and Objectives: Vascularized lymph node transfer (LNT) is gaining popularity in the treatment of lymphedema. However, it is unclear whether vascularization of transferred lymph nodes (LNs) contributes to functional improvement. High endothelial venules (HEVs) are specialized vessels that allow lymphocytes to enter LNs. In this study, we compared the numbers of HEVs and lymphocytes in LNs after vascularized and non-vascularized LNT.

Methods: Fifty mice were divided into three groups (group 1, pedicled vascularized LNT; group 2, pedicled non-vascularized LNT; group 3, free non-vascularized LNT). Afferent lymphatic reconnection was confirmed by patent blue staining. The transferred LNs were harvested 4 weeks after surgery. HEVs, B-cells, and T-cells were subjected to immunohistochemical staining and quantified.

Results: Afferent lymphatic reconnection was observed in 13/20 transferred LNs in group 1, 11/15 in group 2, and 7/15 in group 3. The ratio of dilated/total HEVs in transferred LNs with afferent lymphatic reconnection was significantly higher in group 1 than in groups 2 and 3. No significant differences in numbers of B-cells and T-cells were found in the transferred LNs.

Conclusions: We found that more functional HEVs were preserved in cases with successful afferent lymphatic reconnection after vascularized LNT than after non-vascularized LNT.

Keywords: laboratory animal model; lymph nodes; mice; transplantation; vascularized composite allotransplantation

1 INTRODUCTION

The lymphatic system is a one-way transport network that returns interstitial fluid to the blood circulation. Lymphatic vessels also import soluble molecules and activated antigen-presenting cells from the periphery to the lymph nodes (LNs), where immune responses are triggered, and immune effector cells and humoral response factors are exported to the blood circulation [1]. Defects in lymphatic function can lead to impaired immune responses, accumulation of lymph in tissues, and persistent soft tissue swelling known as lymphedema.

Lymphedema can be either primary or secondary to another condition, such as cancer treatment with lymphadenectomy and/or radiotherapy, trauma, or infection [2]. Current management of lymphedema is based on complex decongestive therapy that consists of meticulous skin care, manual lymph drainage, compression bandaging, and remedial exercises [3,4]. Surgery can be an option for symptomatic patients resistant to conservative management, and may be a debulking or physiologic procedure [5]. In recent years, debulking procedures have given way to physiologic procedures, such as lymphovenous anastomosis and vascularized lymph node transfer (LNT), which have gained popularity with advances in microsurgical techniques [6]. Vascularized LNT is considered more effective than lymphovenous anastomosis for advanced lymphedema [7-10].

The physiologic function of vascularized LNT involves lymphangiogenesis and lymphovenous communications within the LNs [11,12]. Lymphangiogenesis generates new lymphatic vessels from pre-existing lymphatics or lymphatic endothelial progenitors, allowing recanalization of lymphatic vessels between the recipient site and the flap. Lymphovenous communications can pump lymph into the venous circulation [13-15]. Lymphovenous communications within the LNs are located at the high endothelial venules (HEVs) and allow lymphocytes to enter the LNs from the blood circulation [16]. Lymphatic

flow is important to maintain the immune and hemodynamic functions of the LN and its microarchitecture [12,17-21]. Inflammatory mediators transported via afferent channels to the LN are important for maintaining the phenotype of the plump cuboidal endothelial cells in the HEVs [22].

Using a murine model inoculated with B16F10 melanoma cells, we recently demonstrated the immune-mediated antitumor effect of non-vascularized LN autotransplantation through activation of T-cells, showing the expansion of lymphocytes and a change in the lymphocyte population [23]. In that study, the function of the transplanted LN was affected by the change in HEVs brought about by decreased vascularity [23], so maintaining vascularization may also be important for regeneration of HEVs to maintain the immune functions of the LN. Accordingly, we evaluated the feasibility of pedicled vascularized LNT in a murine model with postoperative assessment of lymphatic flow using indocyanine green lymphography [24].

Although vascularized LNT is becoming widely used for treating lymphedema [25-28], experimental studies comparing vascularized and non-vascularized LNT are still lacking in the literature [29]. Furthermore, despite promising clinical results in reducing episodes of infection after vascularized LNT [30], the immune aspects of transferred LNs have not been thoroughly investigated histologically. The aim of this study was to determine the structural characteristics of intranodal HEVs after vascularized and non-vascularized LNT in our established murine model.

2 MATERIALS AND METHODS

2.1 Study design

All animal experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals at Hokkaido University. All procedures were performed with the

approval of the Institutional Animal Care and Use Committee at Hokkaido University. Fifty 8-week-old male C57BL/6N mice (Japan SLC, Inc., Hamamatsu, Japan) were used to create the murine model, and were housed in a controlled environment with food and water ad libitum. All experiments were performed under general anesthesia with isoflurane inhalation at 2.5%. Three experimental groups were created that differed only in the way the LNs were transferred: group 1, pedicled vascularized LNT (n = 20); group 2, pedicled non-vascularized LNT (n = 15); and group 3, free non-vascularized LNT (n = 15). Each transferred LN was named as follows: group 1, vascularized LN (VLN); group 2, non-VLN; and group 3, free non-vascularized LN (FLN). Sample size was determined based on similar animal studies [31,32], as well as our previous experience of pedicled vascularized LNT in a murine model [24].

2.2 Surgical procedures and tissue harvesting

The procedures followed in group 1 were as described previously [24]. In brief, an inguinal lymph node (ILN)-bearing flap with a vascular pedicle containing the superficial caudal epigastric vessels was transferred into the ipsilateral popliteal fossa after excision of the popliteal lymph node (PLN). The fat tissue around the transferred LN was then sutured to the biceps femoris muscle at 3 points using 10-0 nylon. In group 2, the procedures were essentially the same as in group 1, but the vascular pedicle was ligated using 10-0 nylon before being transferred to test the influence of the pedicle flap, which consisted of inguinal fat tissue. In group 3, an excised ILN was transferred into the ipsilateral popliteal fossa as a free graft without revascularization after excision of the PLN. The muscles covering the popliteal fossa were then sutured using 10-0 nylon to keep the transferred LN in place. Four weeks postoperatively, 5 μL of 2% patent blue solution was injected subcutaneously into the ipsilateral hindlimb paw, and each transferred LN was observed for staining to confirm

afferent lymphatic reconnection. The transferred LN (n = 50) and the contralateral intact ILN (n = 10) were then collected with the surrounding soft tissue. Each transferred LN was subcategorized as with or without afferent lymphatic reconnection based on the staining of the LN.

2.3 Histology and immunohistochemistry

Specimens were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned at 4 μm throughout the LN. The sections were stained using hematoxylin and eosin and immunohistochemical stains for MECA-79 (HEVs), B220 (B-cells), and CD3 (T-cells). Sections were deparaffinized in xylene (two changes, 5 min each), and rehydrated in graded ethanol solutions (100%, 95%, 80%, and 70%; 3 min each). Antigen retrieval was performed by incubating the sections in pH 6 citrate buffer (Dako REAL™ Target Retrieval Solution; Dako, Glostrup, Denmark; code S2031, 1:10) or pH 9 buffer (Nichirei Biosciences, Tokyo, Japan; code 415211, 1:10) for 20 min in a 95°C water bath.

The following primary antibodies were used: rat monoclonal MECA-79 antibody (clone MECA-79; Santa Cruz Biotechnology, Santa Cruz, CA; code sc-19602, 1:50) overnight at 4°C; purified rat monoclonal CD45R/B220 antibody (clone RA3-6B2; Abgent, San Diego, CA; code WTA1265, 1:3000); and rabbit monoclonal CD3 antibody (clone EPR4517; GeneTex, Irvine, CA; code GTX62682, 1:1500) for 60 min at room temperature (RT).

For MECA-79 and CD3 staining, the sections were then incubated with goat anti-rat or anti-rabbit secondary antibody conjugated with horseradish peroxidase (Histofine® Simple Stain™ Mouse MAX PO (Rat), code 414311F; and Histofine® Simple Stain™ Mouse MAX PO (R), Nichirei Biosciences, code 414341F, respectively) for 30 min at RT. For B220 staining, sections were incubated with biotinylated rabbit anti-rat IgG secondary antibody (Vector Laboratories, Burlingame, CA, code BA-4000, 1:200) for 30 min at RT, and then

incubated with VECTASTAIN® *Elite*® ABC Kit (Vector Laboratories, code PK-6100) for 30 min at RT. All the sections were finally developed using 3,3′-diaminobenzidine chromogen (Histofine SAB-PO (M) Kit, Nichirei Biosciences, code 425011) for 5 min at RT, counterstained with hematoxylin for 1.5 min, dehydrated, and mounted.

2.4 Histologic and immunohistochemical analysis

All histologic slides were scanned using a whole slide scanner (NanoZoomer S210; Hamamatsu Photonics, Hamamatsu, Japan), and the digital images were analyzed using NDP.view2 software. The size of each LN was measured in the largest hematoxylin and eosin-stained cross-sections. The numbers of total HEVs (defined as having an outline >100 μm^2) and dilated HEVs (defined as having a lumen >80 μm^2) [33,34] were counted for each LN. We defined 80 μm^2 as the minimum luminal cross-sectional area for functional HEVs, consistent with a previous report [33]. The ratio of the number of dilated HEVs to the total number of HEVs was also calculated [34].

After JPEG images were captured at 5× magnification, the percentages of B-cells and T-cells in the total LN area were quantified using ImageJ software (version 1.52; National Institutes of Health, Bethesda, MD). The acquired images were split into three-color channels (red, green, and blue). The blue image was then binarized by applying a threshold. The Analyze Particles settings were set to pixel size 4-infinity and circularity 0.00–1.00.

2.5 Statistical analysis

Continuous data are presented as the median (interquartile range). The transferred LNs with and without afferent lymphatic reconnection in each group were compared using the Wilcoxon rank-sum test. Multiple pairwise comparisons between the transferred LNs with afferent lymphatic reconnection and the contralateral intact ILN were performed using the

Steel-Dwass test. The statistical analysis was performed using JMP software (version 14.1.0; SAS Institute Inc., Cary, NC). Statistical significance was set at $P < 0.05$ (two-tailed).

3 RESULTS

All mice tolerated the procedures without postoperative complications until sacrifice at 4 weeks postoperatively. As shown in Figure 1, afferent lymphatic reconnection was observed in 13/20 (65.0%) transferred LNs in group 1, 11/15 (66.7%) in group 2, and 7/15 (46.7%) in group 3. The measurements are summarized in Table 1 with comparisons within each group.

3.1 Size of the LNs

The sizes of the transferred LNs with or without afferent lymphatic reconnection were as follows: group 1, 0.33 (0.21-0.46) mm^2 vs 0.75 (0.34-2.13) mm^2, $P = 0.048$; group 2, 0.23 (0.21-0.45) mm^2 vs 0.46 (0.11-0.93) mm^2; and group 3, 0.50 (0.21-0.93) mm^2 vs 0.30 (0.25-0.47) mm^2, respectively. The size of the ILN was 2.76 (2.15-3.88) mm^2. All the transferred LNs with afferent lymphatic reconnection showed a significant regression in size when compared with the ILN (all, $P < 0.01$).

3.2 Numbers of HEVs in the LNs

The numbers of total HEVs/mm^2 in the transferred LNs with or without afferent lymphatic reconnection were as follows: group 1, 50.9 (36.9-61.0) vs 14.6 (5.8-24.4), $P = 0.002$; group 2, 54.3 (18.1-78.6) vs 29.8 (4.5-63.4); and group 3, 49.7 (20.1-53.9) vs 12.3 (7.3-15.9), $P = 0.009$, respectively. The number of total HEVs/mm^2 in the ILN was 22.9 (21.3-27.6).

The numbers of dilated HEVs/mm^2 in the transferred LNs with or without afferent lymphatic reconnection were as follows: group 1, 16.5 (9.7-22.9) vs 2.4 (0-2.8), $P < 0.001$; group 2, 2.5 (0-14.6) vs 4.1 (0.3-9.1); and group 3, 2.0 (0-16.7) vs 0 (0-1.5), respectively. The number of

dilated HEVs/mm^2 in the ILN was 4.7 (3.4-7.3).

The numbers of total and dilated HEVs/mm^2 in the transferred LNs with afferent lymphatic reconnection of group 1 were significantly higher than those in the ILN ($P < 0.001$ and $P = 0.006$, respectively), as shown in Figure 3.

3.3 Ratio of dilated/total HEVs of the LNs

The ratio of dilated/total HEVs in the transferred LNs with or without afferent lymphatic reconnection was as follows: group 1, 0.33 (0.25-0.41) vs 0.07 (0-0.14), $P = 0.01$; group 2, 0.05 (0-0.19) vs 0.13 (0.03-0.14); and group 3, 0.10 (0-0.21) vs 0 (0-0.09), respectively. The ratio of dilated/total HEVs in the ILN was 0.21 (0.15-0.31).

The ratio of dilated/total HEVs in the transferred LNs with afferent lymphatic reconnection was significantly higher in group 1 than that in group 2 and group 3 ($P = 0.004$ and $P = 0.049$, respectively), as shown in Figure 4.

3.4 Percentages of B-cells and T-cells in the LNs

No significant differences in the percentages of B-cells and T-cells were found between the transferred LNs (Table 1). However, clear follicle formation was observed in the cortex of the transferred LNs with afferent lymphatic reconnection but not in the cortex of those without afferent lymphatic reconnection (Figure 5).

4 DISCUSSION

In this murine model, we found that vascularized LNT was more likely to preserve functional HEVs in the transferred LN than non-vascularized LNT 4 weeks after surgery. As in the clinical reports, this restoration of lymphovenous communications within LNs can likely be attributed to better lymph drainage from peripheral tissues and immune function in the

transferred LNs. To our knowledge, this is the first experimental study to compare changes in the quantitative histology of transferred LNs with or without vascularization in an animal model of vascularized LNT.

4.1 Afferent lymphatic reconnection in transferred LNs

Afferent lymphatic reconnection in transferred LNs with surrounding lymphatic vessels occurs spontaneously, at reported rates of 22% to 57% [19,32]. In a rat model of vascularized ILN transfer to the popliteal fossa, Rabson et al. reported that the ideal method of dissection was to skeletonize and expose the donor ILN and excise the native PLN and all surrounding fat from the recipient site, which had a 57% success rate of afferent lymphatic reconnection [32]. In our murine model, which involved skeletonizing the donor ILN and excising the PLN without the surrounding fat tissue, the success rate was 65.0%–66.7% for pedicled vascularized LNT (group 1) and for non-vascularized LNT (group 2), but was lower at 46.7% for free non-vascularized LNT (group 3). This finding is in contrast with that of another study that showed 100% afferent lymphatic reconnection of free non-vascularized PLNs transplanted into their original position [35]. This discrepancy may reflect the ectopic site of transplantation and transfer of LNs of different sizes and shapes to the recipient sites.

4.2 Regeneration of transferred LNs

The regeneration of LNs has been a major concern after transplantation, although most of the studies transferred LNs as non-vascularized free grafts [19,36,37]. In early rat models of LNT, VLNs showed preserved normal histology, whereas non-VLNs became fibrotic or necrotic within 6 weeks [31,32]. However, in rat and murine models of LNT, non-VLNs with or without fragmentation regenerated within 3–8 weeks [38,39]. These differences may reflect the numbers of transferred LNs, the sites of transplantation, or the timing of regeneration

[40,41]. Our finding of more regression in size with clear follicles in the transferred VLNs with afferent lymphatic reconnection than in those without suggests remodeling and immune competence in these LNs. Thus, maintaining vascularization may contribute to their survival but not always to their functional regeneration.

4.3 Regeneration of HEVs in transferred LNs

Naive lymphocytes recirculate through the secondary lymphoid organs, including LNs, in search of their cognate antigens. Antigen and/or antigen-presenting cells are transported to the LNs from peripheral tissues through afferent lymphatic vessels, whereas naive lymphocytes enter LNs from the blood via HEVs and exit via efferent lymphatic vessels (and sometimes via HEVs) [42]. Therefore, HEVs are important structures for immune defense against pathogens [43]. Although the importance of lymphatic flow for the maintenance of HEVs is well documented [22,44-47], evidence supporting the need for vascularization of transferred LNs is still lacking for the maintenance of HEVs.

The regeneration of HEVs in transferred LNs has been studied in various animal models [39,48-50]. Liu et al. showed less prominent HEVs with decreased follicles 1 month after vascularized mesenteric LNT without afferent lymphatic reconnection, and barely detectable HEVs at 3 months using a rat allograft model [48]. In contrast, Sasaki et al. found that HEVs appeared 10 days after non-vascularized mesenteric LNT with fragmentation, and the regeneration of transplanted LN fragments was structurally complete with normal HEVs on day 28 in a rat autograft model [50]. Using a murine model inoculated with B16F10 melanoma cells, we recently demonstrated that the lumens of HEVs in a non-vascularized autotransplanted PLN were not dilated when compared with those in an intact PLN 6 weeks after surgery in the premetastatic phase [23].

In our study, the numbers of total and dilated HEVs per unit area in the VLN with afferent

lymphatic reconnection were significantly higher than those in the ILN. These results indicate that VLN with afferent lymphatic reconnection preserved HEVs against regression in size. As for the ratio of dilated/total HEVs, VLN with afferent lymphatic reconnection was preserved more in dilated HEVs than non-VLN and FLN with afferent lymphatic reconnection. The results of our study indicate that both vascularization and afferent lymphatic reconnection of the transferred LNs may be essential for their complete functional regeneration, because these two routes are entry sites for immune cells and soluble molecules [39].

4.4 Limitations

This study has some limitations. First, our murine model was not of lymphedema and the size of murine LNs was smaller than those in humans. However, vascularized LNT is performed clinically in patients with moderate to advanced lymphedema as a LN-bearing flap. Second, we evaluated the transferred LNs at a single time point 4 weeks after surgery, and a longer period may be necessary to observe regeneration of transferred LNs. Third, we histologically evaluated the numbers of HEVs/mm^2 with a definition of luminal area > 80 μm^2 as functional HEVs, because functional analyses of LNs, such as cytotoxicity assay, make it impossible to confirm their histology. Fourth, antigenic stimulation may be needed to evaluate immune cells in transferred LNs. Further research with longer follow-up is required to clarify the functional significance of vascularization of transferred LNs and for comparison with exogenous stimulation by means of functional analyses of LNs.

5 CONCLUSIONS

Using a murine model, we found that vascularized LNT preserved more functional HEVs within the transferred LN than did non-vascularized LNT when spontaneous afferent

lymphatic reconnection was obtained. Both vascularization and afferent lymphatic reconnection were needed for functional regeneration of the transferred LN.

DISCLOSURES

The authors declare that there are no conflicts of interest and nothing to disclose.

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ABBREVIATIONS

FLN, free non-vascularized lymph node

HEVs, high endothelial venules

ILN, inguinal lymph node

LN, lymph node

LNT, lymph node transfer

PLN, popliteal lymph node

VLN, vascularized lymph node

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Figure Legends

Figure 1. Schematic drawings of each group of lymph node transfers and subcategorization based on lymph nodes (LNs) with (+) and without (-) afferent lymphatic reconnection (denoted by an asterisk). FLN, free non-vascularized LN; VLN, vascularized LN.

Figure 2. Immunohistochemical staining of high endothelial venules (HEVs) using MECA-79 antibody in the transferred lymph nodes (LNs) harvested 4 weeks after surgery. **A-D**, gross inspection at 5× magnification. Bars, 250 μm. **E-H**, 40× magnifications of the boxed region in the panel. Bars, 50 μm. FLN, free non-vascularized LN; VLN, vascularized LN; (+), with afferent lymphatic reconnection; (-), without afferent lymphatic reconnection.

Figure 3. Numbers of total and dilated high endothelial venules (HEVs)/mm^2 in the contralateral intact inguinal lymph node (ILN) and the transferred lymph nodes (LNs) with afferent lymphatic reconnection (+). Data are presented as box-and-whisker plots. Boxes represent interquartile ranges (25th to 75th quartiles), the horizontal line across a box shows the median, and whiskers extend from the box to the highest and lowest non-outlier values. Outliers are shown as a circle. *, $P = 0.006$ and **, $P < 0.001$ (Steel-Dwass test). FLN, free non-vascularized LN; VLN, vascularized LN.

Figure 4. The ratio of dilated/total high endothelial venules (HEVs) in the contralateral intact inguinal lymph node (ILN) and the transferred lymph nodes (LNs) with afferent lymphatic reconnection (+). Data are presented as box-and-whisker plots. Boxes represent interquartile ranges (25th to 75th quartiles), the horizontal line across a box shows the median, and

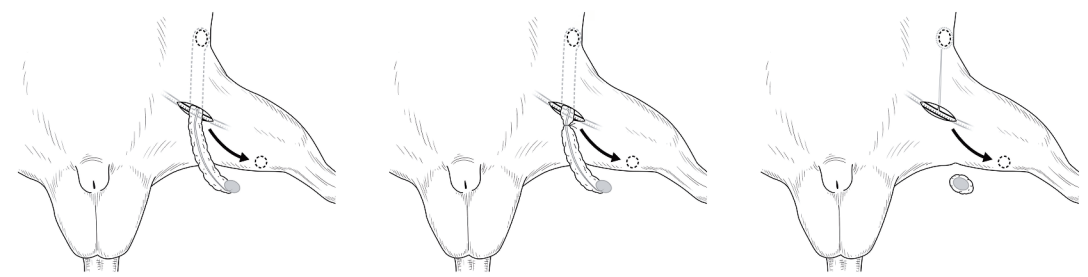
whiskers extend from the box to the highest and lowest values. *, $P = 0.049$ and **, $P = 0.004$ (Steel-Dwass test). FLN, free non-vascularized LN; VLN, vascularized LN.

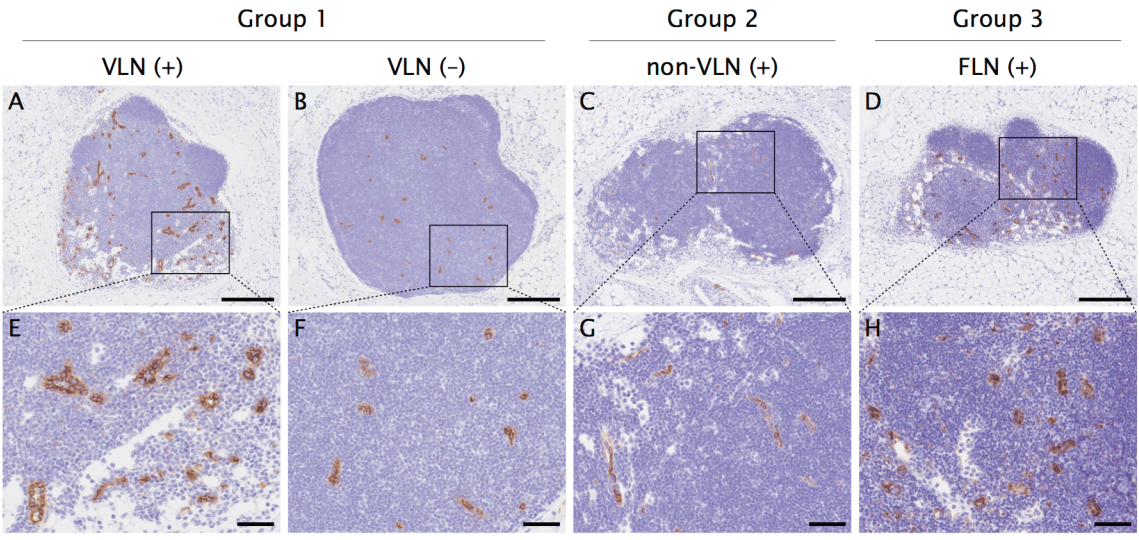
Figure 5. Immunohistochemical staining of B-cells and T-cells using B220 (**A**-**D**) and CD3 (**E-H**) antibodies, respectively, in the transferred lymph nodes (LNs) harvested 4 weeks after surgery. Gross inspections at 5× magnification. Bars, 250 μm. FLN, free non-vascularized LN; VLN, vascularized LN; (+), with afferent lymphatic reconnection; (-), without afferent lymphatic reconnection.

TABLE 1 Histologic evaluation of lymph nodes

	Control	Group 1			Group 2			Group 3		
	ILN	VLN (+)	VLN (-)	*P*	non-VLN (+)	non-VLN (-)	*P*	FLN (+)	FLN (-)	*P*
n	10	13	7		11	4		7	8	
Size (mm^2)	2.76	0.33	0.75	0.048	0.23	0.46	0.84	0.50	0.30	0.52
	(2.15-3.88)	(0.21-0.46)	(0.34-2.13)		(0.21-0.45)	(0.11-0.93)		(0.21-0.93)	(0.25-0.47)	
Numbers of HEVs/mm^2										
Total (A)	22.9	50.9	14.6	0.002	54.3	29.8	0.21	49.7	12.3	0.009
	(21.3-27.6)	(36.9-61.0)	(5.8-24.4)		(18.1-78.6)	(4.5-63.4)		(20.1-53.9)	(7.3-15.9)	
Dilated (B)	4.7	16.5	2.4	< 0.001	2.5	4.1	0.89	2.0	0	0.08
	(3.4-7.3)	(9.7-22.9)	(0-2.8)		(0-14.6)	(0.3-9.1)		(0-16.7)	(0-1.5)	
Ratio of HEVs (B/A)	0.21	0.33	0.07	0.01	0.05	0.13	0.95	0.10	0	0.19
	(0.15-0.31)	(0.25-0.41)	(0-0.14)		(0-0.19)	(0.03-0.14)		(0-0.21)	(0-0.09)	
Cell percentages										
B220+ (B-cells)	26.4	27.9	41.4	0.11	21.6	34.4	0.21	36.2	47.6	0.18
	(24.1-31.9)	(19.6-31.2)	(19.7-53.2)		(13.2-38.7)	(24.7-59.4)		(27.9-43.3)	(29.8-61.9)	
CD3+ (T-cells)	21.6	12.7	24.0	0.08	24.6	22.7	0.65	23.9	35.7	0.12
	(17.4-30.2)	(3.5-23.2)	(15.3-43.9)		(2.1-29.9)	(2.2-55.0)		(12.0-32.2)	(20.8-53.8)	

Continuous data are presented as the median (interquartile range). Comparisons between the transferred lymph nodes (LNs) in each group were performed using the two-tailed Wilcoxon rank-sum test. FLN, free non-vascularized LN; HEV, high endothelial venule; ILN, inguinal LN; VLN, vascularized LN;(+), with afferent lymphatic reconnection; (-), without afferent lymphatic reconnection.


Group 1
(n = 20)
+
*
–
VLN (+)
VLN (–)
n = 13
n = 7
Group 2
(n = 15)
+
*
–
non-VLN (+)
non-VLN (–)
n = 11
n = 4
Group 3
(n = 15)
+
*
–
FLN (+)
FLN (–)
n = 7
n = 8


Group 1
Group 2
Group 3
VLN (+)
VLN (–)
non-VLN (+)
FLN (+)
A
B
C
D
E
F
G
H

Numbers of total and dilated HEVs (/mm²)

100
80
60
40
20
0

**
*

Total HEVs
Dilated HEVs

ILN
Control

VLN (+)
Group 1

non-VLN (+)
Group 2

FLN (+)
Group 3

The ratio of dilated/total HEVs

0.5
0.4
0.3
0.2
0.1
0

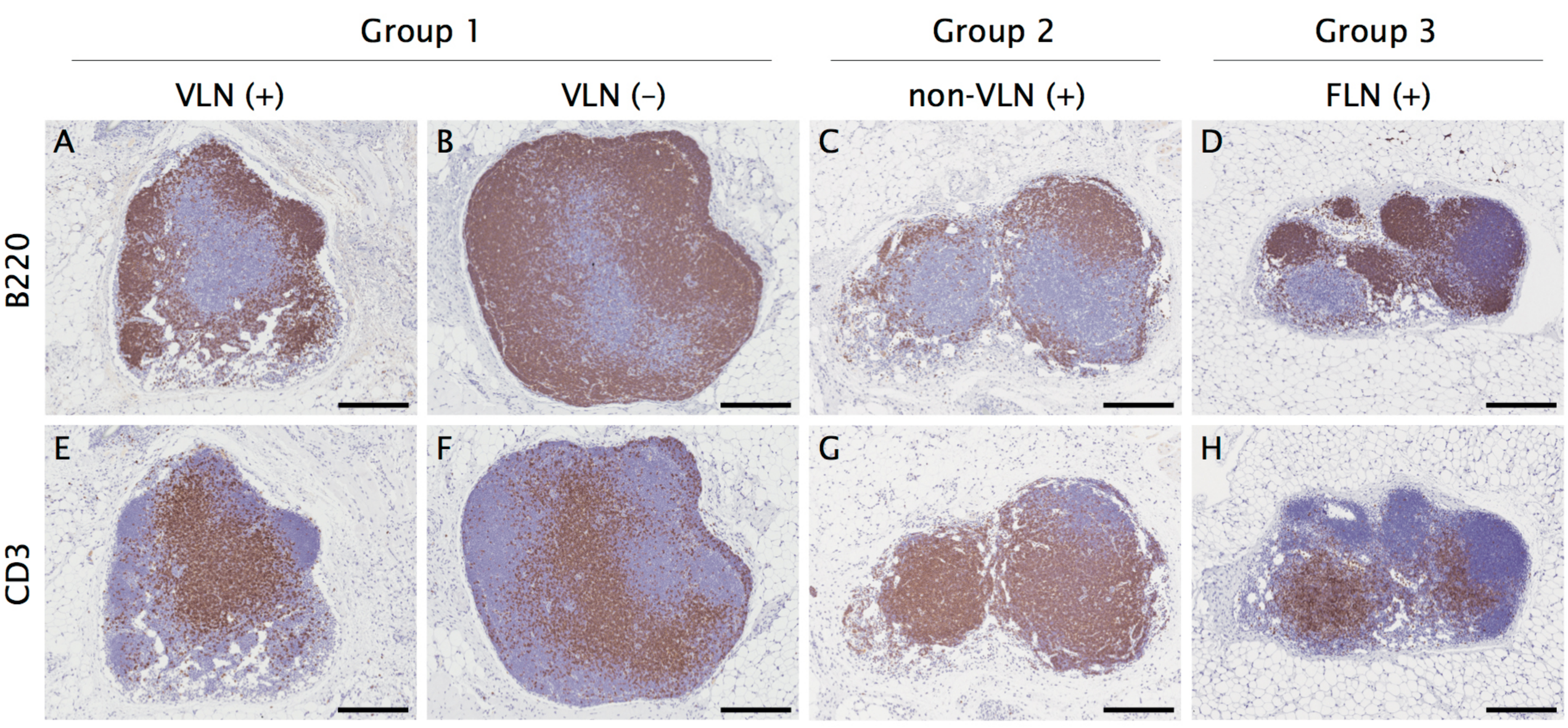
*
**

ILN
Control

VLN (+)
Group 1

non-VLN (+)
Group 2

FLN (+)
Group 3


Group 1
Group 2
Group 3
VLN (+)
VLN (–)
non-VLN (+)
FLN (+)
B220
CD3
A
B
C
D
E
F
G
H