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Regular Article

Distribution of Contractile Structures in a Mouse and Human Lymph Node Associated with Lymph Flow

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Lymph nodes have contractile structures, but their distribution in a lymph node has been less considered in terms of facilitation of lymph flow. Axillary, inguinal, and mesenteric lymph nodes were collected from mice and human cadavers, and their sections were immunostained for alpha-smooth muscle actin (αSMA) and high molecular weight caldesmon (H-caldesmon). The αSMA-positive cells were localized in the capsule beneath the ceiling epithelium on the afferent side in both mice and humans. We found an additional layer of the αSMA-positive cells in the human lymph node, surrounding the inner layer perpendicularly. H-caldesmon was expressed only in these cells of the outer layer. In some human lymph nodes highly containing fat tissue in the medulla, the capsule disappeared on the efferent side, resulting in a disrupted sinusoidal lymph pathway. These findings suggest that human lymph nodes have additional smooth muscles in the outer region of the capsule to facilitate lymph flow. The αSMA-positive cells in the outer and inner layers of human lymph nodes probably have different functions in contraction. The presence of lipomatosis in a human lymph node will reduce its contribution to the lymph flow.

Key words: lipomatosis, lymphedema, lymph node, smooth muscle

I. Introduction

Lymphatic fluid needs to flow from the peripheral tissues towards a specific site, the venous angle, at the bottom of the neck without a central pump like the heart. Lymphadenectomy in the axillary or inguinal region causes damage to the lymphatic pathway and stagnation of the lymph flow, resulting in lymphedema in the ipsilateral limb. Additionally, the lymph node (LN) itself helps the lymph flow by smooth muscles within it [10, 12, 19], and transplantation of the LN is a treatment option for lymphedema [15]. Lymphatic fluid is collected through some afferent lymphatic vessels to an LN and gets out of the LN to several efferent lymphatic vessels at the hilum. Color or power Doppler ultrasound can detect the flow in LNs and distinguish malignant LNs [9, 30, 33, 34]. Therefore, it is crucial to understand the contractile ability of the LN to generate lymph flow.

It is well known that the lymph flow is primarily created by the pumping action of skeletal muscles surrounding lymphatic vessels and of smooth muscles present in the lymphatic vessels. However, the amount of skeletal muscles in the upper and lower limbs varies, and the visceral lymphatic systems cannot rely on the action of the skeletal muscles. Therefore, it is hypothesized that the contractile ability of the lymphatic system varies depending on its anatomical location in a body. A previous physiological study reported the differences in the contractile ability of lymphatic vessels in different body regions, including contraction frequency, diameter change, and outflow resistance [14]. However, there is limited information on the differ-

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ences in the contractile ability of LNs. Although one study reported that inguinal LNs contained more smooth muscle cells [4], they focused on the occurrence of a rare benign neoplasm called palisaded myofibroblastoma.

Previous studies using computed tomography, magnetic resonance imaging, and ultrasound found that even normal human LNs have fat accumulation in the medulla [1, 3, 5, 9, 11, 16], known as lipomatosis. Lipomatosis is more common in aged LNs [17, 26], and a recent study showed that the lipomatosis starts in the medulla by transdifferentiation from interstitial cells to fat tissues [2]. However, its relationship with the contractility of the LN is unclear.

In this study, we investigated the distribution of contractile cells expressing alpha smooth muscle actin (αSMA) and high molecular weight caldesmon (H-caldesmon) in the LNs collected from the axillary, inguinal, and mesenteric regions of the mouse and human cadaver to better understand the characteristics of human LNs.

II. Materials and Methods

This study was approved by the Institutional Review Board of Tokyo Medical University (study approval no. T2020-0050) for using human cadavers and by Institutional Animal Care and Use Committee of Tokyo Medical University (Permission #R3-0060) for using mice. C57BL/6J mice were purchased from Japan SLC, Inc. and maintained at a specific pathogen-free facility at Tokyo Medical University. They were kept at 21–25 degC and 40%–60% relative humidity with a 12-hour light-dark cycle.

Human cadavers (male: 3, female: 3, age: 76–98) that were fixed by perfusion of 3.8% (w/v) formaldehyde (Maskedform B®, Japan Tanner Corporation) were dissected, and three LNs were removed in each anatomical region (superficial axillary LN, superficial inguinal LN, and mesenteric LN of the small intestine). The LNs were also collected from six male mice at ten weeks old and fixed in modified Davidson's fluid [20] overnight at ambient temperature (20–25°C). According to a previous study [28], these tissues were embedded in paraffin and were cut into 5-μm-thick. These sections were used for the detection of αSMA and H-caldesmon. Immunohistochemistry was performed as in the previous study [28]. The primary antibody was anti-αSMA (dilution at 1:400, ab5694, RRID: AB_2223021, Abcam) and anti-H-caldesmon, clone hHCD (dilution at 1:1,000, C4562, RRID: AB_258866, Sigma-Aldrich). The slides were digitized with a virtual slide scanner (Panoramic MIDI II, 3DHISTECH) at 20× magnification.

III. Results

Distribution of αSMA-positive cells in the murine LNs

To determine the basic distribution of αSMA-positive cells in an LN, we first examined mouse LNs as a model. The LNs in the mouse can be divided into four histological regions; the capsule, cortex, paracortex, and medulla. The immunopositive reaction for αSMA was found in the capsule at the afferent side and paracortex, except for vessels (Fig. 1a–c). The capsule consisted of the "ceiling" epithelial cells covering the subcapsular sinus and the surrounding mesenchymal cells in the connective tissues. The αSMA-positive cells were found beneath the ceiling epithelial cells in two or three layers (Fig. 1d–f). The cortex containing the lymphatic follicle was negative for αSMA (Fig. 1d–f), but in the paracortex "dendritic" positive reactions, indicating the reticular cells, were observed (Fig. 1g–i). The medulla was negative, except for vessels (Fig. 1j–l). The capsule near the efferent lymphatic vessel showed negative for αSMA, although immunopositive reactions were found around the efferent lymphatic vessels (Fig. 1m–o). There were no differences in the distribution of αSMA-positive cells among LNs collected from different body sites.

Distribution of αSMA-positive cells in the human LNs

The connective tissue in the capsule of human LNs was thicker compared to that of mice, and the αSMA-positive cells were scattered in the capsule, although their orientation was parallel, similar to that in mice (Fig. 2a–f). The layer of the positive cells was also observed in the trabecula (Fig. 2d–f). We found additional clusters of αSMA-positive cells in the outer region of the capsule, perpendicular to the inner layers (Fig. 2d–f). Positive reactions were also observed in the reticular cells in the cortex and paracortex (Fig. 2d–i). However, the lymphatic follicle was negative, consistent with previous studies that showed the follicle containing the germinal center was negative for actin or desmin in human LNs [4, 6, 29, 32]. The medulla and capsule near the efferent lymphatic vessels were negative for αSMA, similar to the findings in mice (Fig. 2j–o).

Distribution of H-caldesmon-positive cells in the human and mouse LN

We examined the distribution of another marker protein, H-caldesmon, which is more specific to smooth muscle cells rather than nonmuscle cells like reticular cells [21, 31]. In human LNs, the number of H-caldesmon-positive cells were obviously smaller than that of αSMA-positive cells (Fig. 3a). The reticular cells were negative for H-caldesmon (Fig. 3b–e), and the positive reactions were found in the capsule (Fig. 3b). Unlike the distribution of αSMA, H-caldesmon was localized only in the outer layer of the capsule that surrounded the inner layer perpendicularly, although the inner layer showed negative on both afferent and efferent side (Fig. 3b, e). Similarly, there were less positive cells in the mouse LNs compared to the αSMA-positive cells (Fig. 3f). Although smooth muscle cells around the artery showed positive for H-caldesmon (Fig. 3g), no positive cells were found in the capsule even in the afferent side (Fig. 3h).

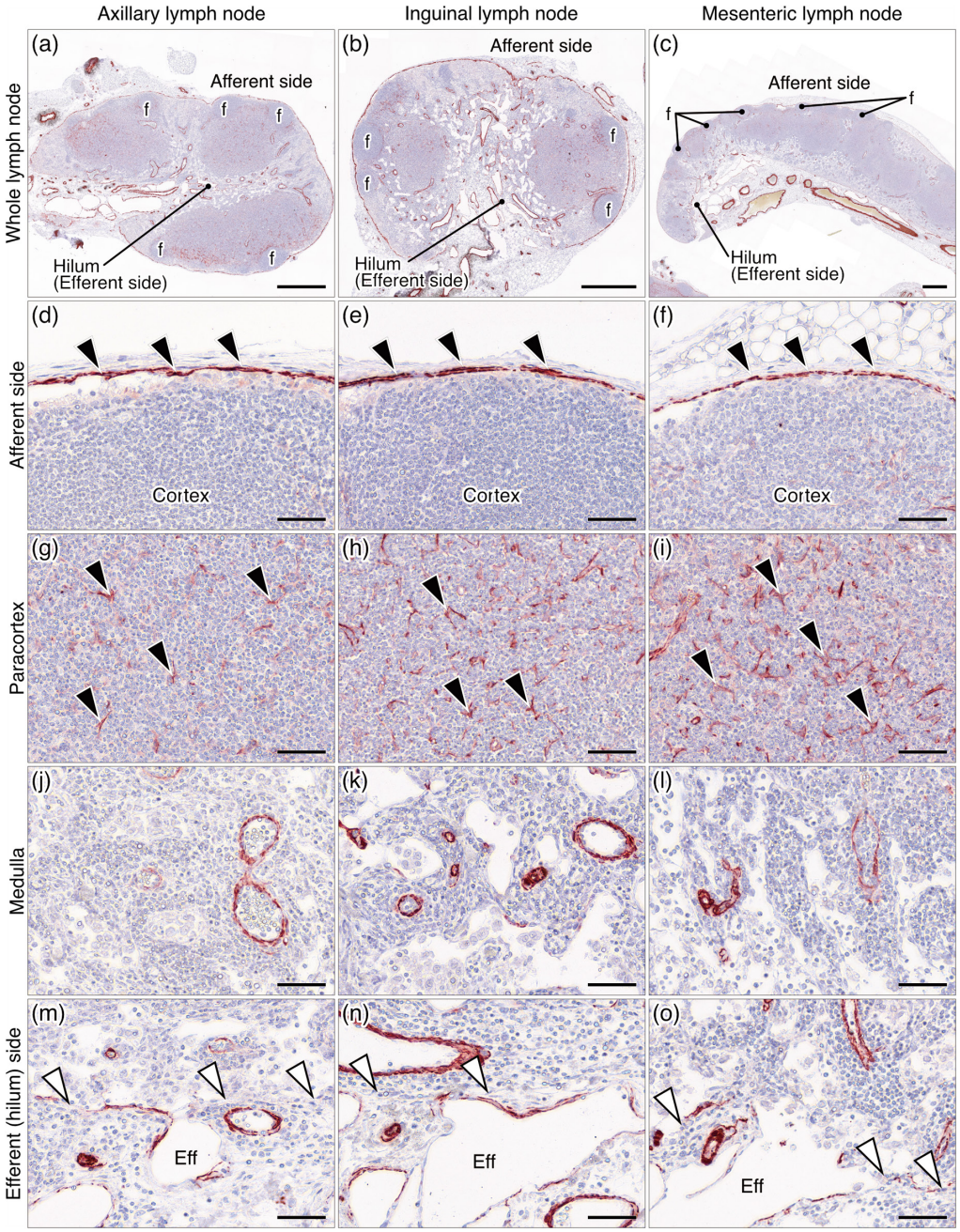

Fig. 1. Representative images for distribution of immunopositive cells for alpha-smooth muscle actin in mouse lymph nodes. (**a–c**) An image of the whole lymph node. f: Lymphatic follicle (**d–i**) Immunopositive cells (black arrowheads) are found in the capsule on the afferent side (**d–f**) and paracortex (**g–i**). (**j–o**) There are no positive cells except for blood vessels in the medulla (**j–l**) and the capsule on the efferent side (**m–o**). White arrowheads indicate the negative area beneath ceiling epithelial cells. Eff: Efferent lymphatic vessel. Bar = 500 μm (**a–c**), 50 μm (**d–o**).

Distribution of αSMA-positive cells in the human LNs with lipomatosis

We noted that some axillary and inguinal LNs had fat accumulation in the medulla, varying degrees of severity (Fig. 4). These LNs were identified as showing lipomatosis. Their capsule on the efferent side could not be identified (Fig. 4a), and lymphocytes were also present outside the capsule in some cases (Fig. 4b). In the most severe case, the medulla was replaced by fat tissue, and the cortex was thin (Fig. 4c). Although there was a capsule-like structure visible due to the alignment of the αSMA-positive cells, the ceiling epithelium was indistinguishable, and the subcapsular sinus was absent (Fig. 4d). The area without the capsule on the efferent side was wider in these LNs than in the ones with mild lipomatosis (Fig. 4e). We did not find any correlation between the degree of lipomatosis and the amount of subcutaneous adipose tissues.

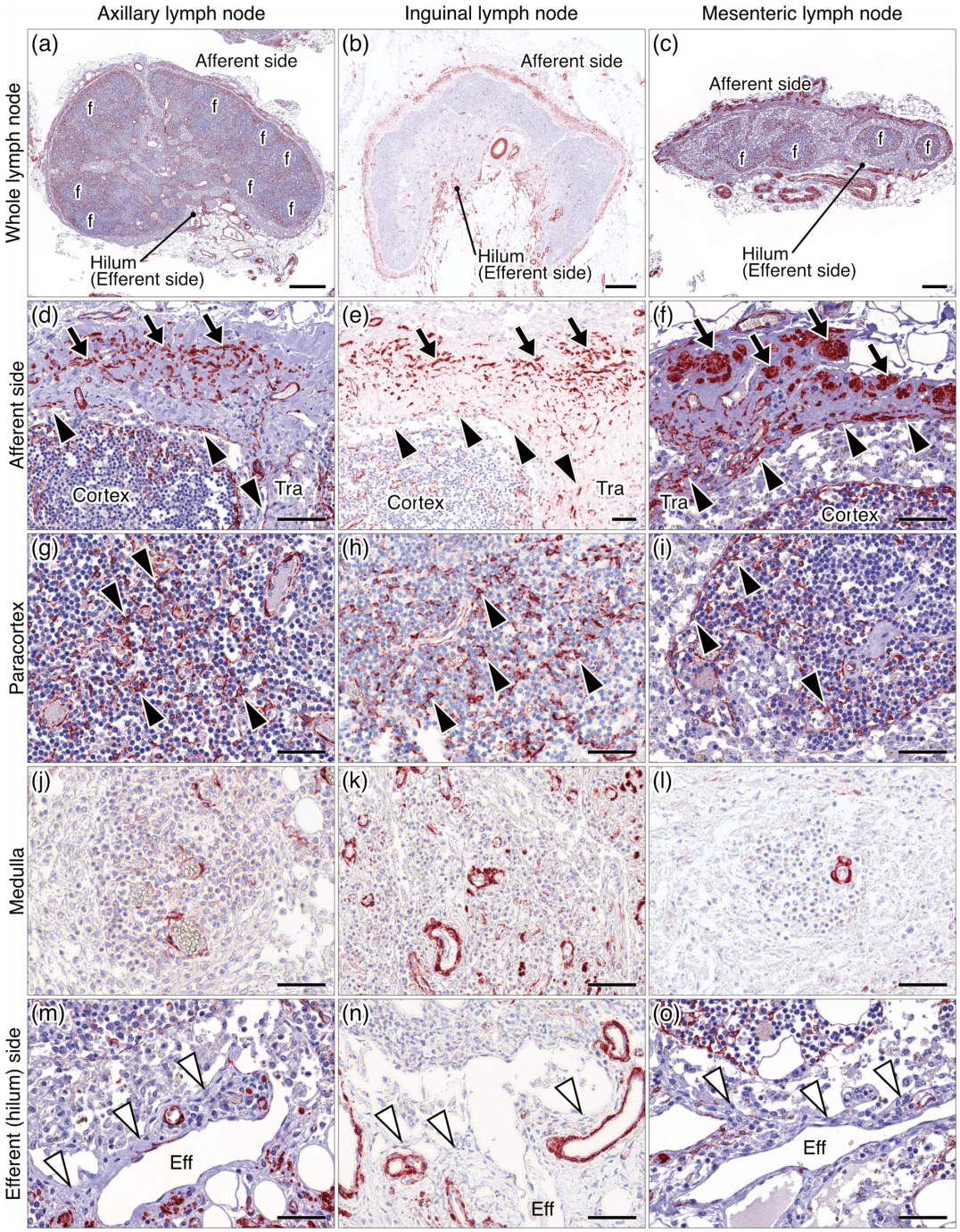

Fig. 2. Representative images for distribution of immunopositive cells for alpha-smooth muscle actin in human lymph nodes. (**a–c**) An image of the whole lymph node. f: Lymphatic follicle (**d–f**) Mesenchymal cells beneath the ceiling epithelium in the capsule on the afferent side and trabecula (Tra) show immunopositive (black arrowheads). Additional clusters of the immunopositive cells (arrows) are found in the outer region perpendicular to the inner layers. (**g–i**) Mesenchymal cells (black arrowheads) in the paracortex show immunopositive. (**j–o**) In the medulla (**j–l**) and the capsule on the efferent side (**m–o**), there are no positive cells except for blood vessels. White arrowheads indicate the negative area beneath ceiling epithelial cells. Eff: Efferent lymphatic vessel. Bar = 500 μm (**a–c**), 50 μm (**d–o**).

IV. Discussion

This study investigated the characteristics of LNs in the mouse and human with a special focus on contractile structures. Key findings of the present study are as follows: 1) αSMA-positive cells are present in the paracortex and capsule on the afferent side; 2) there is an outer layer of the αSMA-positive cells surrounding inner layer perpendicularly in human LNs; 3) H-caldesmon-positive smooth muscle cells are localized only in the outer layer of the capsule

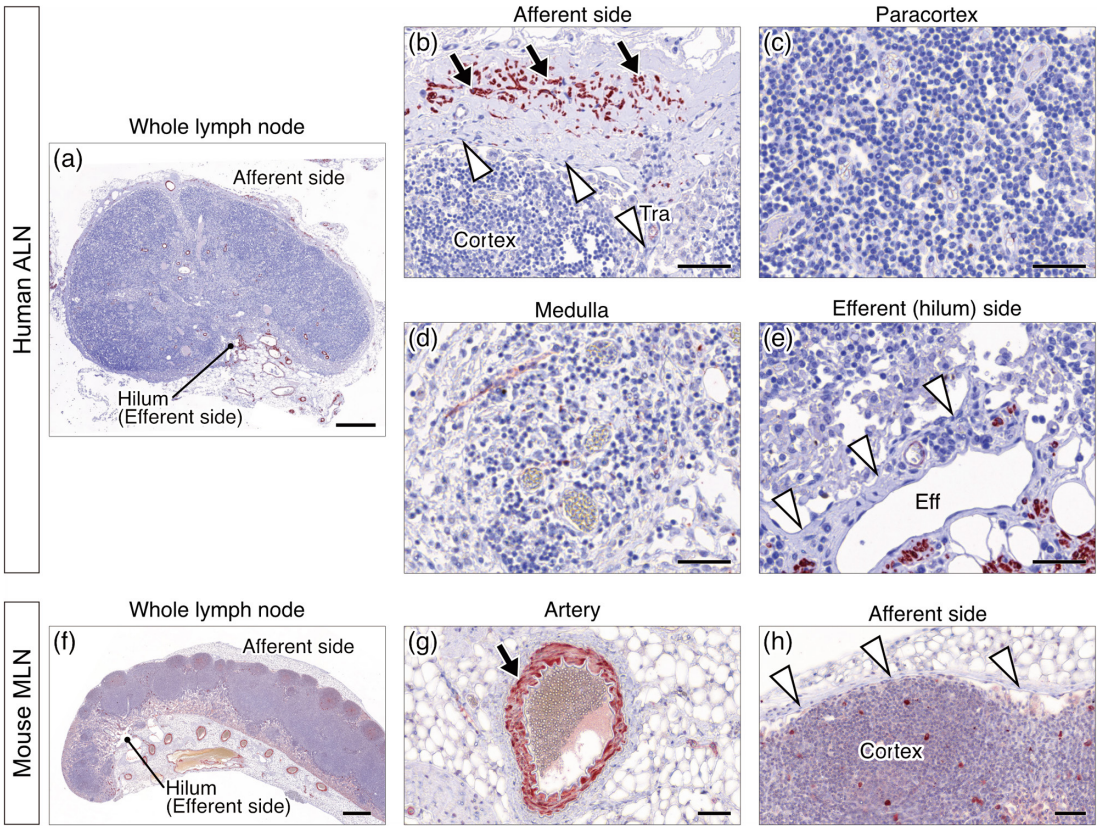

Fig. 3. Representative images showing immunopositive cells for H-caldesmon in the human axillary lymph node (ALN) and mouse mesenteric lymph node (MLN). In the MLN, there are some false-positive cells by using anti-mouse IgG antibody as a secondary antibody. Arrows and arrowheads indicate positive and negative cells, respectively. Eff: Efferent lymphatic vessel. Bar = 500 μm (**a, f**), 50 μm (**b–e, g, h**).

in human LNs; 4) the distribution of αSMA-positive cells is consistent across all body locations examined; and 5) lacking the capsule on the efferent side and sinusoidal lymph pathway is observed in LNs with lipomatosis.

This study showed the presence of αSMA-positive but H-caldesmon-negative cells in the paracortex of LNs. Lymphocytes in the paracortex adhere to the reticular fiber produced by reticular cells. It has been reported that reticular cells are similar to the myofibroblast containing actin and myosin [27], and another study has shown that interstitial cells in LNs are positive for actin [4]. By using αSMA as a marker for smooth muscle-like cells, it was found that reticular cells in the paracortex have a contractile structure, suggesting that the reticular cells contribute to the release of lymphocytes into the lymph flow [27] rather than the generation of the lymph flow itself.

The αSMA-positive cells were also found in the capsule on the afferent side. H-caldesmon, a marker for smooth muscle cells, was also detected in some of these cells in the human LN, consistent with a previous study [32]. Other tissues, such as the spleen and testis, also possess a capsule that contains smooth muscle cells [8, 27]. It has been reported that smooth muscle cells in the LN's capsule are independent of smooth muscle cells surrounding the lymphatic and vascular vessels [10, 12]. Therefore, the smooth muscles in the capsule are probably a specific structure that produces lymph flow in the LN. In fact, physiological studies have shown the contractility of the LN's capsule [23–25]. The capsule of the LNs was observed to be thick on the afferent side but loose on the efferent side, consistent with previous studies [13, 18]. We found that the capsule on the afferent side contained more αSMA-positive cells, suggesting that the contraction of the smooth muscles on the afferent side makes an LN shrunk, pushing the lymphatic fluid to the efferent lymphatic vessels in the hilum.

We discovered the αSMA-positive cells in the outer layer of the human LN capsule but not in mice. These cells were also positive for H-caldesmon, but those in the inner layer and in the capsule in the mouse LN were negative. Previous studies also reported the presence of contractile cells in the capsule of several species [10, 12, 19, 29], but their orientation was only parallel, similar to the inner layer of the human LNs shown in the present study. As far as we know, no report has clearly shown the outer layer of smooth muscle cells in the capsule of LNs. Although two previous studies reported "randomly oriented" smooth muscle cells in the caprine, bovine, and human [10, 12], We cannot confirm their similarity to the outer layer of the αSMA-positive cells in the present study. Therefore, the additional smooth muscles in the outer layer may have

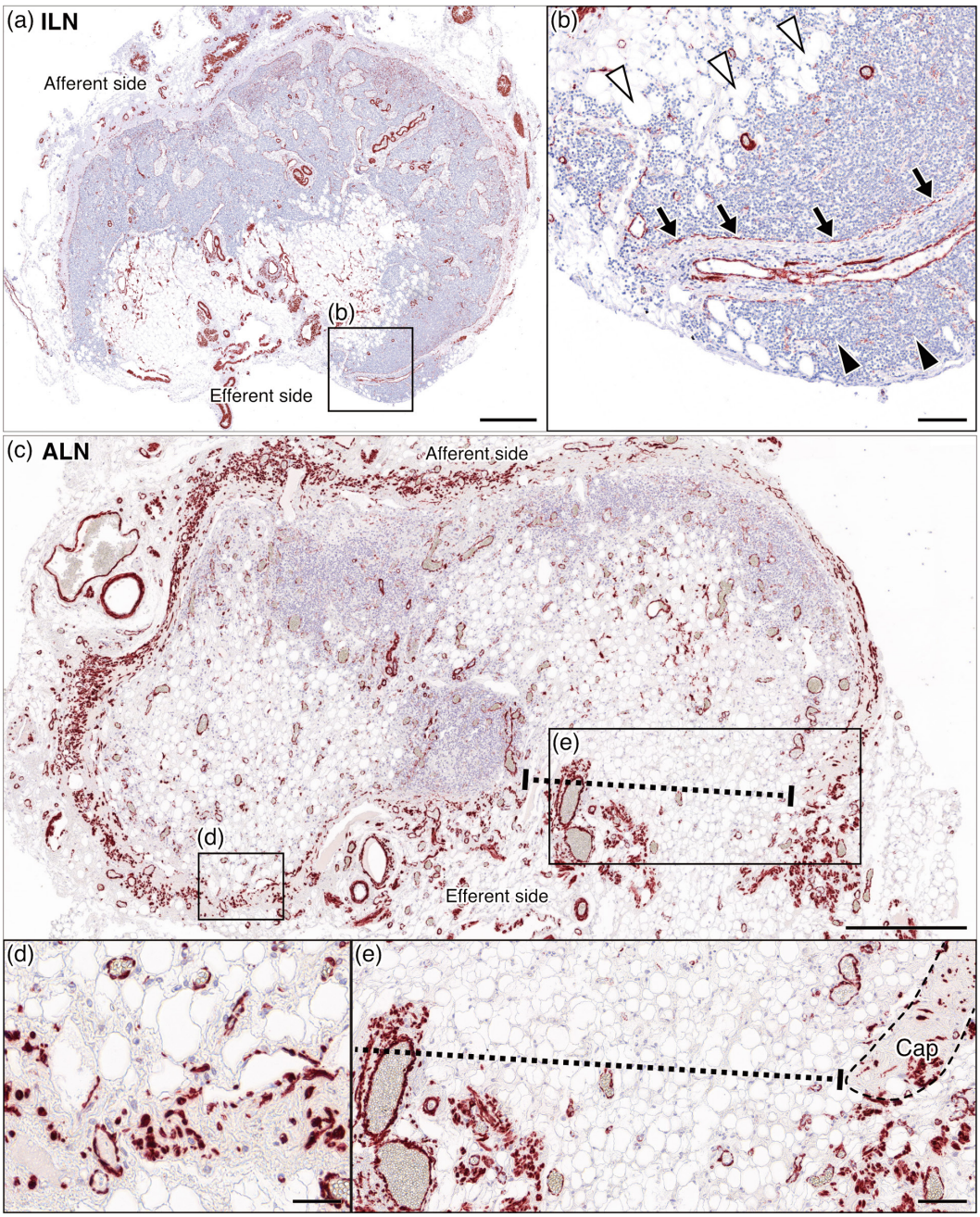

Fig. 4. Representative images of human lymph nodes with fat accumulation. (**a**) A whole image of the inguinal lymph node (ILN) with small fat accumulation. (**b**) A magnified view of the boxed area in (**a**) shows a lack of the capsule and the presence of fat tissues (white arrowheads) and lymphocytes outside of the capsule (black arrowheads). Arrows indicate the ceiling epithelium on the capsule. (**c**) A whole image of the axillary lymph node (ALN) with severe fat accumulation. The dashed line indicates the area without a capsule on the efferent side. (**d**) A magnified view of the boxed area in (**c**) shows the absence of the ceiling epithelium and subcapsular sinus. (**e**) Another magnified view of the boxed area in (**c**) shows a wide area without the capsule (Cap). Bar = 500 μm (**a, c**), 100 μm (**b, e**), 50 μm (**d**).

developed during human evolution and be essential for adequate contraction in human LNs. In the future, it should be addressed whether the smooth muscle cells in the outer layer of the capsule are specific to human or not by comparative studies on other large mammals and non-human primates.

A recent study revealed sympathetic nerve distribution in the outer region of the capsule in human inguinal LNs [7], suggesting that contraction of the capsule is controlled by the autonomic nerve system. This study revealed two types of αSMA-positive cells in the capsule of the human LN, distinguished by the expression of H-caldesmon. These results suggest that αSMA-positive but H-caldesmon-negative cells in the inner layer of the human LN and in the

capsule of the mouse LN are smooth muscle-like cells rather than typical smooth muscle cells. Furthermore, these cells can possess the similar function in contraction. On the other hand, because H-caldesmon-positive and negative contractile cells probably have different gene expression patterns, it is natural to hypothesize that responsibility to stimuli from the autonomic nerve system differs between the outer and inner layer. Therefore, in the future studies, it is worth investigating physiologic differences between the αSMA-positive cells in the outer and inner layer of the capsule to better understand the role of the LN on the lymph flow and the potential for dysfunction that could lead to edema.

Another possibility is that the outer layer of αSMA-positive cells in human LNs is a result of aging or a medical history of inflammation. Previous studies on human LNs collected from surgery and autopsy did not report the presence of smooth muscles in the outer layer of the capsule [4, 12, 32]. However, our study, which examined LNs from cadavers aged from 76 to 96 found the outer layer of the smooth muscles in almost all examined LNs. A recent physiological study on the capsule of the bovine LNs reported that aged LNs showed reduced extensibility and contractility compared to young LNs [22], implying that smooth muscles decrease as animals age. Indeed, the smooth muscles in the capsule shown in this study are less in appearance compared to the previous study using LNs collected from younger humans [32]. Thus, the smooth muscles in the outer region of the capsule are probably formed in the normal development of human LNs. However, their amount may decrease with age, which will affect the lymph flow.

In the present study, we could not find any differences in the distribution of αSMA-positive cells across different body sites. This observation contrasts with a previous study that reported inguinal LNs having more smooth muscles than other regions [4]. However, their conclusion was mainly derived from interstitial smooth muscles. Smooth muscles in the capsule are probably a primary source of flow production, as described above. Transplantation of LNs can be a treatment for edema [15], but this microsurgery does not always yield positive results. Because we observed no difference in the distribution of smooth muscles in the capsule between various body sites, it is suggested that any LN can be chosen for transplantation from the axillary to the inguinal region (or vice versa). However, future studies should investigate contractile differences of LNs among various body sites because physiological features of contractility vary in the lymphatic vessels depending on their location [14].

We found the axillary and inguinal LNs with lipomatosis. Lipomatosis can be recognized by ultrasound, computed tomography investigation, and magnetic resonance imaging [1, 3, 5, 9, 11, 16]. A recent study revealed that lipomatosis starts in the medulla by transdifferentiation from fibroblasts to fat tissues [2]. This study found that LNs with lipomatosis lacked the capsule on the efferent side, with lymphocytes present even outside the capsule, suggesting that proceeding of lipomatosis leads to the rupture of the ceiling epithelium on the efferent side. This lack of the capsule will allow lymphocytes to migrate from inside to outside the LN. Furthermore, the lymph pathway, such as subcapsular and medullary sinus, was difficult to be identified, particularly in LNs with severe lipomatosis. A previous study found compensatory collecting-like lymph vessels in the fat tissues in LNs with severe lipomatosis [2]. However, because LNs can store lymphatic fluid in the vast sinus and contribute to the generation of lymph flow by pushing the fluid with contractile structures in the capsule, the LNs with lipomatosis probably contribute little to lymph flow. Thus, to select LNs for transplantation, the extent of fat tissues should be examined in advance, such as through ultrasound diagnosis.

In conclusion, the capsule of the LNs is a specific structure to produce lymph flow by pushing the sinusoidal lymphatic pathway from the afferent to the efferent side, especially in humans. Lipomatosis in the LNs will lead to a reduction of lymph flow. This study provides basic but essential information on the role of the LNs in lymph flow, which is related to edema.

V. Acknowledgments

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VI. Conflicts of Interest

The authors declare that there are no conflicts of interest.

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