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Malnutrition-Induced Involution of Lymph Nodes in Mice

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Immune suppression is one of the major factors affecting the pandemic outbreak of infectious diseases in societies where malnutrition is common. The thymus and spleen are known to respond to starvation via reductions in their size and functions, which is called thymic or splenic involution. However, almost no reports have been published on the response of lymph nodes, other secondary immune organs, to starvation. Therefore, we here examined the histological characters of lymph nodes of a mouse dietary restriction model. Dietary restriction for 48 h reduced the size of inguinal lymph nodes by 48%. Immunoreactivity to anti-immunoglobulin G antibody was reduced by the dietary restriction, along with a normal level of immunoglobulin M-immunoreactivity, suggesting inhibited immune capacity. Because splenic involution involves macrophages, we immunostained the lymph node sections to detect macrophages. The immunoreactivity to anti-ionized calcium-binding adaptor molecule 1 (IBA1)/allograft inflammatory factor 1 (AIF1) antibody was not changed by the dietary restriction. In contrast, anti-F4/80 antibody reactivities in medullary cord macrophages, interfollicular macrophages, and the macrophages located along trabeculae in the subcapsular sinus were reduced by the dietary restriction. These results indicate that, in addition to thymus and spleen, lymph nodes are also susceptible to starvation. Specific subpopulations of macrophages are reduced in the lymph nodes of starved mice.

Key words lymph node, macrophage, starvation

INTRODUCTION

Starvation is the state of an organism that is completely deprived of food, has an insufficient food intake, or suffers from disturbances in its assimilation of food. Between 10 and 15 million people starve to death worldwide each year. Even when not resulting in death, the insufficiency of calories or alteration of nutritional status impacts on the immune system in mammals and increases susceptibility to bacterial and viral infections, causing severe health problems.[1,2] The thymus, a primary immune organ crucial to T-cell development, undergoes immediate reductions of size and functions upon various biological stresses, including nutritional deficiency. Such stress-induced histological and functional defects of the thymus are referred to as thymic involution or thymic atrophy. Malnutrition also causes morphological changes in thymic epithelial cells, decreases thymic hormone production, and increases thymocyte apoptosis.[3] Our previous study suggested that the polarization of naïve T cells is affected by starvation through the enhanced production of prostanoids.[4] Malnutrition also affects the spleen. The spleen has bilateral functions: hematopoietic and immune functions. As a hematopoietic tissue, the spleen filters old or damaged red blood cells in the circulating blood. It also immediately responds to bacteria, viruses, and other pathogens upon their infection as a secondary immune organ. Involution of the spleen has been reported to occur upon food deprivation in mice.[5] The major splenic change under malnutrition was found to be the shrinkage of red pulp, where old, damaged, and dead red blood cells and opsonized bacteria are phagocytosed and removed, while there was no obvious histological change in the white pulp, which is an important lymphatic tissue.

The splenic involution induced by dietary restriction involves the activation of macrophages.[5] Macrophages are a ubiquitous cell type present in all tissues. They play important roles in the ablation of biological waste and in innate immunity. When microbial infection or tissue injury occurs, macrophages are activated and secrete various inflammatory cytokines. In addition to this general role of macrophages in inflammation, tissue-resident macrophages have tissue-specific homeostatic functions. For example, liver-specific macrophage Kupffer cells adhere to liver blood vessels and play an important role in the first liver path effect and lipid metabolism, while lung-specific alveolar macrophages reside in the alveolar lumen as a first line of defense for the respiratory tract.[6,7] The macrophages in the cardiovascular system contribute to construction of the vascular wall and maintenance of cardiac rhythms.[8,9] Splenic macrophages are classified into at least four types: red pulp macrophages, tangible body macrophages, marginal zone macrophages (MZMs), and marginal metallophilic macrophages (MMMs).[10] Malnutrition in mice selectively activates MZMs and MMMs, whose activity is involved in splenic involution.[5]

Lymph nodes are other peripheral immune organs that contribute to both innate and adaptive immunity. Although malnutrition causes the systemic suppression of immune reactions, it does not cause obvious defects to the immune center of the spleen, white pulp, suggesting another hypothesis that lymph

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nodes are affected by malnutrition and lose their immune functions. To understand the mechanisms behind the systemic immunosuppressive effect of malnutrition, in this study we examined how it affects the histological characters of lymph nodes. The results indicated that nutritional deficiency also caused the involution of lymph nodes. Immunohistochemical study suggested the potential role of some macrophage subsets in the functional defect of the immune system in the lymph nodes.

MATERIALS AND METHODS

Animals and Dietary Restriction Male ICR mice were purchased from Japan SLC Inc. (Shizuoka, Japan). They were housed in individual cages and acclimated for at least 1 week before the experiment in the animal facility at Kobe Pharmaceutical University and used at 16 weeks old. Dietary restriction was performed by taking the mice into new cages without food pellets, where they were housed for 48 h.[4] Water was freely accessible throughout the dietary restriction period. All animal experiments were conducted in accordance with the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan. All protocols were approved by the Kobe Pharmaceutical University Committee for Animal Care and Use.

Analysis of Inguinal Lymph Nodes Mice were deeply anesthetized with isoflurane and sacrificed by cervical dislocation. The inguinal lymph nodes were dissected and observed under an SZX7 stereoscopic microscope (Olympus Scientific Solutions, Tokyo, Japan) and a WRAYCAM-NOA2000 digital camera (Wraymer, Inc., Osaka, Japan). Their volumes were calculated using an ellipsoid volume formula (1/2 × major axis × minor axis^2) from the overview pictures. The statistical significance of differences in the average absolute volume and volume normalized by body weight between control normal fed and dietary restricted mice was evaluated using Student's t-test, after the equal variances of two populations were confirmed by f-test.

Histochemical Analyses The dissected inguinal lymph nodes were fixed in 4% paraformaldehyde in PBS at 4°C for 6 h and cryoprotected in 30% sucrose in PBS at 4°C overnight. Immunohistochemistry was performed as described in our previous study with minor modifications.[11] Briefly, the fixed lymph nodes were embedded in O.C.T. compound (Sakura Fintech, Tokyo, Japan) and 20-μm-thick sections were prepared in a cryostat (SLEE Medical GmbH, Mainz, Germany). The sections were re-fixed in 4% paraformaldehyde in PBS at room temperature for 5 min, washed with PBS, and incubated in 10 mM citrate buffer (pH 6.0) at 65°C for 40 min for antigen retrieval. They were then cooled to room temperature, washed three times in PBS, and blocked with 1.5% fetal bovine serum in PBS. For the detection of immunoglobulin G (IgG) and immunoglobulin M (IgM), Alexa Fluor 568- or Cy3-conjugated antibody against rabbit IgG (A10042; Thermo Fisher Scientific, Wilmington, DE, USA), mouse IgG (A11019; Thermo Fisher Scientific), or mouse IgM (715-165-140; Jackson ImmunoResearch Inc., West Grove, PA, USA) was applied to the sections and incubated at room temperature for 4 h. To detect macrophage markers, the tissue sections were incubated with antibody against ionized calcium-binding adaptor molecule 1 (IBA1)/allograft inflammatory factor 1 (AIF1) (019-19741; Fujifilm Wako Pure Chemical Co., Osaka, Japan), F4/80 (14-4801-82; Thermo Fisher Scientific), or MARCO (MCA1849; Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 4°C overnight. They were then washed with PBS and incubated with secondary antibody, Alexa Fluor 568-conjugated anti-rabbit IgG (for IBA1/AIF1) or anti-rat IgG (for F4/80 and MARCO, A11077; Thermo Fisher Scientific), at room temperature for 3 h. They were washed with PBS and mounted with Fluoromount-G (SouthernBiotech, Birmingham, AL, USA). All images were captured under an Axio Scope A1 fluorescent microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) and processed using the GNU Image Manipulation Program, an open-resource software program for manipulating images.

RESULTS

Involution of Lymph Nodes by Dietary Restriction To reveal the effect of starvation on the lymph nodes, we examined the size of lymph nodes of mice with normal feeding and 48-h dietary restriction. The whole view of the inguinal lymph nodes showed that the dietary restriction caused an apparent reduction in size (Fig. 1A). Calculation of lymph node volume indicated that the difference between normally fed and dietary restricted mice was statistically significant (Fig. 1B). The dietary restriction induced a reduction of body weight; the average difference of body weight between before and after 48 h was −0.9% in normally fed mice and −13.0% in dietary restricted mice. There was also a significant reduction in the volume of inguinal lymph nodes normalized by body weight (Fig. 1C). These findings indicated that lymph nodes undergo involution by starvation.

Reduction of Immune Capacity in the Lymph Nodes by Dietary Restriction In order to know the effect of dietary restriction on lymph node properties, we performed histological analyses. The brief view of tissue structure of mouse lymph nodes was presented in Fig. 2. In the spleen, dietary restriction induces the increased accumulation of IgM, but not IgG, in the tissues, suggesting that the initial inflammatory reaction occurs in the spleen.[5] To examine whether a similar initial inflammatory response also occurs in starved lymph nodes, we stained lymph node sections for IgM. IgM localization was found in the subcapsular sinus (SCS) and medullary sinus (MS) in the inguinal lymph nodes of control mice with normal feeding (Fig. 3A, C). In contrast to the findings in the spleen, the staining pattern and signal intensity for IgM were not affected in the lymph nodes (Fig. 3B, D). The IgM-immunoreactivity was specific, as no signal was observed with anti-rabbit IgG antibody (Fig. 3E, F).

IgG was detected mainly in the SCS, MS, and cortex (CS) in the lymph nodes of normally fed control mice (Fig. 4A). The IgG-positive signals were observed in round and filamentous cells (yellow and white arrows in Fig. $4A_1$, respectively). B-cell follicle and paracortex also contained IgG-positive cells of similar shapes, but the number of signals appeared to be lower than in the SCS, MS, and CS regions (Fig. $4A_2$). The IgG-immunoreactive signals were significantly weaker throughout the lymph nodes of dietary restricted mice (Fig. 4B). This was further confirmed by magnified images of SCS, B-cell follicles, interfollicular zone, and paracortex (Fig. $4B_1$, $4B_2$). Thus, the immune capacity appears to be reduced in the lymph nodes after dietary restriction.

Identification of Tissue Macrophages and Their Responses to Dietary Restriction Our previous study revealed that MZMs and MMMs are activated upon dietary restriction in the

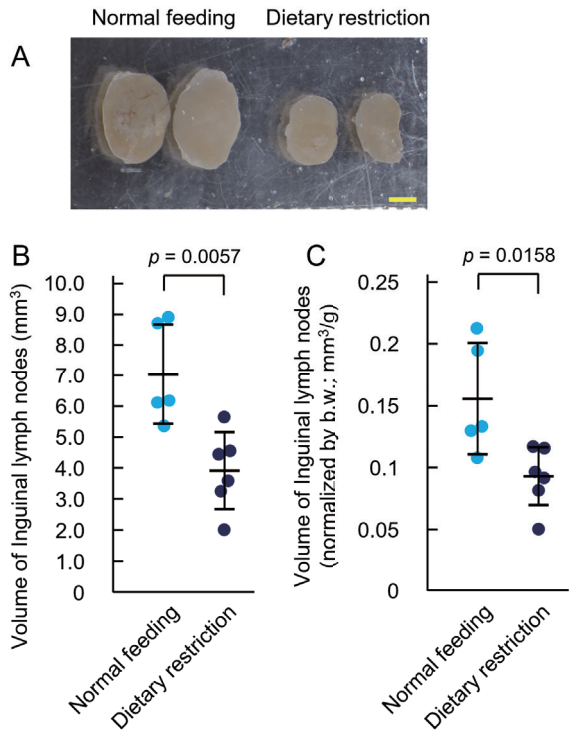

Fig. 1. Involution of Lymph Nodes by Dietary Restriction

A: Overall image of inguinal lymph nodes of normally fed or dietary restricted mice. Scale bar: 1 mm. B, C: The absolute volume (B) and volume normalized by body weight (C) of the inguinal lymph nodes of normally fed and dietary restricted mice (n = 5 and 6, respectively). Error bars indicate standard deviation. P values were calculated by Student's t-test, as described in Materials and Methods.

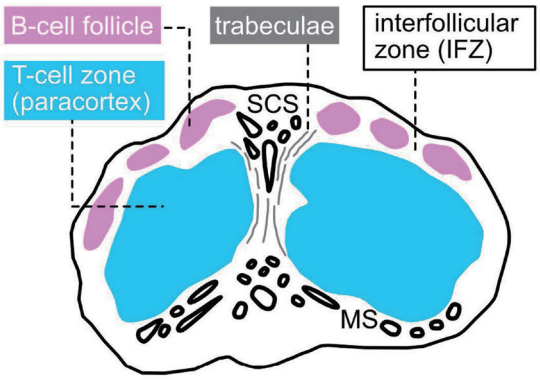

Fig. 2. Tissue Stricture of Mouse Lymph Node

An outline of mouse lymph node structure. MS, medullary sinus; SCS, subcapsular sinus.

spleen.[5] Therefore, we examined macrophage activation in the lymph nodes. Under conditions of normal feeding, many IBA1/AIF1-positive signals were found in the inguinal lymph nodes (Fig. 5A). The IBA1/AIF1-positive macrophages were distributed throughout the entire lymph nodes, especially in the SCS and paracortex (Fig. 5A$_1$, 5A$_2$, 5A$_3$). B-cell follicles contained less numbers of IBA1/AIF1-positive cells (Fig. 5A$_2$). The number and intensity of IBA1/AIF1-positive signals were not apparently changed by the dietary restriction in all regions (Fig. 5B, 5B$_1$, 5B$_2$, 5B$_3$).

We then stained the sections for other macrophage markers, F4/80 and MARCO, to examine the subsets of macrophages. As described in previous reports, F4/80-positive signals in the lymph nodes were observed in the cells with a round amoeboid shape around efferent lymphatics, which are medullary sinus macrophages (MSMs, yellow arrowheads in Fig. 6C), and extended cells in the medullary cord, which are medullary cord macrophages (MCMs, blue arrows in Fig. 6C) in the control mice with normal feeding. The macrophages in the SCS, subcapsular sinus macrophages (SSMs), were mostly negative for F4/80, but some amoeboid-like cells and cells with an extended shape lining the trabeculae were F4/80-positive (yellow and white arrows in Fig. 6A, respectively). In addition, interfollicular macrophages (IFMs) in the interfollicular zone between B-cell follicles and paracortex were also positive for F4/80 (Fig. 6E). Dietary restriction reduced the number of F4/80-positive signals along the trabeculae (white arrows in Fig. 6B). The F4/80 signals in MCMs (open blue arrows in Fig. 6D) and in IFMs in the interfollicular zone (Fig. 6F) were disappeared after the dietary restriction. Thus, a significant population of F4/80-positive macrophages, but not all of them, is sensitive to starvation. The F4/80-positive population in the SCS and MSMs kept their reactivity to anti-F4/80 antibody, even under dietary restriction. Anti-MARCO antibody specifically stained the endothelial cells of lymphatic vessels, but not macrophage-like cells, in the control lymph nodes (Fig. 7A, C). Dietary restriction did not affect the MARCO-positive signals (Fig. 7B, D).

DISCUSSION

The immune organs are vulnerable to various biological events, including infection, aging, mental stress, and malnutrition, which result in them changing their histological and functional characters.[12–14] Among these events, malnutrition has been relatively overlooked compared with other events, although it is a worldwide problem associated with economic inequalities within and between countries and an inability of take up nutrition in the elderly. The involution of the thymus upon starvation or dietary insufficiency has been investigated by many researchers and is regarded as a marker of malnutrition. However, it remains largely unknown how the other lymphatic organs, such as the spleen and lymph nodes, are affected by malnutrition. Our recent study revealed that splenic involution is induced by malnutrition with a significant reduction in the size of red pulp.[5] During the course of splenic involution, macrophages play a key role.

It has been reported that the involution of lymph nodes occurs upon infection with human immunodeficiency virus (HIV), with the selective depletion of CD4$^+$ helper T cells and disorganization of B-cell follicles.[15,16] In HIV-infected human lymph nodes with atrophy, the paracortex is considerably decreased and florid follicular hyperplasia is observed.[15,16] Our analyses presented here did not show such a pathological change in the involuted lymph nodes by dietary restriction (Fig. 4, data not shown). Thus, the mechanism responsible for the starvation-induced involution of lymph nodes differs from that for HIV-related involution. This is probably due to the difference of direct target between the events. That is, dietary restriction suppresses the development of both B and T cells with insufficiency of both energy and nutrients, but does not kill mature B and T cells, whereas HIV infection indirectly affects B-cell development by inhibiting the activities of T follicular helper cells.[17–19]

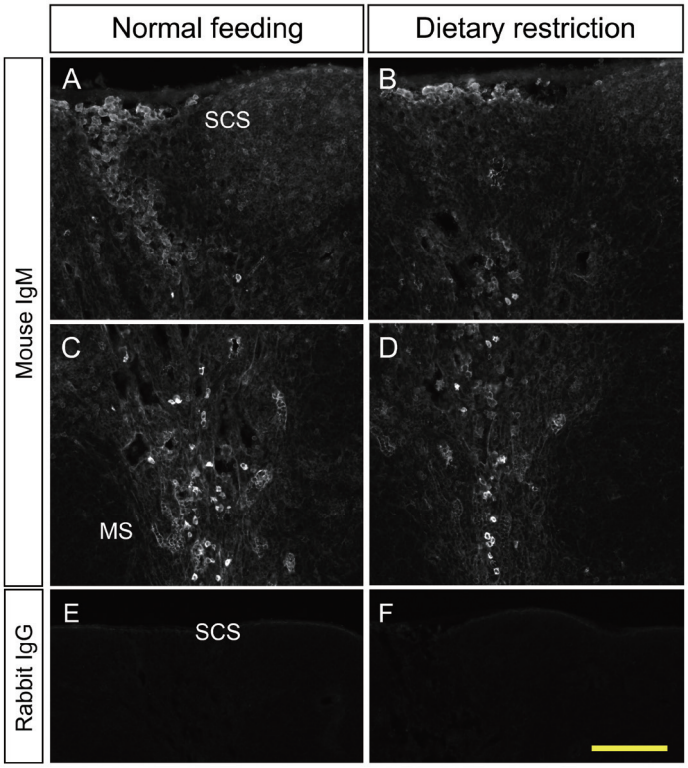

Fig. 3. Comparable Localization of IgM in the Lymph Nodes of Dietary Restricted Mice

A–D: The inguinal lymph node sections of normally fed (A, C) and dietary restricted (B, D) mice were stained by anti-mouse IgM antibody. **E, F:** The adjacent sections were stained by anti-rabbit IgG as negative controls. Representative images of four mice were presented. MS, medullary sinus; SCS, subcapsular sinus. Scale bar: 100 μm.

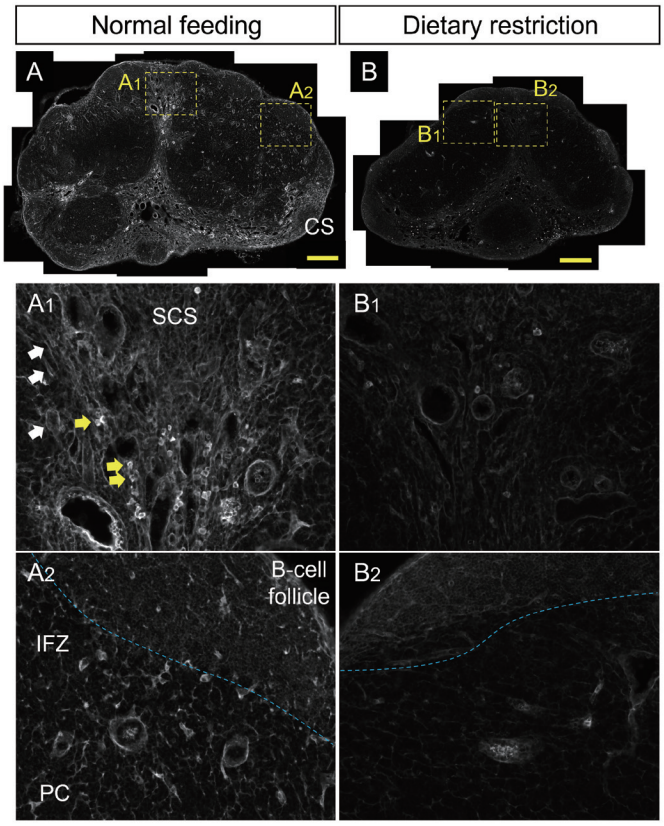

Fig. 4. Reduction of IgG-Immunoreactivity in the Lymph Nodes by Dietary Restriction

Inguinal lymph node sections of normally fed (A) or dietary restricted (B) mice were stained by anti-mouse IgG antibody. Representative images of four mice were presented. Yellow dotted squares are enlarged in panels A_1, A_2, B_1, and B_2. White and yellow arrows indicate examples of filamentous and round IgG-positive signals, respectively. Blue dotted lines in panels A_2 and B_2 indicate the boundary between B-cell follicle and interfollicular zone. CS, cortex; IFZ, interfollicular zone; PC, paracortex; SCS, subcapsular sinus. Scale bars: 300 μm.

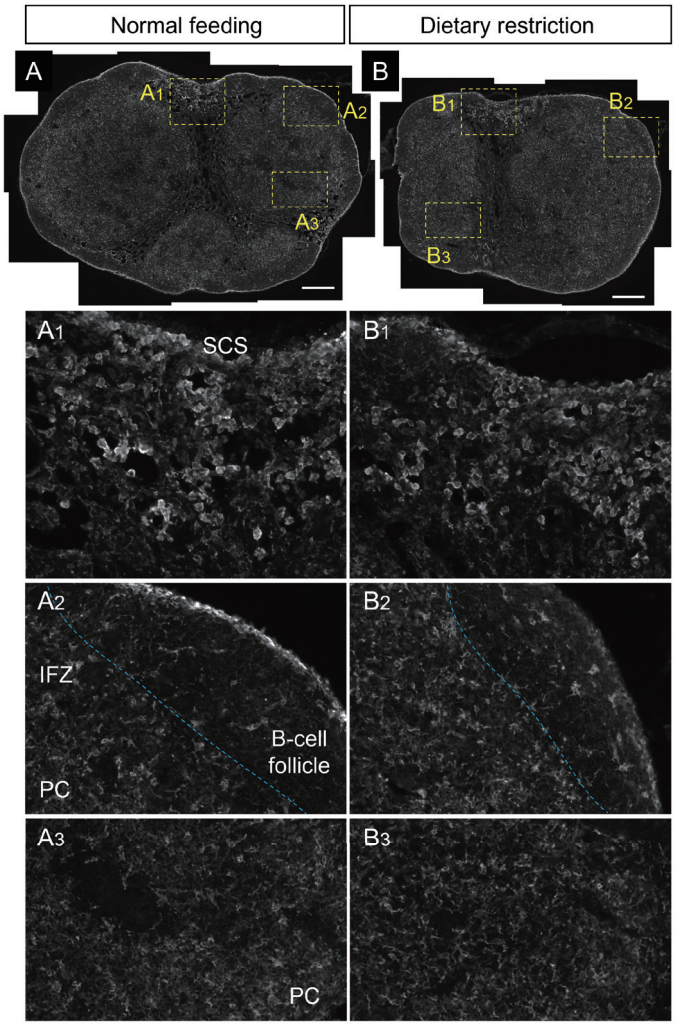

Fig. 5. No Detectable Change of the Localization of IBA1/AIF1 Signals in the Lymph Nodes by Dietary Restriction

A, B: Inguinal lymph node sections of normally fed (A) or dietary restricted (B) mice were stained by anti-IBA1/AIF1 antibody. Representative images of four mice were presented. Yellow dotted squares are enlarged in panels A_1, A_2, A_3, B_1, B_2, and B_3. Blue dotted lines in panels A_2 and B_2 indicate the boundary between B-cell follicle and interfollicular zone. IFZ, interfollicular zone; PC, paracortex; SCS, subcapsular sinus. Scale bars: 300 μm.

Aging also induces lymph node involution, resulting in a decline of skin immunity.[20] In aged lymph nodes, the size of the T-cell zone (paracortex) and interfollicular zone is reduced, so the relative size of B-cell follicles is increased, similarly to the case of HIV-infected lymph nodes.[21] Thus, the mechanisms by which starvation causes lymph node involution appear to be completely different from those involved in infection- or aging-induced involution. Although lymph nodes respond to immune challenge to form a germinal center, which is a transiently formed structure within B-cell follicles for the immediate activation of B cells, this germinal center formation is diminished in the elderly with decreased antibody and memory cell responses.[22] Kwok *et al.* suggested that lymph nodes in aged mice display increased fibrosis, resulting in impaired T-cell motility.[23] Future studies should examine how the stromal structure and functional responses of lymph nodes are affected during starvation-induced lymph node involution.

Lymphatic macrophages have been classified into four populations: SSMs, MSMs, MCMs, and IFMs. SSMs localize in the SCS, which is the outermost layer of lymph nodes and the region where afferent lymphatics enter lymph nodes.[24] They play a role in capturing and transferring antigens to the B cells in B-cell follicles.[25] They are recognized as a CD169-hi, Mac1-positive, and F4/80-negative population. Our results of immunostaining for IBA1/AIF1 and F4/80 are consistent with the above reports and showed IBA1/AIF1-positive and F4/80-negative cells in the SCS (Figs. 5 and 6). However, some F4/80-positive cells were also found in the afferent lymphatic-rich region near trabeculae (Fig. 6A, yellow arrows). These cells may be a subpopulation of SSMs or a different subtype of lymphatic macrophages. MSMs are an F4/80-positive population.[26] Their reactivity to anti-F4/80 antibody was not affected by the dietary restriction in this study. MSMs express the macrophage marker genes Mac1 (CD11b/CD18) and Siglec-1 (CD169) and have a gene expression profile similar to that of MSMs, with the exception of F4/80.[27] Both SSMs and MSMs are proinflammatory macrophages, although they have different functions: SSMs have a strong capacity to produce inflammatory cytokines, whereas MSMs contain a high level of lysozyme and are responsible for the processing of antigens.[28] It should be examined whether the F4/80-positive subpopulation in the SCS has a similar character to MSMs. The F4/80-positive population along trabeculae became few and lost its strong expression of F4/80 upon dietary restriction (white

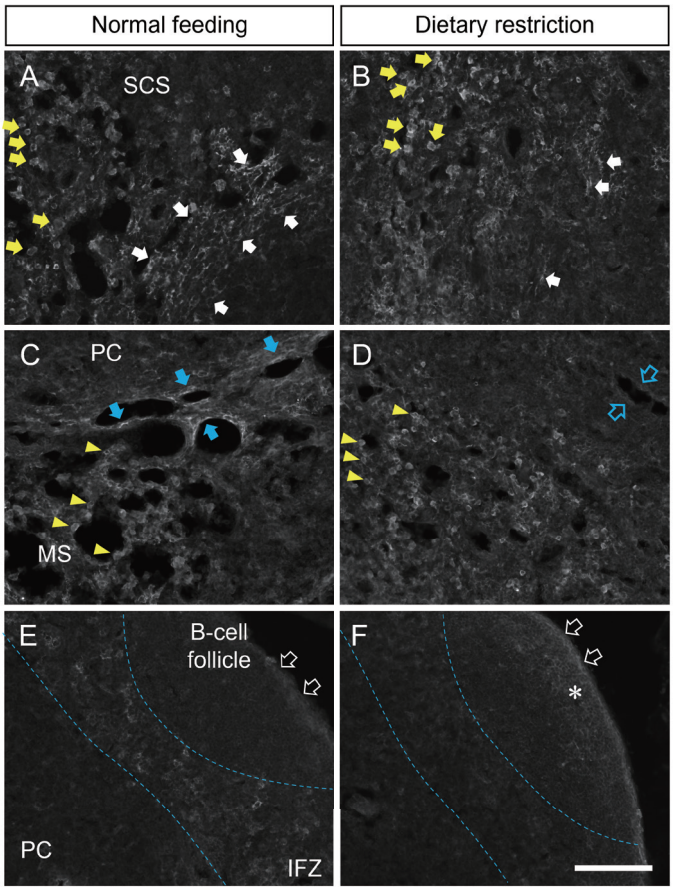

Fig. 6. Disappearance of the F4/80-Positive Macrophage Subsets in Dietary Restricted Inguinal Lymph Nodes

Inguinal lymph node sections of normally fed (A, C, E) or dietary restricted (B, D, F) mice were stained by anti-F4/80 antibody. Representative images of four mice were presented. Yellow, white, and blue arrows indicate the amoeboid F4/80-positive cells accumulated in the SCS, F4/80-positive signals lining afferent lymphatic vessels, and F4/80-positive signals surrounding efferent lymphatics in the medullary sinus (MS), respectively. Open blue arrows in panel D indicate the absence of signals surrounding efferent lymphatics in the MS. Yellow arrowheads in panel C indicate examples of MSMs. The boundaries between B-cell follicle/interfollicular zone and interfollicular zone/paracortex were indicated by blue dotted lines in panels E and F. An asterisk and white open arrows in panels E and F indicate background signals in the outer region of B-cell follicle. MS, medullary sinus; IFZ, interfollicular zone; PC, paracortex; SCS, subcapsular sinus. Scale bar: 100 μm.

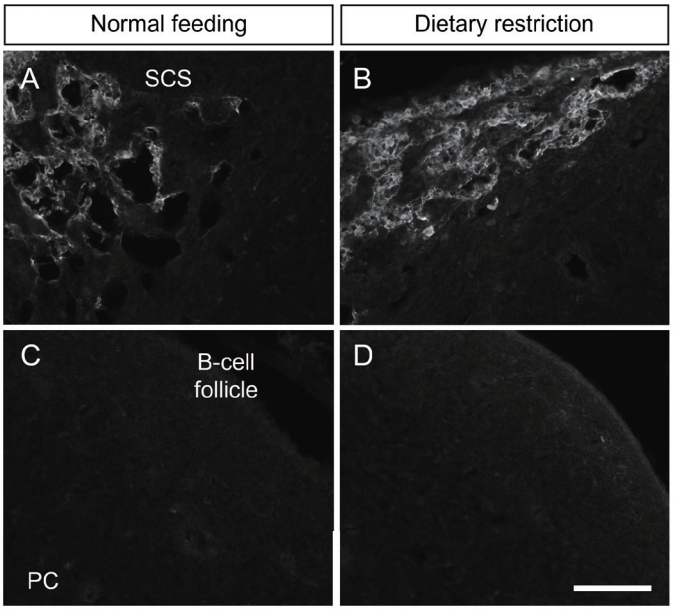

Fig. 7. Unchanged Localization of MARCO in Lymphatic Vessel Endothelial Cells upon Dietary Restriction

Inguinal lymph node sections of normally fed (A, C) or dietary restricted (B, D) mice were immunostained by anti-MARCO antibody. Representative images of four mice were presented. PC, paracortex; SCS, subcapsular sinus. Scale bar: 100 μm.

arrows, Fig. 6B). This population has not been precisely documented in previous reports. These cells may be related to MCMs, considering the similarities in shape and F4/80-expression. MCMs themselves also lost their reactivity to anti-F4/80 antibody. The identification of these MCM-like cells near the trabeculae should be pursued to deepen our understanding of the diversity of macrophages in lymph nodes. The IFMs, another population affected by the dietary restriction, have also not been well characterized so far. The translocation of SSMs from SCS to the interfollicular region has been reported, although the marker gene expression pattern of IFMs is similar to that of MSMs rather than SSMs.[26] These MCMs, MCM-like population, and IFMs may have disappeared in response to the dietary restriction. Alternatively, considering that the IBA1/AIF1-positive signals were not apparently affected by the dietary restriction (Fig. 5), it is more likely that they changed their characters upon malnutrition, as macrophages are highly plastic and change their phenotype depending on the local tissue microenvironment.[29] Future studies characterizing the diverse subtypes of lymph node macrophages should be performed to obtain a better understanding of the immune response of lymph nodes and facilitate the development of a method to cure the malnutrition-induced dysfunction of lymph node immunity.

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Conflict of interest The authors declare no conflict of interest.

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