Immunofluorescent characterization of non-myelinating Schwann cells and their interactions with immune cells in mouse mesenteric lymph node

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Abstract

The central nervous system (CNS) influences the immune system in a general fashion by regulating the systemic concentration of humoral substances, whereas the autonomic nervous system communicates specifically with the immune system according to local interactions. Data concerning the mechanisms of this bidirectional crosstalk of the peripheral nervous system (PNS) and immune system remain limited. To gain a better understanding of local interactions of the PNS and immune system, we have used immunofluorescent staining of glial fibrillary acidic protein (GFAP), coupled with confocal microscopy, to investigate the non-myelinating Schwann cell (NMSC)-immune cell interactions in mouse mesenteric lymph nodes. Our results demonstrate: i) the presence of extensive NMSC processes and even of cell bodies in each compartment of the mouse mesenteric lymph node; ii) close associations/interactions of NMSC processes with blood vessels (including high endothelial venules) and the lymphatic vessel/sinus; iii) close contacts/associations of NMSC processes with various subsets of dendritic cells (such as $CD4^+CD11c^+$, $CD8^+CD11c^+$ dendritic cells), macrophages ($F4/80^+$ and $CD11b^+$ macrophages), and lymphocytes. Our novel findings concerning the distribution of NMSCs and NMSC-immune cell interactions inside the mouse lymph node should help to elucidate the mechanisms through which the PNS affects cellular- and humoral-mediated immune responses or vice versa in health and disease.

Introduction

Recent studies have demonstrated that the nervous system and immune system collaborate with each other to maintain homeostasis and to protect the host against infectious and non-infectious diseases.[1-2] The nervous system regulates hematopoiesis, priming, migration, and the cytokine production of immune cells.[2-6] Consecutively, the immune response can have surprising effects on homeostatic neural circuits such as those controlling hypertension, metabolism, and inflammatory reflex.[2,7-8] The central nervous system (CNS) influences the immune system in a general fashion by regulating the systemic concentration of humoral substances, such as cortisol and epinephrine, whereas the autonomic nervous system communicates specifically with the immune system according to local conditions.[2-3,6,9] The main immune organs (bone marrow, thymus, spleen, and lymph nodes) are supplied with an autonomic efferent (mainly sympathetic) innervation and afferent sensory innervation, and both classic (catecholamines and acetylcholine) and peptide neurotransmitters probably participate in neuroimmune modulation.[2,10-15] However, despite these above-mentioned studies that indicate the occurrence of functional interconnections between the immune and nervous systems, data available on the mechanisms of this bidirectional crosstalk of the peripheral nervous system (PNS) and immune system are frequently incomplete, and do not always focus on their relevance to neuroimmune modulation in infection and immunological diseases. Therefore, we have become interested in the characterization of the "thread" (hardwiring) of the connections between the PNS (*e.g.*, sympathetic and parasympathetic nerve systems) and the immune system (*e.g.,* secondary lymphoid tissues/organs).

Several studies have demonstrated that the majority of nerve fibers in peripheral nerves are unmyelinated and these fibers account for approximately 80% of the peripheral nerves.[15] The Schwann cells of the unmyelinated nerve fibers, namely the non-myelinating Schwann cells (NMSCs), although ensheathing the axons, have many axonal lengths embedded within grooves of their plasma membrane.[16,17] All axons of the PNS are unmyelinated along some of their lengths, specifically at regions proximal to neuromuscular junctions, at the most distal segments of sensory and autonomic neurons, and at specialized sensory endings.[16] The NMSCs include the Schwann cells of Remak fibers, the specialized terminal Schwann cells at the neuromuscular junctions, and the Schwann cells of some sensory transducers.[15] The Remak fibers (usually in the range of 0.5-1.5 μm in diameter), whose Schwann cells form the main populations of NMSCs, have small axons that include small nociceptive (C-type) axons, the postganglionic sympathetic axons, and some preganglionic sympathetic/parasympathetic fibers.[15] Remak NMSCs have territories that can extend longitudinally for 50-100 μm.[18] Considering the location and functions of NMSC, we think that NMSCs are highly suitable for the study of the local interactions/communications of PNS and secondary lymphoid tissues/organs.

Schwann cells, including NMSCs, play important roles not only in neural regulation but also in immunomodulation.[19,20] Schwann cells not only can induce an immune response within the PNS via pattern-recognition receptors, but can also trigger the T cell response via the presentation of antigen fragments on MHC class II molecules in the

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context of costimulatory molecules.[21,22] Through the release of immunomodulators, Schwann cells can regulate the immune reaction and even terminate an ongoing immune response by inducing apoptosis.[23] Some recent studies have also demonstrated that Schwann cells interact with immune cells such as T cells and macrophages during health and disease.[24-26] The local interaction of NMSCs and dendritic cells (DCs)/lymphocytes has also been described in our previous study and the studies of other investigators.[27,28] In the present study, we have used immunofluorescent staining and confocal microscopy to investigate the distribution of NMSCs and NMSC-immune cell interactions *in situ* in the mouse mesenteric lymph node in order to gain a better understanding of the interconnections of the PNS and immune system.

Materials and Methods

Animals

C57BL/6 female mice (8-12 weeks old) were obtained from the Animal Resources Centre (Perth, Australia). All animal experiments were performed in accordance with the Australian code for the care and use of animals for scientific purposes at Murdoch University, Perth, Australia, with local animal ethics committee approval. In total, ten mice were used for the study.

Section preparation

Glass coverslips (Best circular, 13-mm diameter, 0.08-0.12 mm thick) were purchased from Thermo Fisher Scientific (Scoresby, Australia). After a brief rinse with 70% ethanol, the coverslips were coated with 0.01% poly-L-lysine solution (PLL; Sigma-Aldrich, St. Louis, MP, USA) followed by air-drying overnight at room temperature.

Mice were killed by carbon dioxide followed by cervical dislocation. Their mesenteric lymph nodes were then removed, embedded in Tissue-Tek® O.C.T. COMPOUND (ProSciTech, Kinwan, Australia), and snap-frozen in liquid nitrogen. Cryosections (15 μm thick) were prepared by using a Leica CM1850 UV Cryostat (Leica Biosystems, Nussloch, Germany) and mounted on the above-mentioned PLL-treated glass coverslips.

Antibodies

Monoclonal rat anti-mouse B220 (CD45R), rat anti-mouse CD31, rat anti-mouse CD4, rat anti-mouse CD8a, rat anti-mouse F4/80, and rat anti-mouse CD11b (Mac1) were purchased from Australian Biosearch (Karrinyup, Australia). Polyclonal rabbit anti-glial fibrillary acidic protein (GFAP) was purchased from (DAKO, North Sydney, Australia). Hamster (Armenian) monoclonal IgG antibody anti-mouse CD11c was purchased from STEMCELL Technologies (Tullamarine, Australia).

Goat anti-Armenian hamster IgG H&L (Alexa Fluor® 647), Goat F(ab')$_2$ anti-rabbit IgG - H&L (DyLight® 488)- pre-adsorbed, and Goat anti- rat IgG - H&L (Alexa Fluor® 555) were purchased from Abcam Australia (Melbourne, Australia).

Immunofluorescent staining

Sections were washed in phosphate-buffered saline (PBS) for 5 min and then fixed in 4% paraformaldehyde (PFA, Electron Microscopy Sciences, Hatfield, PA, USA) for 10 min at room temperature. All washes (3x10 min) between stages were performed in PBS. After the sections had been permeabilized with 0.2% Triton X-100 (Sigma) in PBS for 5 min, potential non-specific binding sites were blocked with antibody dilution buffer (2% goat serum (Sigma) and 1% IgG-free bovine serum albumin (Sigma) in PBS) for 20 min at room temperature. Sections were then incubated with primary antibodies overnight at 4°C. In negative control experiments, primary antibodies were omitted. After being washed, the sections were then incubated with secondary antibodies for 1 h at room temperature. Following the final washing step, the glass coverslips were mounted upside down on clean microscope slides with Fluorescence Mounting Medium (DAKO).

Confocal microscopy

Confocal imaging was performed with a Nikon Instruments C2 Plus Confocal Microscope (Nikon Instruments, Melville, NY, USA) equipped with three lasers (excitation wavelength at 488 nm, 561 nm, and 633 nm). A Plan Apochromat λ 20x objective lens and a Plan Apochromat λ 40x objective lens were used for the imaging. For some micrographs, Tile Scan was performed to obtain an image of a whole lymph node at high resolution. After the acquisition, the images were adjusted and analyzed by using NIS-Elements Advanced Research (AR) of the confocal system. Maximal intensity projection of a Z-Stack was performed by using the "Maximal intensity projection" function of the NIS-Elements AR program. The images obtained were then exported as TIFF files and further edited in Jasc Paint Shop Pro 9 (Corel Corporation, Ottawa, Canada).

Colocalization analysis

Colocalization analysis was performed by using a Colocalization Plugin integrated into ImageJ. Two images from two channels (blue and red) were imported into ImageJ and converted into two 8-bit images for colocalization analysis. The colocalized points appeared white in merged images.

Results

Distribution of NMSCs in mouse mesenteric lymph nodes

GFAP is an intracytoplasmic filamentous protein that forms part of the cytoskeleton of glial cells,[29] and can also be expressed in perivascular cells (including stellate cells), kidney cells, chondrocytes, keratinocytes, and other cells.[30,31] GFAP has been used as a cellular marker for NMSCs in the PNS.[32] Moreover, GFAP-cre mice have been utilized as a powerful tool for studying NMSCs, and the colocalization of GFAP-GFP/ S100 has also demonstrated the reliability of GFAP as a suitable marker for NMSCs.[33] Furthermore, no convincing evidence of the expression of GFAP in myelinating Schwann cells has been observed.[33] Therefore, anti-GFAP antibodies can be utilized as a suitable marker for NMSCs.[32,33]

The rabbit polyclonal antibody from DAKO has been used extensively to study glial cells within both the CNS and PNS.[6,29,33] We have applied this antibody for immunofluorescent staining in a variety of tissues including brain, lung, trachea, skin, intestine, spleen, and lymph nodes. We observed brightly stained cells with astrocyte morphology in the brain and what would be expected for the morphology and distribution for NMSCs in these tissues (except for the lymph node, other data not shown here). In our previous study, a rabbit anti-GFAP antibody from STEMCELL Technologies was successfully applied to cryosections to characterize the NMSCs in the mouse intestine and Peyer's patches.[27] For secondary antibody detecting anti-GFAP, we used a goat F(ab')$_2$ anti-rabbit IgG - H&L (DyLight® 488) - pre-adsorbed (which means a minimal cross activity with mouse and rat IgG). In negative control experiments, no positive signals were observed when the anti-GFAP antibody was omitted (*data not shown*).

At first, 15 μm-thick cryosections were stained with anti-GFAP (from DAKO) and anti-B220 to identify the NMSCs and B cells inside the mouse mesenteric lymph node. The results are shown in Figures 1 and 2. An extensive meshwork of NMSC processes was observed in the various compartments of the lymph nodes, including the cortex (Figure 2a), subcapsular sinus (SCS, Figure 2b), paracortex (Figure 2c), and medulla (Figure 2d). NMSCs were present in B cell follicles including germinal centers, and even some cell bodies of NMSCs were observed in some B cell follicles (Figure 2a). Upon breaching the capsule of the lymph node, the content of the afferent lymphatics is released to SCS, a region between the capsule and cortex. The wall of the SCS is lined with lymphatic endothelial cells and contains peripheral nerves that span the SCS, where they are exposed to the flowing lymph. We observed the close association of NMSCs with the SCS (Figure 2b). NMSCs were also present in the paracortex, and some NMSC processes exhibited a close association with B cells (Figure 2c). In the medulla region, NMSC processes were also found to be closely associated with lymphatic sinus, blood vessels, and B cells inside the medullary cord (Figure 2 d-e). The longest NMSC process that we observed was 95 µm (Figure 2e).

Spatial relationship between NMSCs and blood vessels in mouse mesenteric lymph nodes

To understand the spatial relationship between NMSC and blood vessels, double-immunolabeling with anti-CD31 (a blood vessel endothelial cell marker) and anti-GFAP antibodies was performed. The results are shown in Figure 3. In B cell follicles, the processes of the NMSCs were closely associated with capillaries (Figure 3a). In the paracortex, a similar close association of NMSCs and high endothelial venules (HEVs) was also observed, possibly indicating the neuronal control of the blood flow in HEVs and other blood vessels inside the lymph node (Figure 3b). In the medulla region, we also found a close association of NMSCs with blood vessels, even with lymphatic vessels/sinuses. For a better visualization, 3D reconstruction was performed to reveal the spatial relationship of NMSC/blood vessels, and the results are shown in Figure 3 c-d.

Interaction of NMSCs and DCs in mouse mesenteric lymph nodes

As DCs are the most important antigen-presenting cells, their crosstalk with the PNS is of great interest for an understanding of the microanatomical basis of neuronal control/regulation on antigen presentation. Since a few subsets of DC are present in the lymph node, we also utilized anti-B220, anti-CD4, and anti-CD8a to identify these DC subsets. We obtained several interesting findings concerning the interaction of two types of cells in the paracortex. First, both B220$^-$CD11c$^+$ and B220$^+$CD11c$^+$ DCs were closely associated with NMSC processes (Figure 4 a,b). Secondly, CD4$^-$CD11c$^+$ and CD4$^+$CD11c$^+$ DCs were closely associated with NMSC processes (Figure 4 c-f). We also found that DC (including CD4$^+$CD11c$^+$ and CD4$^-$CD11c$^+$)-T cell (CD4$^+$) clusters had a close association with NMSC processes in the paracortex (Figure 4 d,f). Thirdly, both CD8$^-$CD11c$^+$ and CD8$^+$CD11c$^+$ DCs were closely associated with NMSC processes inside the paracortex in the lymph node (Figure 5). Furthermore, some DC (including CD8$^+$ CD11c$^+$ and CD8$^-$ CD11c$^+$)-T cell (CD8$^+$) clusters also had close associations with NMSC processes in the paracortex (Figure 5c).

Interaction of NMSCs and macrophages in mouse mesenteric lymph nodes

As is known, the macrophage response can be triggered, maintained, and terminated by a two-way interaction of macrophages and Schwann cells. In the present study, the crosstalk of NMSCs and macrophages was investigated.

First, we examined the interaction of NMSCs with one subset of macrophages, namely F4/80$^+$ macrophages. In the cortex, we detected only a very few F4/80$^+$ macrophages (Figure 6a). In the interfollicular region (Figure 6a), paracortex (Figure 6b), and medulla (Figure 6 c,d), several F4/80$^+$ CD11c$^-$ macrophages were seen, and some of them had close associations with NMSC processes. We also observed the presence of F4/80$^+$ CD11c$^+$ and F4/80$^-$ CD11c$^+$ DCs and their close associations with NMSC processes (Figure 6 a-d). In the medulla, many F4/80$^+$ CD11c$^-$ macrophages were located inside the medullary sinus, which had a close association with NMSC processes (Figure 6 c,d).

Secondly, we investigated the interaction of NMSCs with another subset of macrophages, namely Mac1 (CD11b)$^+$ macrophages. The distribution overview of Mac1$^+$ macrophages, DCs, and NMSCs inside mouse lymph node is shown in Figure 7. In the cortex, we saw very few Mac1$^+$ macrophages (Figure 7a). In the paracortex (Figure 7b) and medulla (Figure 7c), several Mac1$^+$ CD11c$^-$ macrophages were observed, some of them having a close association with NMSC processes. We also detected the presence of Mac1$^+$CD11c$^+$ DCs and their close asso-

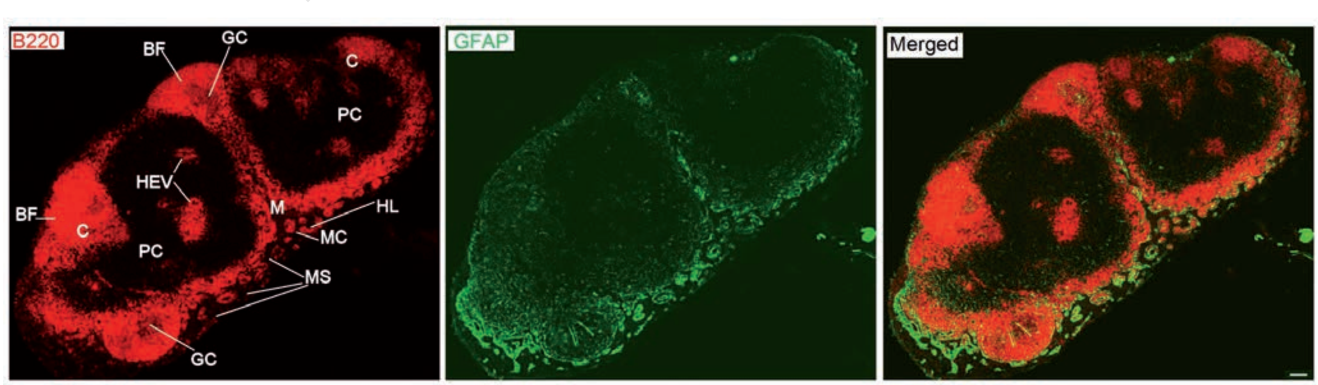

Figure 1. Overview of NMSC distribution in the C57BL/6 mouse mesenteric lymph node. Antibodies against B220 (red) and GFAP (green) label mainly B cells and NMSCs inside the lymph node, respectively; objective lens: 20x; laser scanning mode: tile scan. BF, B cell follicle; GC, Germinal center, C, cortex; PC, paracortex; HEV, high endothelial venules; M, Medulla; MS, medullary sinus; MC, medullary cord; HL, Hilum. Scale bar: 100 µm.

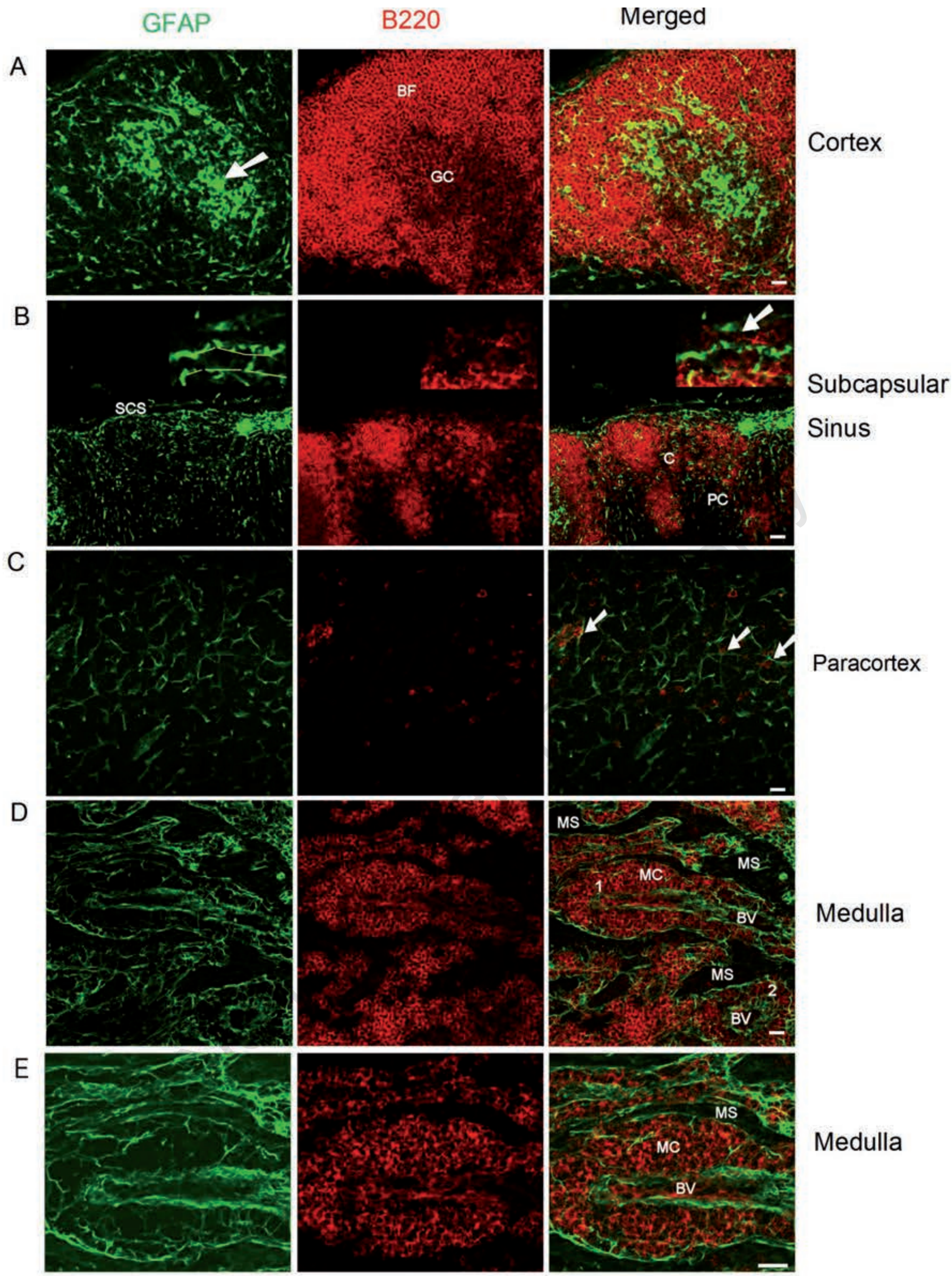

Figure 2. Distribution of NMSCs in the cortex (A), subcapsular sinus (B), paracortex (C), and medulla (D) of mesenteric lymph node from C57BL/6 mouse. Antibodies against B220 (red) and GFAP (green) label mainly B cells and NMSCs inside the lymph node, respectively. BF, B cell follicle; GC, Germinal center; SCS, subcapsular sinus; C, cortex; PC, paracortex; MS, medullary sinus; MC, medullary cord. BV, blood vessel; objective lens: 40x; scale bar: 20 μm. A) The white arrow indicates the soma (cell bodies) of NMSCs. B) High-resolution views of a subcapsular sinus are shown in inserted windows; the yellow lines indicate the border of a subcapsular sinus. The white arrow indicates NMSC processes associated with sinus. C) The white arrows indicate a few B cells that have a close association with NMSC processes. D) The images are maximal intensity projections of Z-Stack images; number 1 indicates a medullary cord that has been sectioned according to its longitudinal axis; number 2 indicates a medullary cord that has been sectioned perpendicular to its longitudinal axis; stack size: 3.5 μm; optical slice interval: 0.25 μm. E) High-resolution views of a medullary cord in (d).

ciations with NMSC processes (Figure 7 a-c). In the medulla, many $Mac1^{+}CD11c^{-}$ macrophages were located inside the medullary sinus, which had close associations with NMSC processes (Figure 7c).

Discussion

We have used immunofluorescent staining and confocal microscopy to investigate the distribution of NMSCs and NMSC-immune cell contacts in the mouse mesenteric lymph nodes in order to improve our knowledge of the microanatomical basis of PNS-immune system interactions inside the

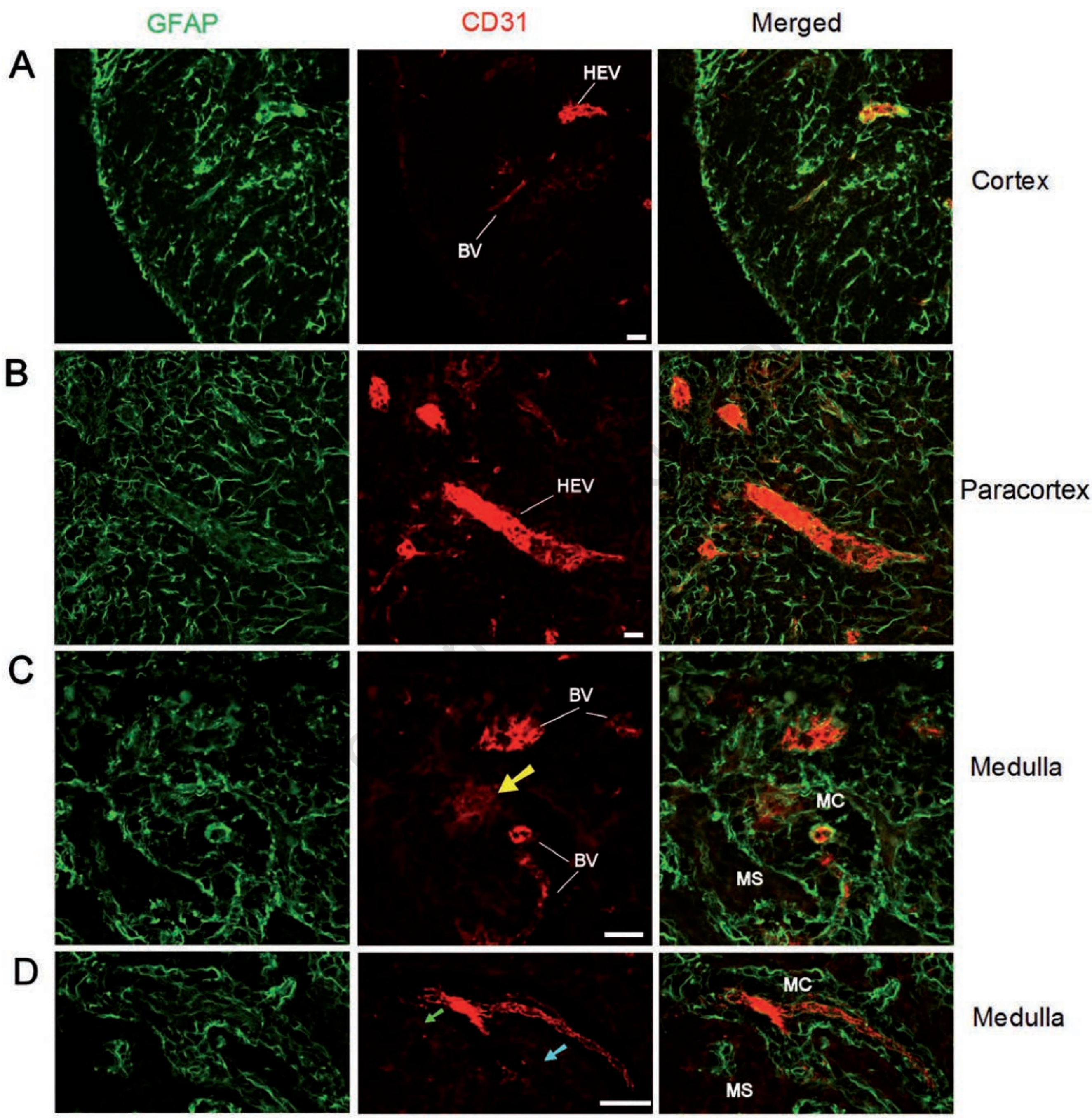

Figure 3. Distribution of NMSCs and blood vessels in the cortex (A), paracortex (B), and medulla (C,D) of a mesenteric lymph node from a C57BL/6 mouse. Antibodies against CD31 (red) and GFAP (green) label mainly blood vessel endothelial cells and NMSCs inside lymph node, respectively; BV, Blood vessel; HEV, high endothelial venules; MS, medullary sinus; MC, medullary cord; objective lens: 40x; scale bar: 20 µm. C) The images are maximal intensity projections of Z-Stack images acquired from medulla region; the yellow arrow indicates a few cells (macrophages or DCs) that are weakly positive for CD31. Stack size: 5.5 µm; optical slice interval: 0.25 µm. D) The images are maximal intensity projections of Z-Stack images; they show a medullary cord that has been sectioned according to its longitudinal axis; the medullary sinus (lymphatic vessels, green arrow) and sinus macrophages (cyan arrow) are weakly positive for CD31; Stack size: 5.4 µm; optical slice interval: 0.25 µm.

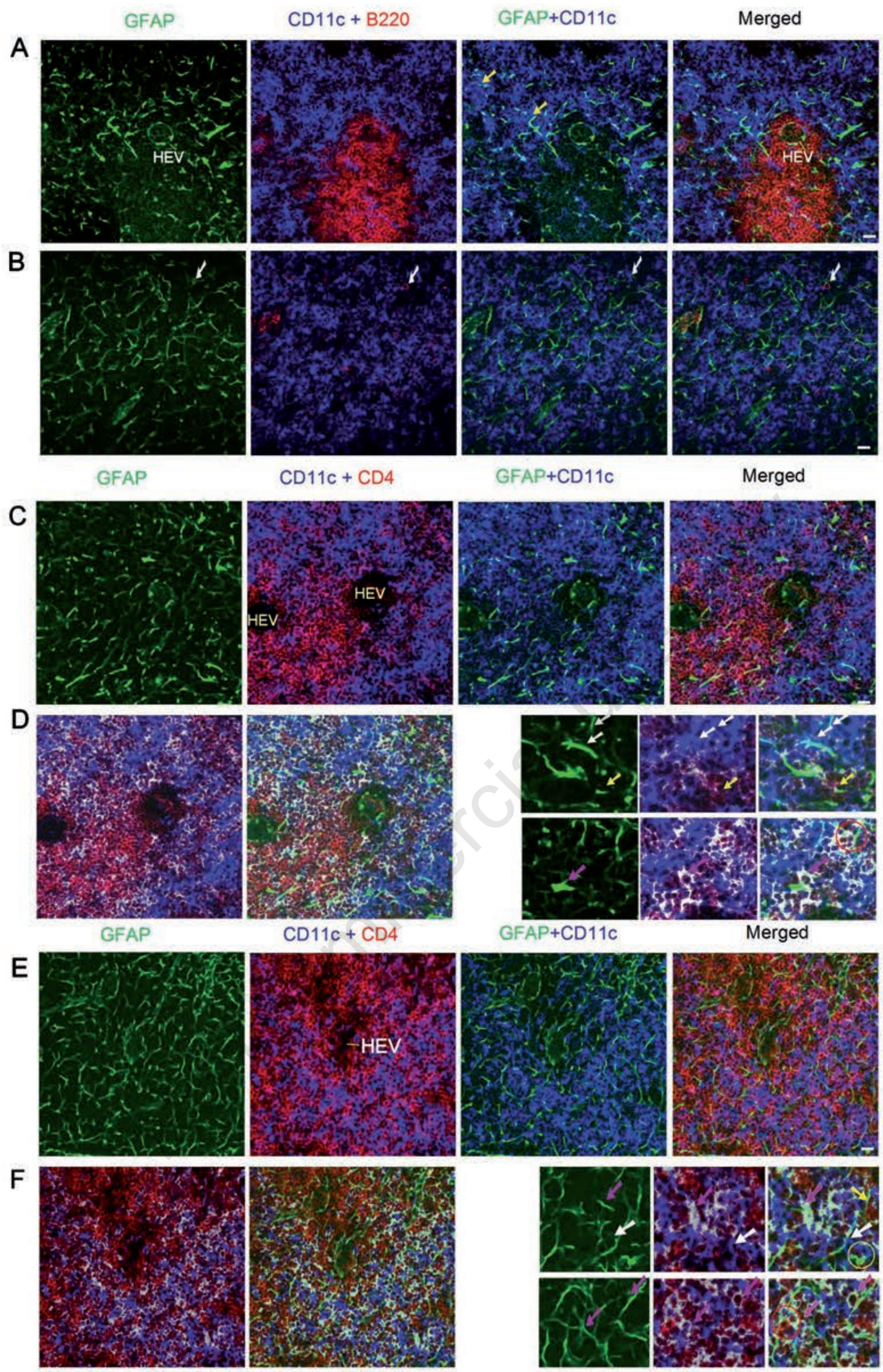

Figure 4. Interaction of NMSCs and DCs in the paracortex of a C57BL/6 mouse mesenteric lymph node. HEV, high endothelial venules; objective lens: 40x; scale bar: 20 µm. A) The yellow arrows indicate a few B220$^-$ CD11c$^+$ DCs that have a close association with the NMSC processes. B) The white arrow indicates a B220$^+$ CD11c$^+$ DC that has a close association with the NMSC processes. C,E) CD4$^+$ CD11c$^+$ DCs appear magenta in the CD4$^+$ CD11c channel. D,F) The colocalized structures (CD4$^+$ CD11c$^+$) are shown in white. In the right panels, parts of the images (left merged images) are shown at higher-resolution to demonstrate the interaction of DCs/T cells and NMSC processes. The white arrows show CD4$^-$ CD11c$^+$ DCs that have a close association with NMSC processes. The magenta arrows indicate CD4$^+$ CD11c$^+$ (appearing white) DCs that have a close association with NMSC processes. The yellow arrows indicate CD4$^+$ T cells that have a close association with NMSC processes. The red circle shows two DCs (CD4$^+$ CD11c$^+$)-T cell (CD4$^+$) clusters that have a close association with NMSC processes. The yellow circle indicates one DC (CD4$^-$CD11c$^+$)-T cell (CD4$^+$) cluster that has a close association with NMSC processes.

mouse mesenteric lymph nodes. To the best of our knowledge, we are the first to report these kinds of local associations/contacts of NMSC and DCs/macrophages/lymphocytes within mouse lymph nodes.

The immune and nervous systems are anatomically and functionally interconnected, with this crosstalk being evidenced by the dense innervation (mainly sympathetic) of the primary (bone marrow and thymus) and secondary (spleen and lymph nodes) lymphoid tissues/organs.[1-2,6,10] These tight microanatomical connections between the cells of the two systems provide the structural support of the complex network of immune responses. Until now, despite some reported studies,[11,13,14,34,35] detailed microanatomical descriptions of innervation of lymph nodes have been limited. By using anti-GFAP as a reliable cellular marker, we have analyzed the distribution of NMSCs in the mesenteric lymph nodes. In our study, an extensive meshwork of GFAP+ NMSC processes was seen throughout the lymph nodes, and the intensity was much higher compared with the GFAP staining in secondary lymphoid tissues such as Peyer's patches in some studies.[12,3] Since NMSCs have been reported to be closely associated with nerve fibers including PGP9.5+ nerve fibers, tyrosine hydroxylase (TH)+ sympathetic nerve fibers, and neurofilament (NF)+ nerve fibers,[6,35] the distribution of NMSCs revealed in our study has also demonstrated the presence of nerve fibers, especially of Remak fibers, inside the lymph nodes. Thus, our GFAP staining indicates some of the innervation inside the lymph node indirectly.

Compared with another study of the sympathetic innervation of rat cervical lymph nodes by using anterograde tracking of nerves with Fluoro-Ruby (a neuronal tracer)[34] revealing the varicosities (nerve endings) of sympathetic fibers only, our study has not only demonstrated the distribution of NMSC/Remak fibers, but also revealed another kind of NMSC-immune cell contact. Compared with another innervation study of the lymph node by means of an anti-NF antibody,[14] we have also observed more NMSCs including cell bodies and processes inside the B cell follicle; this might also indicate the additional presence of more nerve fibers in the cortex region including B cell follicles and their Germinal centers. The vascular and nervous systems undergo crosstalk, and this contributes to the development of some common diseases when dysregulated.[36] Electron microscopy has demonstrated nerve endings on muscular vessels and reticular cells in the mouse lymph node.[37] In our study, we have investigated the distribution of NMSCs related to the blood vessels in order to understand the autonomic control of the circulatory system inside the secondary lymphoid tissues. We have observed a close association of NMSC processes with blood vessels in every compartment of the lymph node. In the cortex, capillaries have a close association with NMSC processes; in the paracortex, the capillaries, blood vessels inside the paracortical cord, and HEVs have a close association with NMSC processes, and in the medullary region, the blood vessels inside the medullary cord also have a close relationship with NMSC processes. The innervation of blood vessels, which might be through Remak fibers (indicated by the presence of NMSCs), is supposed to regulate the blood flow and vascular permeability inside the lymph node.

HEVs are anatomically distinct post-

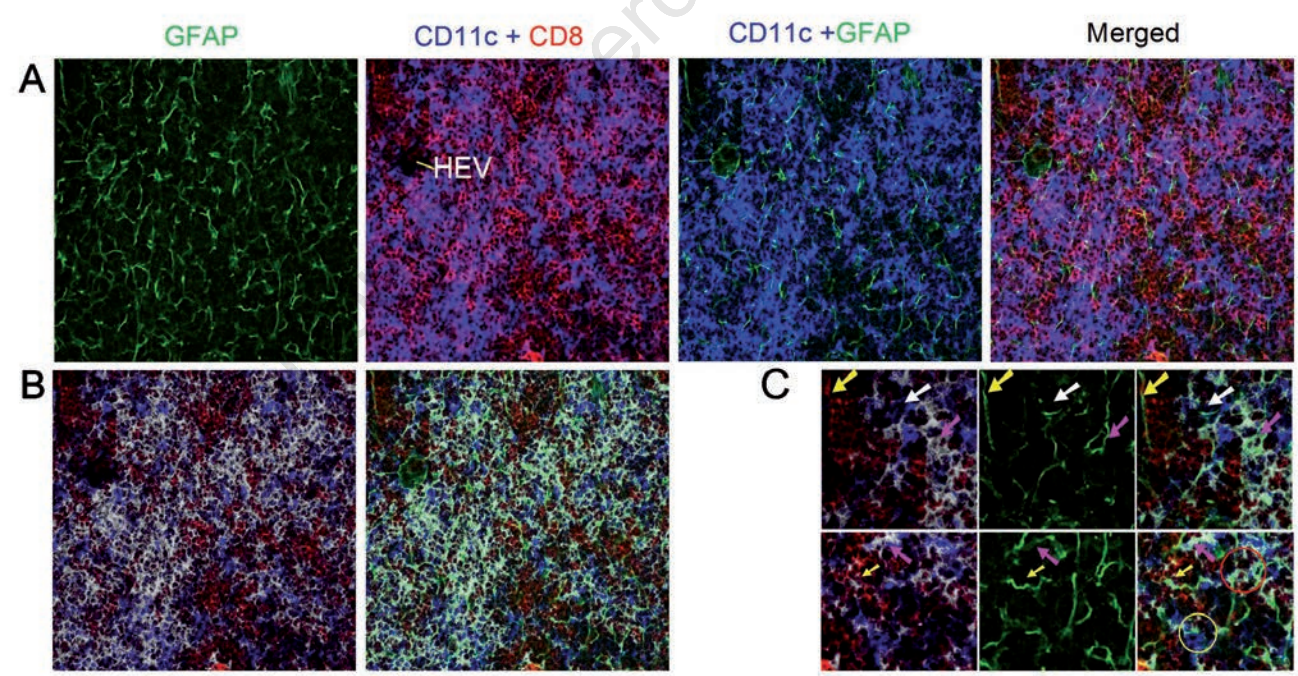

Figure 5. Interaction of NMSCs and DCs in the paracortex of C57BL/6 mouse mesenteric lymph node. HEV: high endothelial venules; objective lens: 40x; scale bar: 20 µm. A) CD8+ CD11c+ DCs appear magenta in the CD8+CD11c channel. B) Colocalized structures (CD8+ CD11c+) are shown in white. C) Parts of (B) -merged image panel- are shown at a higher resolution to demonstrate the interaction of DCs/T cells and NMSCs. The white arrows show two CD8– CD11c+ DCs that have a close association with the NMSC processes. The magenta arrows indicate CD8+ CD11c+ (appearing white) DCs that have a close association with the NMSC processes. The yellow arrows indicate a few CD8+ T cells that have a close association with the NMSC processes. The red circle shows two DC (CD8+ CD11c+) and T cells (CD8+) clusters that have a close association with NMSC processes. The yellow circle indicates a DC (CD8– CD11c+) and T cell (CD8+) cluster that has a close association with NMSC processes.

capillary venules in the lymph node and other secondary lymphoid tissue/organs. Lymphocytes and other cells flow through arteries/arterioles and pass the capillaries before entering HEVs, which are lined with specialized endothelial cells that support leukocyte adhesion and emigration.[38] Therefore, the neuronal regulation of the blood flow and vascular permeability of HEVs might affect the subsequent immune cell dynamics of the lymph node.

Since GFAP can be expressed in perivascular cells (including stellate cells),[30,31] our GFAP staining might also come from the perivascular cells (*e.g.*, pericytes) of blood vessels (including capillaries and HEVs). For example, in Figure 3 a-c, some GFAP+ structure closely associated with blood vessels may be perivascular cells. In Figure 3d, inside the medullary cord, some GFAP+ structures are closely associated with blood vessels, while most of GFAP+ structures nearby are not. Further studies (*e.g.*, colocalization studies by using anti-GFAP antibodies and perivascular cell markers) should be carried out to distinguish the NMSCs from perivascular cells in some organs.

Recent studies have shown that the lymphatic vessels are also innervated, and this innervation contributes to the regulation of lymph flow.[39,40] We have also observed the association of NMSCs and the

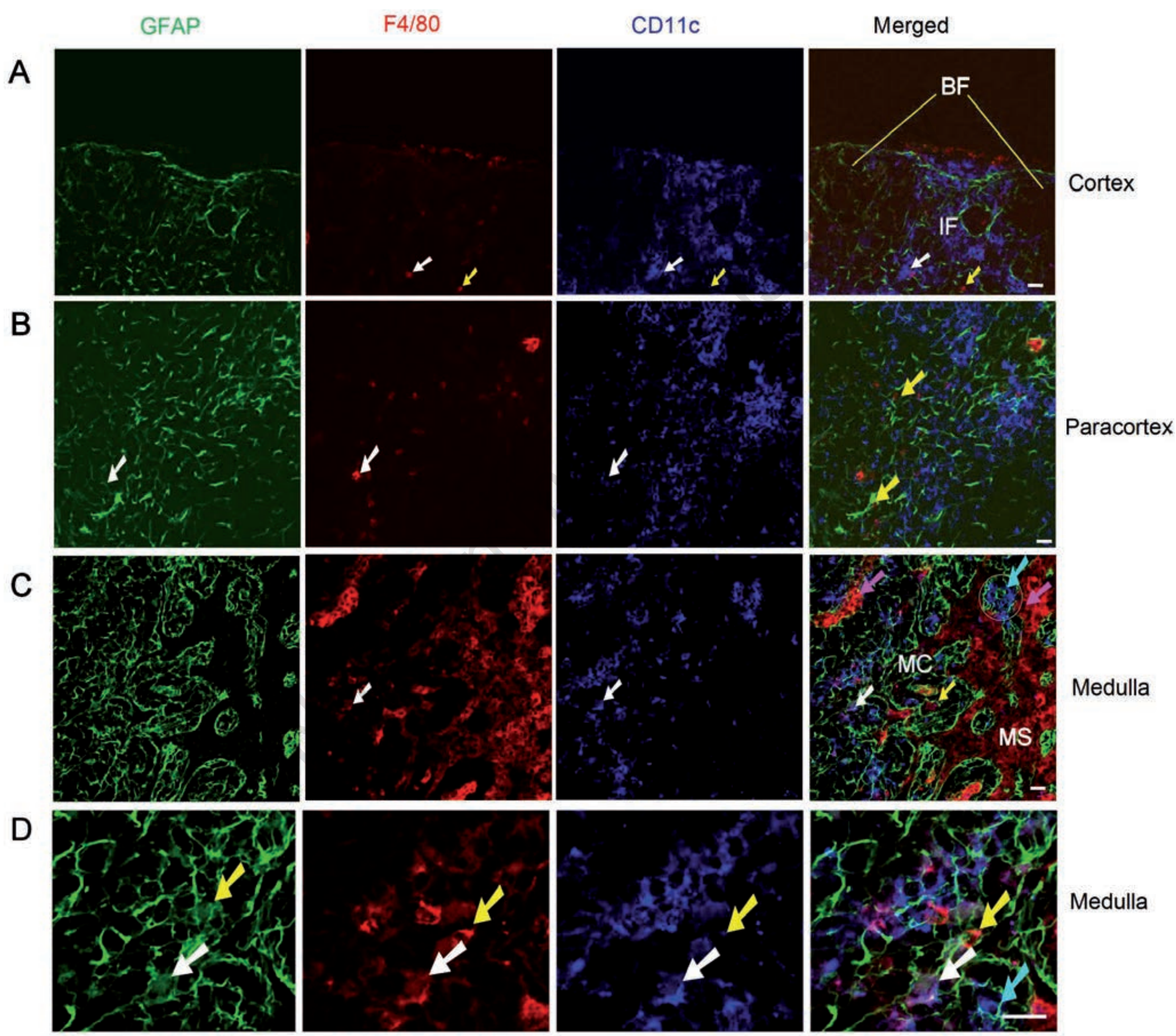

Figure 6. Interaction of NMSCs and F4/80+ macrophages in the cortex (A), paracortex (B), and medulla (C,D) of mesenteric lymph node from C57BL/6 mouse; BF, B cell follicle; MS, medullary sinus; MC, medullary cord; IF, interfollicular region; objective lens: 40x; scale bar: 20 µm. Antibodies against F4/80 (red), CD11c (blue), and GFAP (green) label mainly macrophages, DCs, and NMSCs inside the lymph node, respectively. The white arrows, yellow arrows, and cyan arrows indicate F4/80+ CD11c+ DCs, F4/80+ CD11c− macrophages and F4/80−CD11c+ DCs that have a close association with the NMSC processes inside the lymph node, respectively. The sinus macrophages are shown with magenta arrows. The white circle indicates interaction of F4/80−CD11c+ DCs and NMSC processes inside one medullary cord. (E) Part of (D) shown at higher resolution.

lymphatic vessel/sinus, as identified indirectly by microanatomical features instead of cellular markers. The NMSC processes have close relationships with the SCS and medullary sinus (Figure 2 b,d; Figure 6c; Figure 7c). This kind of interaction may suggest an active neuronal regulation of vessel caliber and functionality of the lymphatic vessel/sinus (*e.g.*, lymph flow) inside the lymph node. Certainly, further morphological, biochemical, and functional studies are needed to define the role of the nerve fibers and NMSCs in the function of lymphatic vessels.

In the present study, the interaction of NMSCs and immune cells inside the lymph node has been extensively studied *in situ*, and our results raise a few interesting points. First, since NMSCs are essential components of Remak fibers, and as recent studies have demonstrated the close association of NMSC and various nerve fibers,[15,33] the local interaction of NMSC processes and immune cells (*e.g.*, DCs and macrophages) may indicate the local interaction of nerve fibers and these immune cells. Secondly, as Schwann cells can be considered as important glial cells that provide life support for axons[41] and that play important roles in the degeneration/regeneration of axons,[16] the local interactions suggest that NMSCs may have effects on the innate/adaptive immune response and vice versa. Thirdly, as Schwann cells can be considered as immune-competent cells that can recognize/present antigen and regulate/terminate the immune response,[21] the local interactions suggest that NMSCs may have effects on the innate/adaptive immune response (*e.g.*, activation of T cells with presented antigen) and *vice versa*.

Recent studies have demonstrated the local interactions of DCs and nerve fibers in the lung, lymph node, and other organs.[14,42] Moreover, the local interactions of DCs and NMSC processes have also been reported.[21,28] We have observed, in the paracortex, extensive interactions of NMSC processes and DCs. Because of the heterogeneity of DCs,[43-44] we have combined the DC marker -CD11c with other CD markers (such as B220, CD4, and CD8a) and performed colocalization analysis to identify the different subsets of DCs. This approach has revealed the local interaction of NMSC processes with DCs subsets including B220$^+$CD11c$^+$, B220$^-$CD11c$^+$, CD4$^+$CD11c$^+$, CD4$^-$CD11c$^+$, CD8$^+$CD11c$^+$, CD8$^-$CD11c$^+$, CD11b$^+$CD11c$^+$, CD11b$^-$CD11c$^+$, and possibly F4/80$^+$CD11c$^+$/F4/80$^-$CD11c$^+$ DCs. Although further studies of the molecular mechanism need to be carried out *in vitro* and *in vivo*, our findings provide a

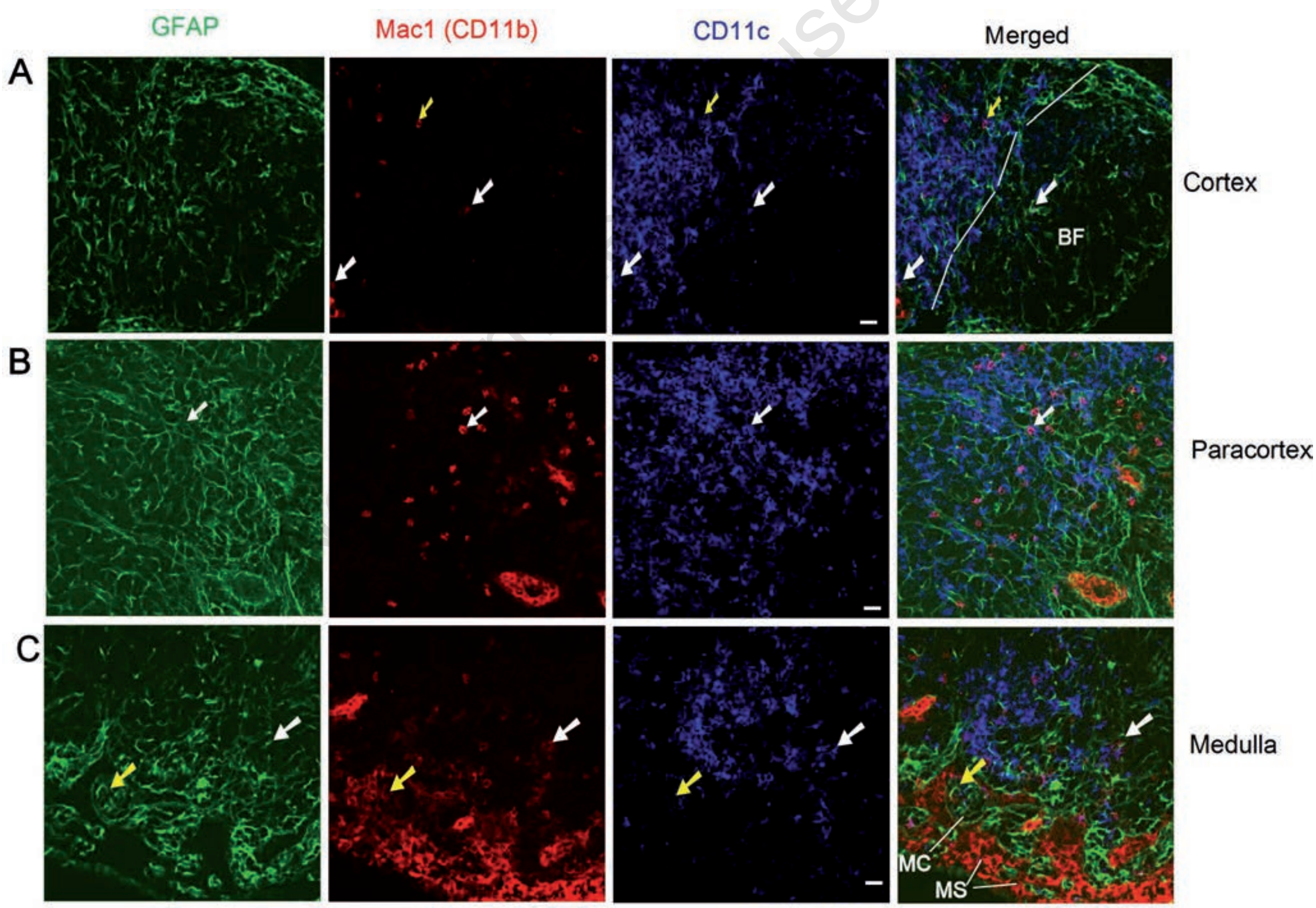

Figure 7. Interaction of NMSCs and Mac1$^+$ macrophages in the cortex (A), paracortex (B), and medulla (C) of mesenteric lymph node from C57BL/6 mouse. BF, B cell follicle; MS, medullary sinus; MC, medullary cord; objective lens: 40x; scale bar: 20 µm. The white arrows and yellow arrows indicate Mac1$^+$ CD11c$^+$ DCs and Mac1$^+$ CD11c$^-$ macrophages that have a close association with the NMSC processes inside the lymph node, respectively. A) The white lines indicate the border between cortex and paracortex. B) Almost all the Mac1$^+$ cells are also CD11c$^+$. C) The yellow arrows indicate a Mac1$^+$ macrophage that has a close association with NMSC processes inside a medullary cord.

reliable microanatomical basis for the NMSC/nerve fibers/DC communications inside the lymph nodes.

Since DCs can form immunological synapses with T cells,[45] we consider it to be of interest to examine the interactions of NMSCs/Remak fibers with DC-T clusters. We have observed that NMSCs interact with the $CD4^+$ T cell-$CD4^+CD11c^+$ DC clusters, $CD4^+$ T cell-$CD4^-CD11c^+$ DC clusters, $CD8^+$ T cell-$CD8^+CD11c^+$ DC clusters, and $CD8^+$ T cell-$CD8^-CD11c^+$ DC clusters. These findings indicate a potential role of NMSC/Remak fibers in T cell activation by DCs.

Recent studies have also demonstrated the local interactions of macrophages and nerve fibers in the lung, lymph node, and other organs.[14] However, the local interactions of macrophages and Schwann cells, which may support macrophage functions after peripheral nerve injury, have not yet been reported. In our study, we have observed the close interactions of NMSCs and with two macrophage subsets ($CD11b^+$and $F4/80^+$)[43] in various compartments of lymph nodes suggesting that NMSC/Remak fibers have effects on macrophage functions such as antigen presentation and cytokine production.

Although some studies have reported that B and T cells do not form contacts with nerve fibers,[14,34-35] we have observed a close association of NMSC with B cells, $CD4^+$ T helper cells, and $CD8^+$ cytotoxic T cells. This kind of close contiguity leads to several suggestions. First, the Remak fibers may affect the antigen presentation of B cells. Secondly, the Remak fibers may have effects on the activation of $CD4^+$/$CD8^+$ T cells. Thirdly, NMSCs as antigen-presenting cells may activate $CD4^+$ or $CD8^+$ T cells. Fourthly, NMSCs as immune-competent cells may regulate the immune response by the production of cytokines.

Since NMSCs and nerve fibers are relatively static, and as immune cells (such as DCs, macrophages, and lymphocytes) are quite mobile, this kind of cell-cell contact/communication should be dynamic. Two types of studies can be carried out to reveal the mechanisms of this kind of cell-cell interactions. The first is to characterize the direct ligand-receptor interactions of two types of cells. For example, it has been reported that Schwann cells express several ligands that are known to interact with receptors expressed by macrophages.[25] The second is to identify the indirect contacts/communications through neurotransmitters, cytokines, or other factors.[23]

In summary, our novel findings of the NMSC distribution and the NMSC-immune cell interaction provide new insights into the bidirectional crosstalk of the PNS and the immune system. Undoubtedly, further studies by using *in vitro* models (e.g., *in vitro* culture of NMSC and DCs/macrophages) and *in vivo* models (infectious and non-infectious diseases) need to be performed to reveal the molecular mechanisms of these kinds of cell-cell communications of the PNS and immune system. These studies of bidirectional crosstalk of the PNS and immune system will greatly facilitate our understanding of the pathogenesis of many neurological, neuroimmune, and infectious/immune diseases.

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