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REGULAR ARTICLE

Immunofluorescent characterization of innervation and nerve-immune cell neighborhood in mouse thymus

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Abstract

The central nervous system impacts the immune system mainly by regulating the systemic concentration of humoral substances, whereas the peripheral nervous system (PNS) communicates with the immune system specifically according to local "hardwiring" of sympathetic/parasympathetic (efferent) and sensory (afferent) nerves to the primary and secondary lymphoid tissue/organs (e.g., thymus spleen and lymph nodes). In the present study, we use immunofluorescent staining of neurofilament-heavy to reveal the distribution of nerve fibers and the nerve-immune cell neighborhood inside the mouse thymus. Our results demonstrate (a) the presence of an extensive meshwork of nerve fibers in all thymic compartments, including the capsule, subcapsular region, cortex, cortico-medullary junction and medulla; (b) close associations of nerve fibers with blood vessels (including the postcapillary venules), indicating the neural control of blood circulation and immune cell dynamics inside the thymus; (c) the close proximity of nerve fibers to various subsets of thymocytes (e.g., $CD4^+$, $CD8^+$ and $CD4^+CD8^+$), dendritic cells (e.g., $B220^+$, $CD4^+$, $CD8^+$ and $F4/80^+$), macrophages ($Mac1^+$ and $F4/80^+$) and B cells. Our novel findings concerning thymic innervation and the nerve-immune cell neighborhood in situ should facilitate the understanding of bi-directional communications between the PNS and primary lymphoid organs. Since the innervation of lymphoid organs, including the thymus, may play essential roles in the pathogenesis and progression of some neuroimmune, infectious and autoimmune diseases, better knowledge of PNS-immune system crosstalk should benefit the development of potential therapies for these diseases.

Keywords Peripheral nervous system · Innervation · Thymocyte · Dendritic cell · Thymus

Introduction

Several studies have demonstrated communication and interaction between the nervous system and immune system in health conditions and diseases (Tian et al. 2012; Kenney and Ganta 2014; Ordovas-Montanes et al. 2015; Gabanyi et al. 2016; Yoo and Mazmanian 2017; Pavlov et al. 2018; Pinho-Ribeiro et al. 2018). The central nervous system impacts the immune system mainly by regulating the systemic concentration of humoral substances (such as cortisol and epinephrine; ThyagaRajan and Priyanka 2012; Soto-Tinoco et al. 2016), whereas the peripheral nervous system (PNS; including sensory and motor divisions) communicates with the immune system specifically according to local

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"hardwiring" of sympathetic/parasympathetic (efferent) and sensory (afferent) nerves to primary and secondary lymphoid tissue/organs (e.g., thymus, spleen and lymph nodes; Kenney and Ganta 2014; Ordovas-Montanes et al. 2015). For example, neurotransmitters/neuropeptides released by the sensory and autonomic nervous systems (including sympathetic/parasympathetic and enteric divisions) bind to their respective receptors on leukocytes and then regulate immune responses (Pacheco et al. 2012; Ordovas-Montanes et al. 2015). Cytokines and other immune/inflammatory factors might also impact the activity and responsivity of sensory and sympathetic/parasympathetic nerves innervating various target organs, including primary and secondary lymphoid tissue/organs (Pacheco et al. 2012; Ordovas-Montanes et al. 2015).

The thymus is an essential primary lymphoid organ in which bone marrow-derived T cell precursors (thymocytes) undergo differentiation, ultimately leading to the migration of selected thymocytes (T cells) to the T cell-dependent areas of peripheral lymphoid organs, such as the spleen and lymph nodes (Klein et al. 2014). In the cortex, positive selection, in which precursors with low reactivity to the major histocompatibility complex are deleted/eliminated, occurs after the T cell precursors enter the thymus. Subsequently, in the medulla, the thymocytes undergo negative selection, whereby cells that have reactivity against self-antigens are deleted/eliminated (Klein et al. 2014). These highly complicated selection processes require precise parenchymal organization and compartmentation in which various subsets of residential and migrating cells interact and a number of signaling pathways occur (Klein et al. 2014). The nerve fibers are considered as essential components of the thymic microenvironment, with the thymic innervation being thought to regulate T cell development and even thymic endocrine function (e.g., secretion of self-hormones, including thymulin, thymopoietin and thymosins; Mignini et al. 2014).

Numerous studies involving the use of retrograde tracers/virus and histochemical/immunohistochemical techniques have demonstrated the sensory and autonomic innervation of the thymus across a few species, including mouse, rat and human (Felten et al. 1985; Tollefson and Bulloch 1990; Mićić et al. 1992; Anagnostou et al. 2007; Trotter et al. 2007; Nance and Sanders 2007; Mignini et al. 2010; Roggero et al. 2011; Mignini et al. 2014; Wülfing et al. 2018). The sympathetic innervation of the thymus originates from postganglionic neurons in the superior cervical ganglion and the stellate ganglion of the sympathetic chain (Mignini et al. 2014). In addition, the thymus is also innervated by the parasympathetic fibers from branches of the vagus nerve (e.g., superior/inferior or recurrent laryngeal nerves; Mićić et al. 1992). Furthermore, branches from the phrenic nerves (containing somatic motor/sensory and sympathetic nerve fibers) innervate the thymic capsule but usually do not enter the thymus itself (Mignini et al. 2014).

However, despite the innervation studies mentioned previously, the accessible data and knowledge concerning the mechanisms of bidirectional cross-talk between the peripheral nerves and immune cells inside the thymus are often insufficient and do not always pay particular attention to their relevance to neuroimmune modulation. In one of our previous studies (Hu et al. 2018), we observed, inside the mouse thymus, an extensive meshwork of non-myelinating Schwann cells (NMSCs) and their processes, which indirectly demonstrate the presence of extensive Remak fibers (consisting of group C nerve fibers (sensory), postganglionic sympathetic fibers and motor nerve terminals at neuromuscular junctions). By using immunofluorescent staining and confocal microscopy/three-dimensional reconstruction, we have now directly characterized the innervation and nerve-immune cell neighborhood in the mouse thymus in order to enhance our understanding of the microscopic basis of the PNS-thymus interaction and communication.

Materials and methods

Animals

C57BL/6 male mice (8–10 weeks old) were bought from the Animal Resources Centre (Perth, Australia). All animal experiments were carried out following the Australian code for the care and use of animals for scientific purposes at Murdoch University, Perth, Australia and with approval from the local animal ethics committee. In total, eight mice were used for the study. Thymic cryosections (20 μm thick; transversely sectioned) were prepared as previously described (Shi et al. 2017).

Antibodies

The specificities and sources of antibodies are described in Table 1.

Immunofluorescent staining

Thymic cryosections were rinsed in phosphate-buffered saline (PBS) for 5 min and then fixed with 4% paraformaldehyde (PFA, Electron Microscopy Sciences, Hatfield, PA, USA) for 10 min at room temperature. After the thymic sections had been treated with 0.2% Triton X-100 in

Table 1 Specificities and sources of primary and secondary antibodies

Target (alternative name) [cat. no.]	Conjugate	Species and isotype	Main cells labeled	Dilution	Company
CD11c [60002]	–	Armenian hamster monoclonal IgG	Dendritic cell	1:500	STEMCELL Technologies (Tullamarine, Australia)
B220 (CD45R) [103202]	–	Rat monoclonal IgG	B cell	1:300	Australian Biosearch (Karrinyup, Australia)
CD31 [102402]	–	Rat monoclonal IgG	Blood vessel endothelial cells	1:300	Australian Biosearch
CD4 [100506]	–	Rat monoclonal IgG	$CD4^+$ Thymocytes	1:300	Australian Biosearch
CD4 [100426]	Alexa Fluor® 647	Rat monoclonal IgG	$CD4^+$ Thymocytes	1:200	Australian Biosearch
CD8a [100802]	–	Rat monoclonal IgG	$CD8a^+$ Thymocytes	1:300	Australian Biosearch
CD8a [100708]	PE	Rat monoclonal IgG	$CD8a^+$ Thymocytes	1:200	Australian Biosearch
Mac1(CD11b) [100302]	–	Rat monoclonal IgG	Macrophages	1:300	Australian Biosearch
F4/80 [123102]	–	Rat monoclonal IgG	Macrophages	1:300	Australian Biosearch
Neurofilament 200 (NF-H) [N4142]	–	Rabbit polyclonal	Neuronal marker	1:2000	Sigma (St. Louis, Missouri, US)
Rabbit IgG H&L [ab150081]	Alexa Fluor® 488	Goat polyclonal	–	1:1000	Abcam Australia (Melbourne, Australia)
Rabbit IgG H&L [ab150078]	Alexa Fluor® 555	Goat polyclonal	–	1:1000	Abcam Australia
Armenian Hamster IgG H&L [ab173004]	Alexa Fluor® 647	Goat polyclonal	–	1:1000	Abcam Australia
Rat IgG H&L [ab150157]	Alexa Fluor® 488	Goat polyclonal	–	1:1000	Abcam Australia

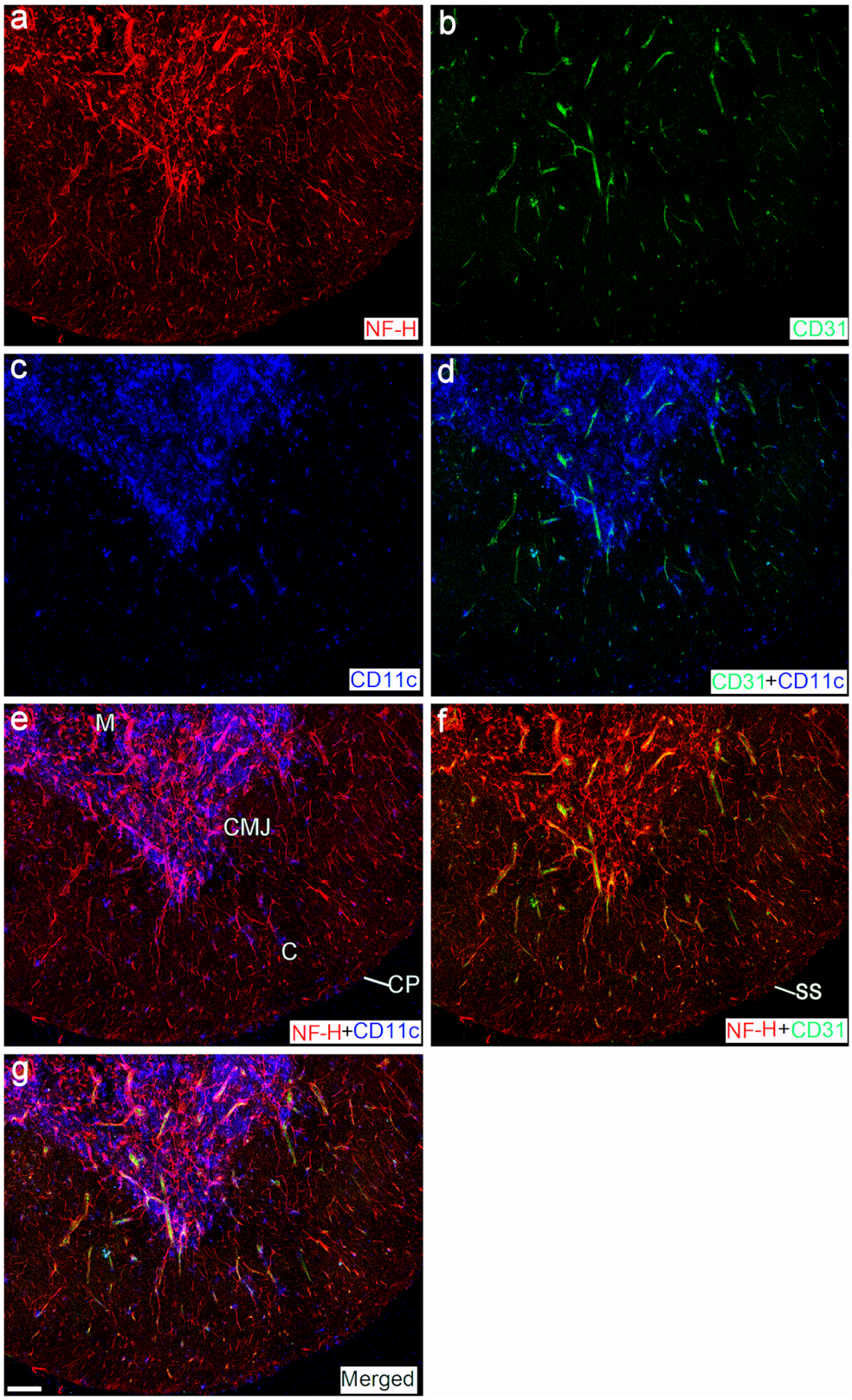


Fig. 1 Distribution of nerve fibers, blood vessels and DCs in the thymus of a C57BL/6 mouse. Antibodies against NF-H (*red*), CD31 (*green*) and CD11c (*blue*) label mainly nerve fibers, blood vessels and DCs, respectively (*C*, cortex; *M*, medulla; *CMJ*, cortico-medullary junction; *CP*, capsule; *SS*, subcapsular sinus). Objective lens: 40×; scanning mode: tile scan; Bar: 100 μm

PBS for 5 min, antibody dilution buffer (2% goat serum in PBS) was applied (20 min at room temperature) to block potential non-specific binding sites. The thymic sections were then incubated with primary antibodies

overnight at 4 °C. After being washed, the thymic sections were incubated with secondary antibodies (pre-incubated with mouse serum (Sigma, 1:1000) for 20 min to avoid their potential cross-reaction with mouse immunoglobulin) for 2 h at room temperature. Following the final washing step, the thymic sections were mounted with coverslips by using Fluorescence Mounting Medium (DAKO, North Sydney, Australia).

Confocal microscopy

Confocal microscopy was undertaken by using a Nikon Instruments C2 plus Confocal Microscope (Nikon Instruments, Melville, NY, USA) with three lasers (488, 561 and 633 nm). A Plan Apo λ 40×/0.95 objective lens and a Plan Apo λ 60×/1.40 oil immersion objective lens were used for the imaging experiments. Tile scan was employed to scan a large area of the thymus. After 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.

Image processing and colocalization analysis

The images obtained from confocal microscopy were exported as BMP files and further processed (adjustment of brightness and contrast)/edited (cropping and labeling) in

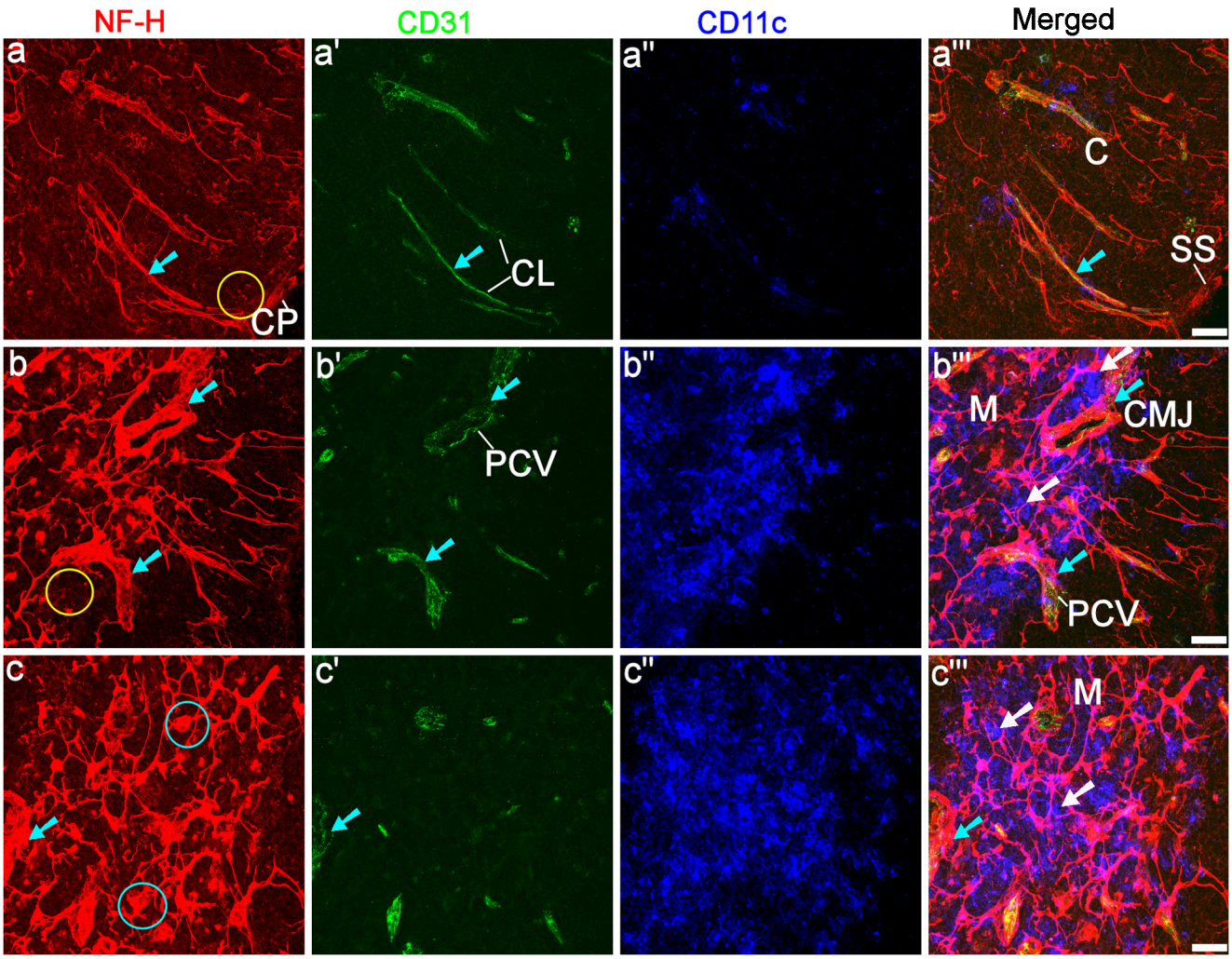
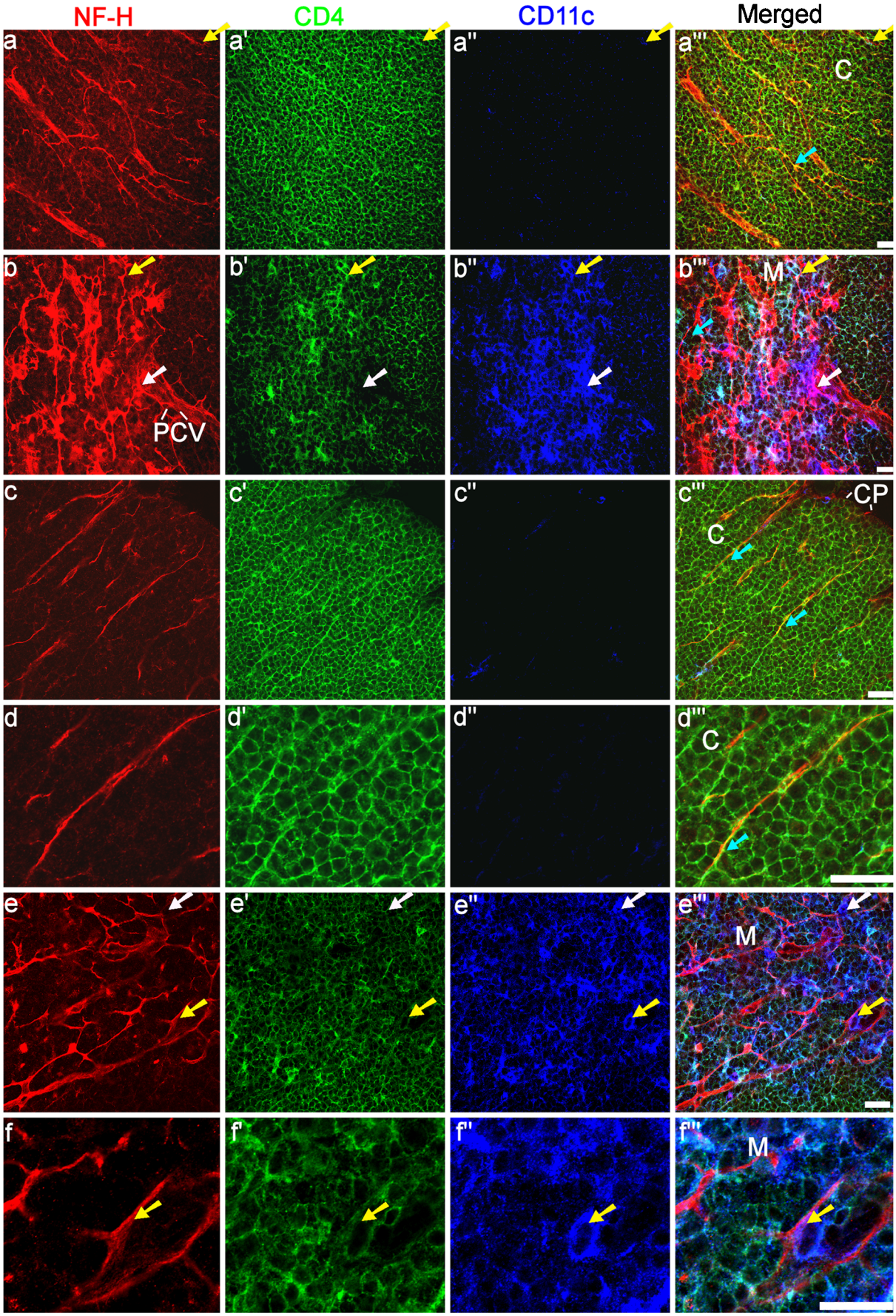


Fig. 2 Distribution of nerve fibers, blood vessels and DCs in the cortex (**a**–**a‴**), cortico-medullary junction (**b**–**b‴**) and medulla (**c**–**c‴**) in the thymus of a C57BL/6 mouse. Antibodies against NF-H (*red*), CD31 (*green*) and CD11c (*blue*) label mainly nerve fibers, blood vessels and DCs, respectively. *Cyan arrows* indicate blood vessels having close associations with nerve fibers. *White arrows* indicate a few DCs having close associations with nerve fibers. (**b**–**b‴**) DCs form a cluster around the PCV in the medulla. *Yellow circles* indicate a few nerve endings that appear as *small red dots*. (**c**) *Cyan circles* indicate two somata. Each image is a maximal intensity projection of a Z-stack (*PCV*, postcapillary venules; *C*, cortex; *M*, medulla; *CMJ*, cortico-medullary junction; *CP*, capsule; *CL*, capillary; *SS*, subcapsular sinus). Stack size: 6.0 μm; optical slice interval: 0.50 μm. Objective lens: 40×. Bar: 20 μm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◀ **Fig. 3** Distribution of nerve fibers, $CD4^+$ thymocytes and DCs in the cortex (**a–a‴**, **c–d‴**) and medulla (**b–b‴**, **e–f‴**) of the thymus of a C57BL/6 mouse. Antibodies against NF-H (*red*), CD4 (*green*) and CD11c (*blue*) label mainly nerve fibers, $CD4^+$ thymocytes and DCs, respectively. *Cyan arrows* indicate a cluster of $CD4^+$ thymocytes that are closely associated with nerve fibers. *White arrows* show $CD4^-CD11c^+$ DCs closely apposed to nerve fibers. *Yellow arrows* indicate $CD4^+$ $CD11c^+$ DCs having a close association with nerve fibers. (**a–b‴**) Each image is a maximal intensity projection of a Z-stack (*PCV*, postcapillary venules; *CP*, capsule; *C*, cortex; *M*, medulla). Stack size: 6.0 μm; optical slice interval: 0.50 μm. Objective lens: 40×. (**c–f‴**) High-magnification images acquired by using a 60x objective lens showing the distribution of nerve fibers and their associations with $CD4^+$ thymocytes and DCs in the cortex (**c–d‴**) and medulla (**e–f‴**). Bar: 20 μm

Corel PaintShop Pro 2018 (Corel, Ottawa, Canada). Colocalization analysis was performed by using the program ImageJ 1.51h as previously described (Ma et al. 2018).

Results

Innervation and nerve-blood vessels associations in mouse thymus

In the present study, neurofilament heavy (NF-H; Yuan et al. 2012) was utilized as a reliable marker to identify the nerve fibers inside the thymus. We previously applied rabbit-anti-NF-H for immunofluorescent staining on a variety of mouse tissues, including brain, trachea, skin, intestine, lymph node and spleen and observed brightly stained cells with the morphology expected for nerves/nerve fibers in these tissues (data not shown). In addition, no staining was observed when anti-NF-H was omitted and only goat anti-rabbit IgG antibodies were applied (Supplementary Fig. 1).

To reveal thymic innervation and to understand the spatial relationship between nerve fibers and blood vessels/dendritic cells (DCs), triple immunostaining with anti-NF-H, anti-CD31 (a blood vessel endothelial cell marker) and anti-CD11c antibodies was performed on 20-μm-thick thymic cryosections from C57BL/6 male mice; the results are shown in Figs. 1 and 2. An extensive meshwork of nerve fibers was observed in all thymic compartments, including the capsule/subcapsular region, cortex, cortico-medullary junction and medulla.

Subcapsular plexi consisting of fluorescent fibers were observed in the outermost layer of the thymic cortex (beneath the capsule; Figs. 1 and 2a–a‴). The parenchyma in the cortex was innervated, with some nerve fibers traveling along blood vessels (including capillaries; Figs. 1 and 2a–a‴). In the cortico-medullary junction, some nerve fibers were associated with the blood vessels, including the postcapillary venules (PCV; Fig. 2b–b‴). The NF-H staining in the medulla (Fig. 2b–c‴) was much stronger than that of the cortex. A few somata (cell bodies of neurons) were seen in the medulla (Fig. 2c). Moreover, in the medulla, nerve fibers formed plexi around the blood vessels and some nerve fibers exhibited close associations with a large number of DCs (Figs. 1 and 2b–c‴).

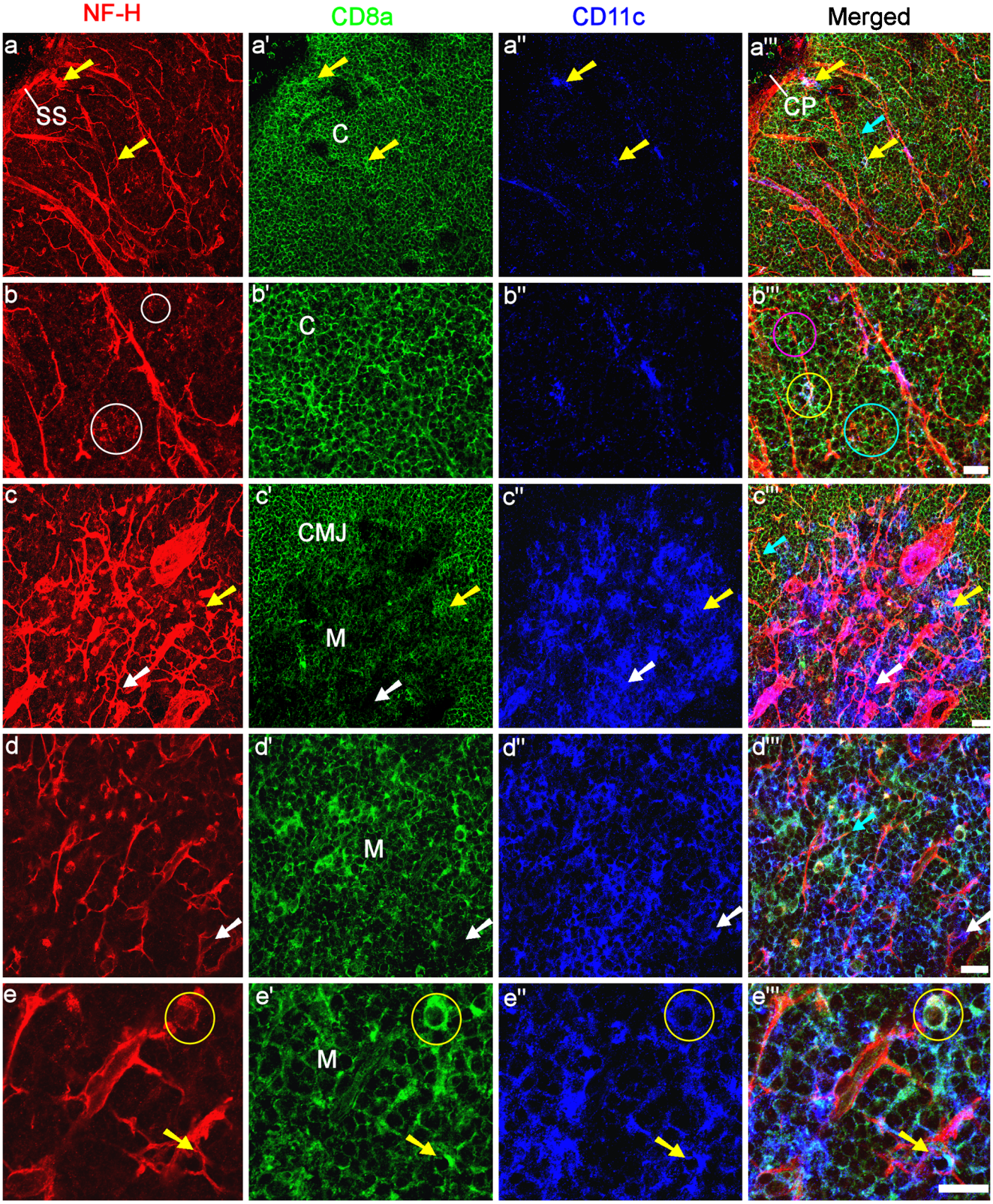
Associations of nerve fibers and thymocytes/DCs

Thymocytes are classified into three distinct maturation stages based on the expression of cluster of differentiation (CD) markers (Pearse 2006). The earliest thymocyte stage is $CD4^-CD8^-$ and the next major stage is $CD4^+CD8^+$. The final stage of maturation is the single-positive stage ($CD4^+CD8^-$ or $CD4^-CD8^+$; Klein et al. 2014).

We first analyzed the distribution of nerve fibers, $CD4^+$ thymocytes (revealed by anti-CD4 staining) and DCs inside the thymus; the results are shown in Fig. 3. In the cortex (Fig. 3a–a‴, c–d‴), most thymocytes were $CD4^+$ and many of them had a close association with nerve fibers. In the medulla region (Fig. 3b–b‴, e–f‴), nerve fibers were thicker than those of the cortex and some fibers demonstrated close associations with $CD4^+$ thymocytes, $CD4^-CD11c^+$ DCs and $CD4^+CD11c^+$ DCs.

We then analyzed the distribution of nerve fibers, $CD8^+$ thymocytes (revealed by anti-CD8a staining) and DCs; the results are shown in Fig. 4. In the cortex (Fig. 4a–b‴), most of the thymocytes were also $CD8^+$ and many of them exhibited close apposition to nerve fibers. In the medulla, we observed that the nerve fibers were closely associated with $CD8^+$ thymocytes, $CD8a^-CD11c^+$ DCs and $CD8a^+CD11c^+$ DCs (Fig. 4c–e‴).

Since no single CD marker, namely CD4 or CD8, could be utilized to identify the maturation stage of the thymocytes exactly, we combined these two markers to characterize the thymocyte subpopulations and their associations with nerve fibers inside the thymus. The results are shown in Fig. 5 (for an overview) and Fig. 6. In the cortex (Fig. 6a–b‴), most of the thymocytes were $CD4^+CD8^+$, many of which had close associations with nerve fibers. Some thymocytes were single-positive ($CD4^+CD8^-$ or $CD4^-CD8^+$) and associations were also seen between these thymocytes and nerve fibers. In the deep cortex and cortico-medullary junctions (Fig. 6c–d‴), most of the thymocytes were again $CD4^+CD8^+$, some of them being closely apposed to nerve fibers. In the medulla (Fig. 6c–d‴), nerve fibers exhibited close proximity to $CD4^+CD8^-$, $CD4^-CD8^+$ and $CD4^+CD8^+$ thymocytes.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◀ **Fig. 4** Distribution of nerve fibers, $CD8^+$ thymocytes and DCs in the cortex (**a–b‴**) and medulla (**c–e‴**) of the thymus of a C57BL/6 mouse. Antibodies against NF-H (*red*), CD8a (*green*) and CD11c (*blue*) label mainly nerve fibers, $CD8^+$ thymocytes and DCs, respectively. *Cyan arrows* indicate $CD8a^+$ thymocytes that are closely associated with nerve fibers. *White arrows* show $CD8a^-CD11c^+$ DCs closely apposed to nerve fibers. *Yellow arrows* indicate $CD8a^+CD11c^+$ DCs having a close association with nerve fibers. *White circles* show a few nerve endings (appearing as *small red dots*), whereas the *cyan circle* shows a few $CD8a^+$ thymocytes that are closely associated with some nerve endings. *Magenta circle* shows a nerve fiber that is closely associated with a few $CD8a^+$ thymocytes. *Yellow circles* show $CD8a^+CD11c^+$ DCs associated with a nerve fiber and/or a few nerve endings. (**a–c‴**) Each image is a maximal intensity projection of a Z-stack (*SS*, subcapsular sinus; *CP*, capsule; *C*, cortex; *CMJ*, cortico-medullary junction; *M*, medulla). Stack size: 6.0 μm; optical slice interval: 0.50 μm. Objective lens: 40×. (**b–b‴**) High-resolution view of cropped region from images in (**a–a‴**) shows two types of neighborhood between nerves and immune cells. (**d–e‴**) High-magnification images acquired by using a 60× objective lens showing the distribution of nerve fibers and their associations with $CD8^+$ thymocytes and DCs. (**e–e‴**) High-resolution view of cropped region from images in (**d–d‴**) shows two types of neighborhood between nerves and immune cells. Bar: 20 μm

Associations of nerve fibers and B cells/macrophages

Although B cells account for only 0.5% of all thymic cells, recent studies have suggested that they might also play a novel role (e.g., antigen presentation) in the maturation of thymocytes (Klein et al. 2014). In the present study, we therefore also analyzed the distribution of B cells and their associations with nerve fibers inside the thymus. The results are shown in Fig. 7. The cortex contained a few B cells and DCs, some of which had close associations with nerve fibers (Fig. 7a–a‴). The nerve fibers in the medulla were thicker than those of the cortex (Fig. 7a–b‴). Furthermore, in the medulla, the nerve fibers exhibited close associations with a large number of DCs ($B220^-CD11c^+$ or $B220^+CD11c^+$) and some B cells (Fig. 7b–b‴).

Macrophages are a type of thymic stromal cell involved in phagocytosis and antigen presentation. Therefore, we analyzed the associations of nerves with two subsets of thymic macrophages, namely $Mac1^+$ macrophages and $F4/80^+$ macrophages; the results are shown in Fig. 8. The thymic cortex contained only a few oval-shaped $Mac1^+$ macrophages (Fig. 8a–a‴) and most of the $Mac1^+$ macrophages were located in the medulla (Fig. 8b–b‴). In addition, some nerve fibers had close associations with $Mac1^-CD11c^+$ DCs and $Mac1^+CD11c^+$ macrophages (Fig. 8b–b‴). Another subset of thymic macrophages was represented by $F4/80^+$macrophages with two shapes, namely oval and plate (Fig. 8c–c‴). Some nerve fibers also had close associations with $F4/80^+CD11c^-$ macrophages, $F4/80^-CD11c^+$ DCs and $F4/80^+CD11c^+$ DCs (Fig. 8c–d‴).

Discussion

We utilized high-resolution multi-color confocal imaging to reveal the innervation and nerve-immune cell neighborhood of the thymus. Our approaches are robust and much easier to perform compared with some other technically difficult methods (e.g., tracing methods with tracer substances or viruses that are transported across synapses; Felten et al. 1985; Tollefson and Bulloch 1990; Nance and Sanders 2007). We observed intensive staining for nerve fibers in each compartment of the thymus (including the capsule, subcapsular region, cortex, cortico-medullary junction and medulla), suggesting the potential neural control of T cell development (including positive and negative selection). To our knowledge, this is the first study to describe such an intensive innervation and close nerve-immune cell neighborhood inside the thymus.

Our results agree with those from one of our previous studies demonstrating the presence of an extensive meshwork of NMSCs (also regarded as a type of antigen-presenting cell) and their processes, a finding that indirectly demonstrated the presence of extensive Remak fibers (non-myelinated nerve fibers; consisting of group C nerve fibers (sensory), postganglionic sympathetic fibers and motor nerve terminals at neuromuscular junctions) in the thymus (Hu et al. 2018). Compared with some previously published studies that have not shown innervation in the cortex or/and medulla of thymus (Mignini et al. 2014; Nance and Sanders 2007; Felten et al. 1985), we observed intensive $NF\text{-}H^+$ nerve fibers in each compartment of the thymus, suggesting the potential neural control of T cell development (including positive and negative selection). In addition, compared with other studies showing only sparse nerve fibers in the thymic parenchyma (Anagnostou et al. 2007; Nance and Sanders 2007; Mignini et al. 2014; Wülfing et al. 2018), we observed much denser nerve fibers in various compartments (including the outer cortex, deep cortex, cortico-medullary junction and medulla) of the thymus from 8- to 10-week-old mice. Since T cell development is closely related to the age of animals and since thymic innervation might have changed in aged animals (Madden et al. 1997), thymic innervation studies of mice at younger/older ages should be carried out in the future.

The cortico-medullary arterioles from thymic arteries branch into capillaries and extend into the cortex and medulla. Capillaries in the cortex are rarely fenestrated (restricting access of circulating antigens to developing cortical

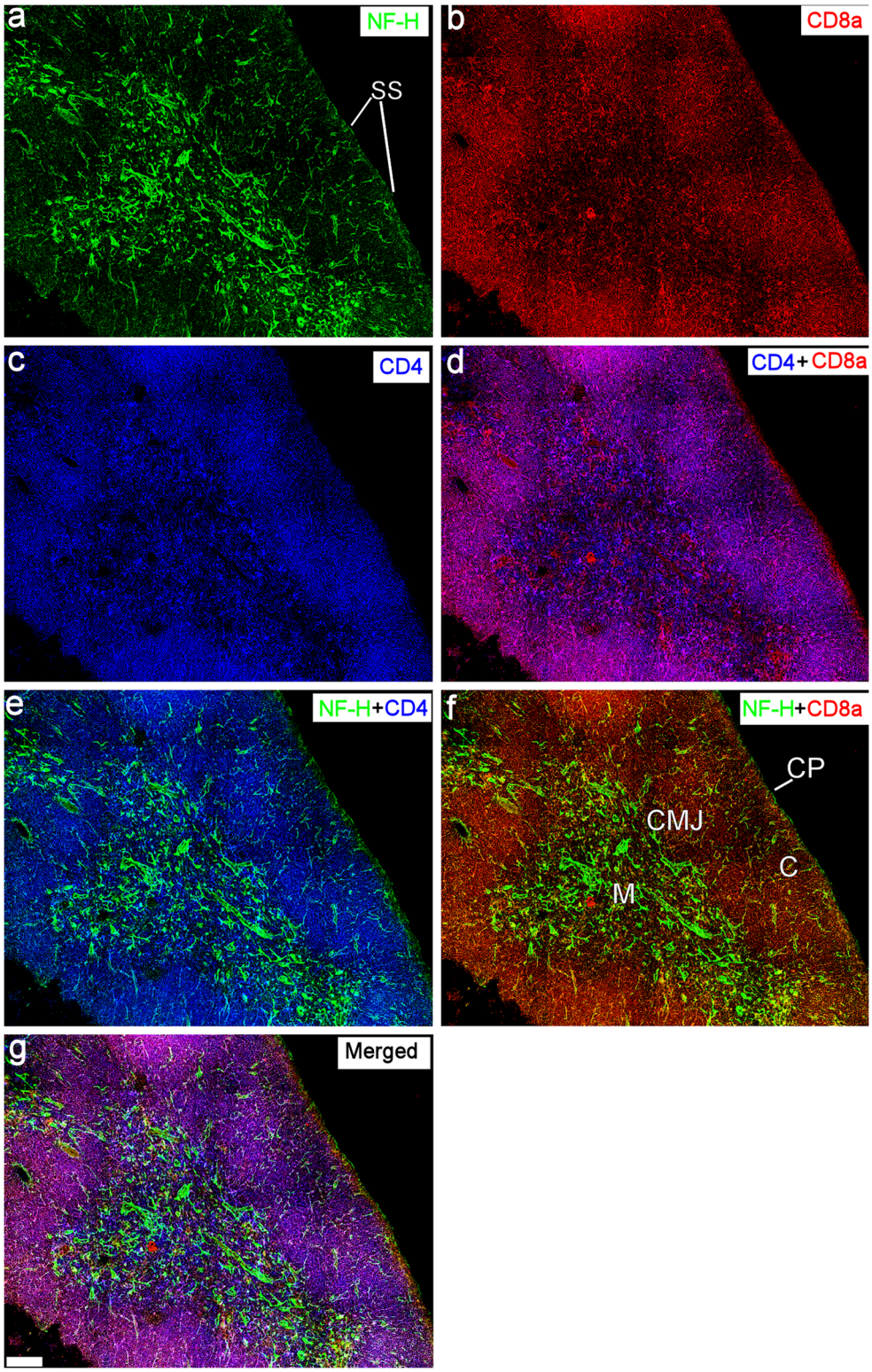


Fig. 5 Overview of the distribution of nerve fibers (NF-H, *green*), CD8a$^+$ thymocytes (*red*, detected by CD8a-PE) and CD4$^+$ thymocytes (*blue*, detected by CD4-Alexa 647) in the thymus from a C57BL/6 mouse (*M*, medulla; *C*, cortex; *CMJ*, cortico-medullary junction; *CP*, capsule; *SS*, subcapsular sinus). Objective lens: 40×; Scanning mode: tile scan. Bar: 100 μm

thymocytes), whereas medullary capillaries are fenestrated (freely permeable to circulating antigens; Pearse 2006). We observed that almost all blood vessels (including capillaries and PCVs) have close associations with nerve fibers, indicating the neural control of blood flow within blood vessels (including capillaries with pericytes and without smooth muscle cells; Hamilton et al. 2010) and vascular permeability (e.g., for circulating antigens) inside the thymus. Since PCVs

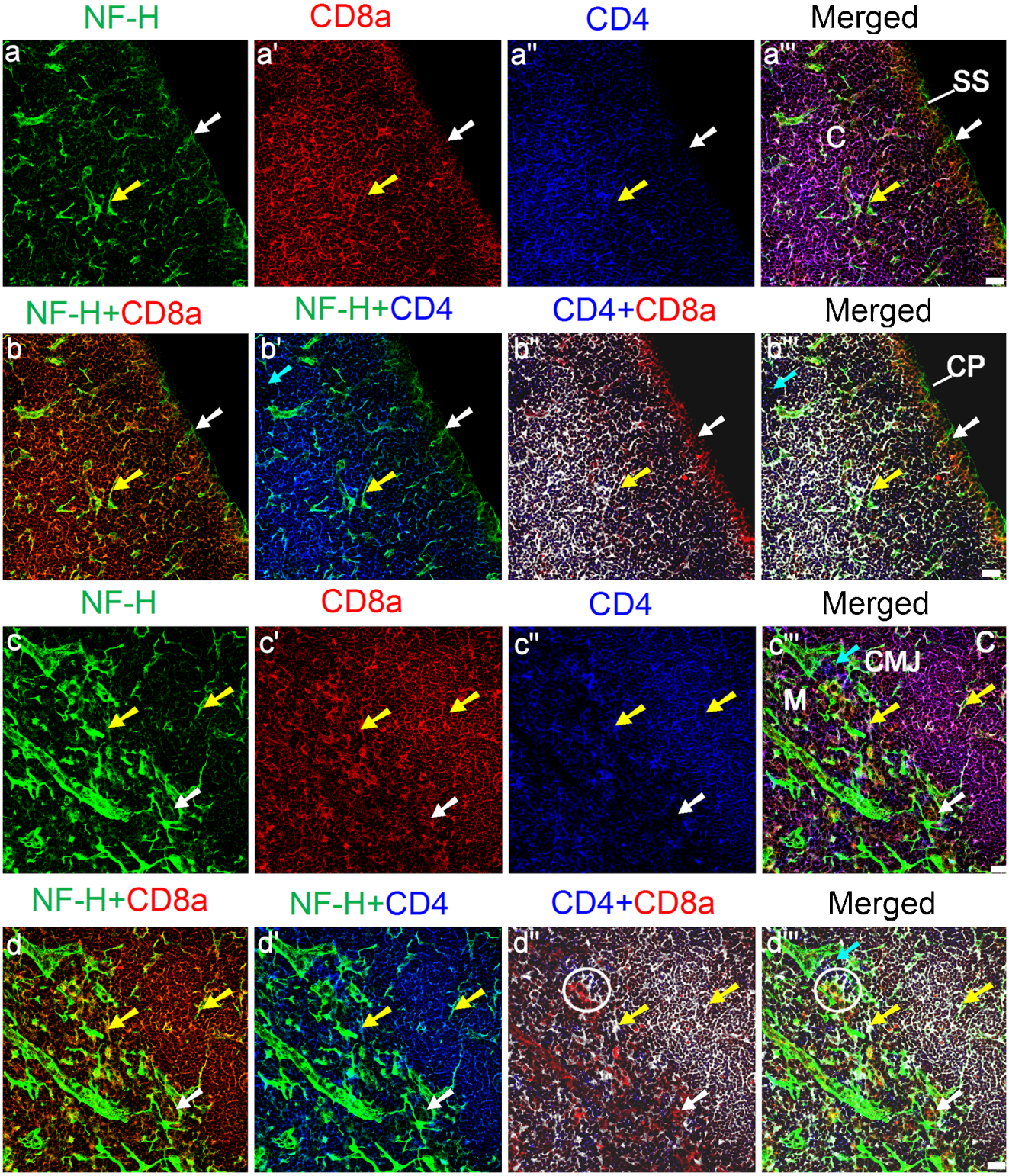
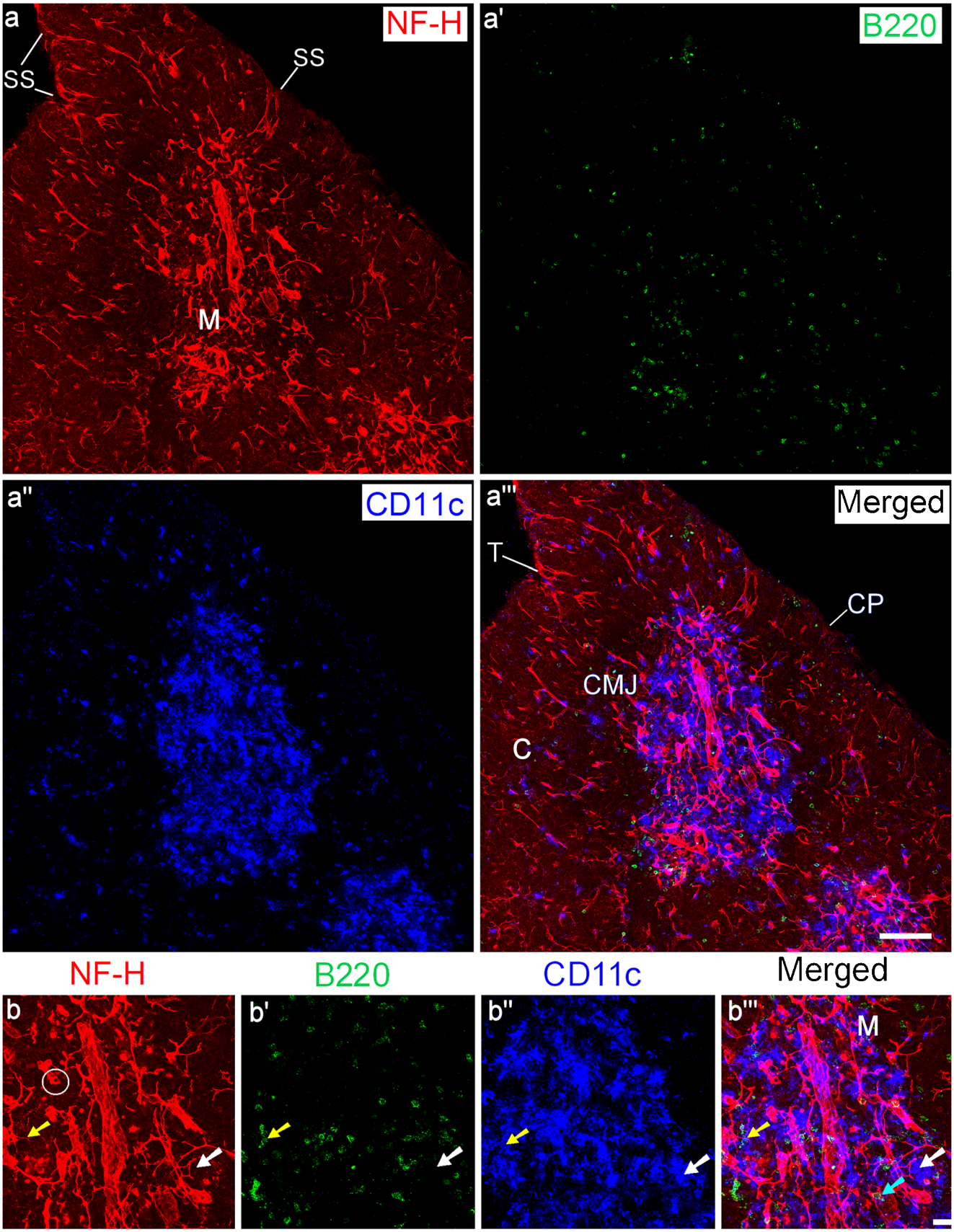


Fig. 6 Distribution of nerve fibers (NF-H, *green*), CD8a$^+$ thymocytes (*red*, detected by CD8a-PE) and CD4$^+$ thymocytes (*blue*, detected by CD4-Alexa 647) in the cortex (**a–b‴**) and medulla (**c–d‴**) of the thymus from a C57BL/6 mouse. Images **b–b‴** and **d–d‴** were generated by combination/colocalization analysis of channels in **a–a″** and **c–c″**, respectively. *Cyan arrows* indicate CD4$^+$CD8$^-$ thymocytes that are closely associated with nerve fibers. *White arrows* show CD4$^-$CD8$^+$ thymocytes closely apposed to nerve fibers. *Yellow arrows* indicate CD4$^+$CD8$^+$ thymocytes having a close association with nerve fibers. (**d″–d‴**) *White circles* show nerve fibers that are closely associated with a CD4$^+$CD8$^+$ thymocyte-CD8$^+$ cell (probably a DC as identified from its morphology) cluster (*M*, medulla; *C*, cortex; *CMJ*, cortico-medullary junction; *CP*, capsule; *SS*, subcapsular sinus). Objective lens: 40×. Bar: 20 μm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◀ **Fig. 7** Distribution of nerve fibers, B cells and DCs in the thymus from a C57BL/6 mouse. Antibodies against NF-H (*red*), B220 (*green*) and CD11c (*blue*) label mainly nerve fibers, B cells and DCs, respectively (*M*, medulla; *C*, cortex; *CMJ*, cortico-medullary junction; *CP*, capsule; *SS*, subcapsular sinus; *T*, trabecula). Objective lens: 40×. (**a**–**a‴**) Scanning mode: tile scan. Bar: 100 μm. (**b**–**b‴**) *Cyan arrow* indicates $B220^{+}CD11c^{-}$ B cells closely associated with nerve fibers. *White arrows* show $B220^{-}CD11c^{+}$ DCs closely apposed to nerve fibers. *Yellow arrows* indicate $B220^{+}CD11c^{+}$ DCs having a close association with nerve fibers. *White circle* indicates one soma. Each image is a maximal intensity projection of a Z-stack. Stack size: 6.0 μm; optical slice interval: 0.50 μm. Bar: 20 μm

are the entry sites for T cell precursors and exit sites for mature/functional lymphocytes, the dense innervation around the PCV suggests the potential neural regulation of the cellular dynamics and trafficking of thymocytes (lymphocytes).

The thymus is separated into two defined regions, namely the cortex and medulla, each of which contains several types of thymic epithelial cells (TECs). T cell precursors from the bone marrow enter the thymus through PCV at the cortico-medullary junction and then undergo processes of positive and negative selection (Klein et al. 2014). Therefore, several types of thymocytes are located in spatially restricted regions of the thymus. For example, the cortex contains densely packed $CD4^{+}CD8^{+}$ thymocytes, whereas the medulla contains sparser single-positive ($CD4^{+}$ or $CD8^{+}$) thymocytes (Klein et al. 2014). In our study, triple-color confocal imaging revealed intensive associations between nerve fibers and the main population (about 80% of all thymic cells) of thymocytes, namely $CD4^{+}CD8^{+}$ thymocytes (mainly in the outer cortex and deep cortex/cortico-medullary junction). We also saw close associations of nerve fibers with some $CD4^{+}CD8^{-}$or $CD4^{-}CD8^{+}$ thymocytes in the medulla and cortex. The close associations of nerve fibers with thymocytes at various developmental stages indicate the potential neural control of T cell development (including positive and negative selection) inside the thymus.

The thymic medulla, which contains medullary TECs and DCs (both are professional antigen-presenting cells), provides a specialized microenvironment suitable for the establishment of T cell tolerance (Lopes et al. 2015). The medulla contains three distinct subsets of DCs, which are as follows: resident conventional DCs ($CD11c^{hi}CD11b^{-}CD8\alpha^{hi}Sirp\alpha^{-}$), migratory conventional DCs ($CD11c^{hi}CD11b^{+}CD8\alpha^{lo}Sirp\alpha^{+}$) and plasmacytoid DCs ($CD11c^{int}B220^{+}PDCA\text{-}1^{+}$; Klein et al. 2014). In this study, we investigated the distribution of DCs and their associations with nerve fibers. We demonstrated that the cortex contains only a few DCs and that most DCs are located in the medullary region. Furthermore, we observed that some nerve fibers are closely associated with various subsets of DCs (including $B220^{+}CD11c^{+}$, $B220^{-}CD11c^{+}$, $CD4^{+}CD11c^{+}$, $CD4^{-}CD11c^{+}$, $CD8a^{-}CD11c^{+}$, $CD8a^{+}CD11c^{+}$, $F4/80^{+}CD11c^{+}$DCs, $F4/80^{-}CD11c^{+}$ and $Mac1^{-}CD11c^{+}$ DCs), indicating the potential neural control of DC activities/functions (including antigen presentation and cytokine production). Although further molecular and functional studies of this type of association need to be carried out in vitro and in vivo, our findings revealed the microanatomical basis for nerve-DC communications and for the neural control of negative selection.

Macrophages are essential cellular components of the thymic microenvironment and are involved in antigen presentation and the phagocytosis of deleted/eliminated thymocytes (Hung et al. 2012). In addition, macrophages secrete cytokines and other immune factors that might impact the proliferation, maturation and differentiation of thymocytes (Wood 1985). We observed close associations of nerve fibers with two types of macrophages, namely Mac1 $(CD11b)^{+}$ and $F4/80^{+}$ macrophages, indicating potential neuroimmune modulation through the thymic macrophages.

This type of nerve-immune cell neighborhood has also been described in our previous studies (Shi et al. 2017; Hu et al. 2018). Instead of being a random event, this sort of close cell-cell neighborhood is intensive in the thymus. Since the nerve fibers and other cellular/structural components of thymic microenvironment are relatively constant and since immune cells such as DCs, macrophages and thymocytes (lymphocytes) are flexible, this type of neighborhood is probably dynamic under a variety of conditions. Further ultrastructural (Straub 2004) and molecular studies are needed to investigate whether this kind of close cell-cell neighborhood is a "neuroimmune synapse" (Dustin 2012) that shares some features of neurological synapses (van Spronsen and Hoogenraad 2010) and immunological synapses (Finetti et al. 2017).

Our novel findings concerning thymic innervation and the nerve-immune cell neighborhood in situ should facilitate our understanding of bi-directional communications between the PNS and primary lymphoid organs. Further in vivo and in vitro molecular and functional investigations need to be performed to reveal the molecular basis of these types of cell-cell communication (Pacheco et al. 2012; Pinho-Ribeiro et al. 2018). Since the innervation of lymphoid organs, including the thymus, might have essential roles in the pathogenesis and progression of some neuroimmune, infectious and autoimmune diseases, better knowledge of PNS-immune system interactions should benefit the development of potential treatments for these diseases (Roggero et al. 2011; Chavan et al. 2017).

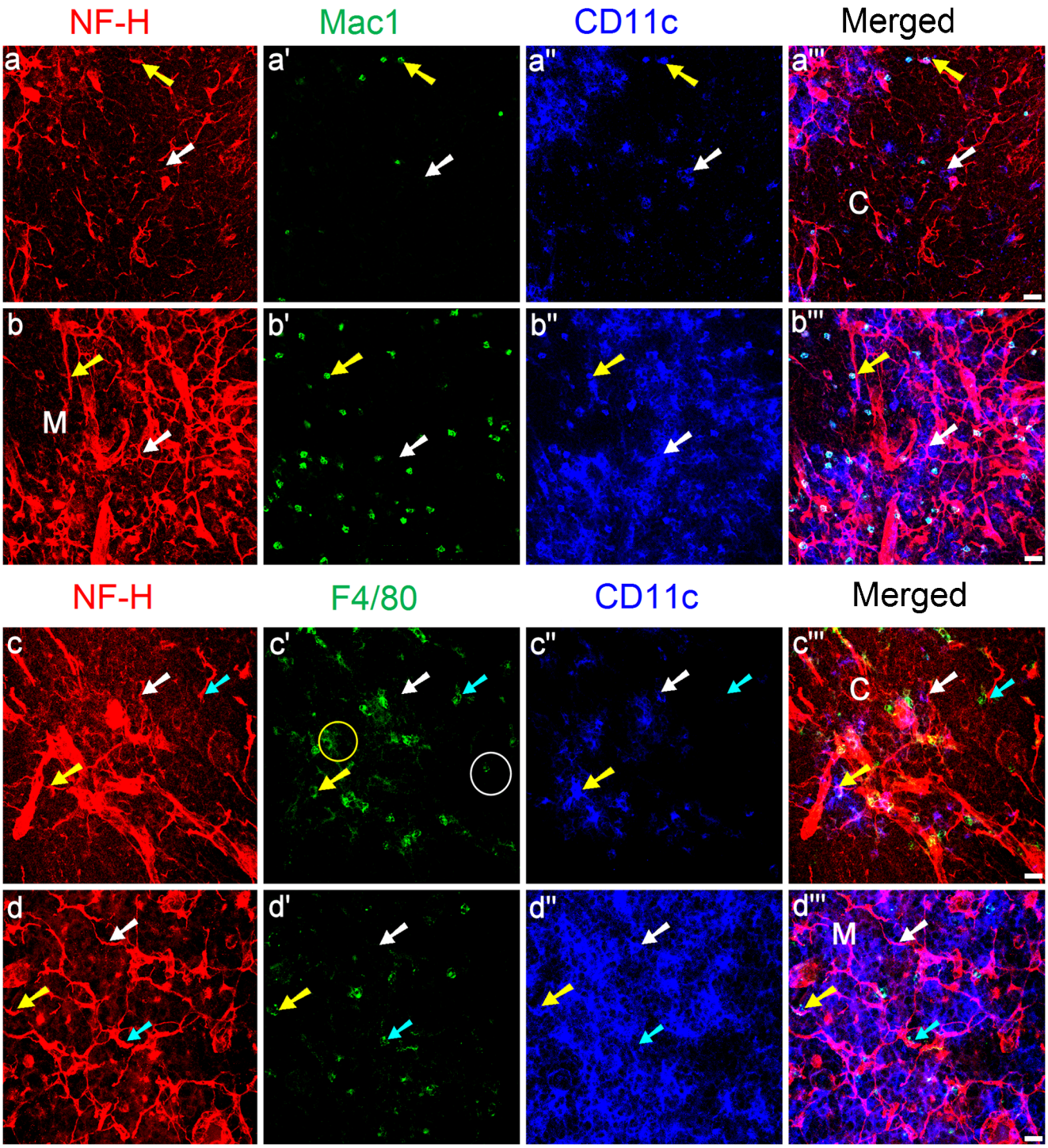

Fig. 8 Distribution of nerve fibers, macrophages and DCs in the cortex (**a–a‴**, **c–c‴**) and medulla (**b–b‴**, **d–d‴**) in the thymus from a C57BL/6 mouse. Antibodies against NF-H (*red*), Mac1 (CD11b) or F4/80 (*green*) and CD11c (*blue*) label mainly nerve fibers, macrophages and DCs, respectively. (**a–b‴**) *White arrows* show $Mac1^{-}CD11c^{+}$ DCs closely apposed to nerve fibers. *Yellow arrows* indicate $Mac1^{+}CD11c^{+}$ macrophages having a close association with nerve fibers. (**c–d‴**) *Cyan arrows* indicate $F4/80^{+}CD11c^{-}$ macrophages that are closely associated with nerve fibers. *White arrows* show $F4/80^{-}CD11c^{+}$ DCs closely apposed to nerve fibers. *Yellow arrows* indicate $F4/80^{+}CD11c^{+}$ DCs having a close association with nerve fibers. The macrophages are of two shapes, namely oval (*white circle*) and plate (*yellow circle*). Each image is a maximal intensity projection of a Z-stack (*M*, medulla; *C*, cortex). Stack size: 6.0 μm; optical slice interval: 0.50 μm. Objective lens: 40×. Bar: 20 μm

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval All animal experiments were carried out following the Australian code for the care and use of animals for scientific purposes at Murdoch University, Perth, Australia and with local animal ethics committee approval.

Informed consent Not applicable.

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