Original Article

Stem Cell INVESTIGATION

CD44 expression in stem cells and niche microglia/macrophages following ischemic stroke

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Background: CD44, an adhesion molecule in the hyaluronate receptor family, plays diverse and important roles in multiple cell types and organs. Increasing evidence is mounting for CD44 expression in various types of stem cells and niche cells surrounding stem cells. However, the precise phenotypes of $CD44^+$ cells in the brain under pathologic conditions, such as after ischemic stroke, remain unclear.

Methods: In the present study, using a mouse model for cerebral infarction by middle cerebral artery (MCA) occlusion, we examined the localization and traits of $CD44^+$ cells.

Results: In sham-mice operations, CD44 was rarely observed in the cortex of MCA regions. Following ischemic stroke, $CD44^+$ cells emerged in ischemic areas of the MCA cortex during the acute phase. Although CD44 at ischemic areas was, in part, expressed in stem cells, it was also expressed in hematopoietic lineages, including activated microglia/macrophages, surrounding the stem cells. CD44 expression in microglia/macrophages persisted through the chronic phase following ischemic stroke.

Conclusions: These data demonstrate that CD44 is expressed in stem cells and cells in the niches surrounding them, including inflammatory cells, suggesting that CD44 may play an important role in reparative processes within ischemic areas under neuroinflammatory conditions; in particular, strokes.

Keywords: CD44; ischemic stroke; microglia; neuroinflammation; stem cell niche

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Introduction

CD44 is an adhesion molecule that plays essential roles in various cells and multiple organs through a number of signaling pathways (1). Although the precise function of CD44 in the central nervous system (CNS) remains unclear, previous studies demonstrate that CD44 plays an essential role in regulating the neural cell functions; such as axon guidance, synaptic transmission, oligodendrocyte and astrocyte differentiation, and brain protection and regeneration after injury (2,3). In the CNS, it is well-documented that CD44 is expressed in various neural cell types, such as neurons (4), astrocytes (4-6), and oligodendrocytes (6,7). However, increasing evidence shows that CD44 is observable in neural stem/progenitor cells (NSPCs) (8,9). NSPCs reside in a perivascular niche (10) and produce all neural cell types, including neurons, astrocytes, and oligodendrocytes (11). In addition, Naruse and colleagues found that CD44 expression is dynamically altered from NSPCs to neurons and astrocytes in the developing brain (4), indicating that CD44 is first expressed in NSPCs rather than mature neural cells.

In various organs outside the CNS, CD44 is expressed in multipotent stem cells, such as mesenchymal stem cells (MSCs) (12,13) and adipose-derived stem cells (ADSCs) (14,15), which localize at the perivascular niche of bone marrows and fat pads, respectively. Although the precious traits of multipotent vascular stem cells in the CNS remain unclear, increasing evidence indicates that brain pericytes are likely multipotent vascular stem cells (16) because of their perivascular localization and multipotency (17-19). In support of this viewpoint, we previously demonstrated that brain pericytes/perivascular cells following ischemia exhibit multipotency (20); thus, ischemia-induced stem cells (iSCs) have the potential to differentiate into neural and also mesoderm lineages (20-29), consistent with the traits of multipotent pericytes (17,30-34). Further, we recently demonstrated that iSCs express CD44 (21,35,36) and that iSCs can differentiate into various neural cell types, including microglia/macrophages lineages (22). As microglia/macrophages function in stem cell niches within the brain (37,38), this suggests that CD44 can be expressed stem cells and the niche cells surrounding them following ischemic strokes.

However, the precise traits of $CD44^+$ cells in brain pathology brains remain unclear. In the present study, using a mouse model of cerebral infarction, we investigated the localization and phenotypes of $CD44^+$ cells in the brain.

Methods

Induction of focal cerebral ischemia

All experimental procedures were approved by the Animal Care Committee of the Hyogo College of Medicine (License number: 17-051, 18-061). We used adult 6–10 week-old male mice (CB-17/Icr-+/+Jcl mice (CB-17 mice; Clea Japan Inc., Tokyo, Japan); B6.Cg-Tg(Nes-EGFP)1Yamm transgenic mice (Nestin-GFP mice) (39). Nestin-GFP mice were provided by the RIKEN BRC (Ibaraki, Japan) through the National Bio-Resource Project of the MEXT/AMED, Japan. Permanent focal cerebral ischemia was produced in the mice by ligation and interruption of the distal portion of the left middle cerebral artery (MCA) (22,24-26,40).

Preparation of samples from brains

CB-17 mice were anesthetized with sodium pentobarbital and transcardially perfused with 4% paraformaldehyde (PFA) as described previously (20,35,41). Next, whole brains were carefully removed, subjected to post-fixation with 4% PFA, and processed for paraffin embedding. Samples were then cut into 8-μm sections, followed by immunohistochemistry. In another experiment, fixed brains obtained from CB-17 mice or Nestin-GFP mice were cryoprotected in 30% sucrose, frozen at –80 ℃, and cut into 16-μm sections using a cryostat for double-staining using immunohistochemistry.

Immunohistochemistry

Immunohistochemistry was performed as previously described (42). In brief, after deparaffinization, sections were subjected to heat treatment by microwave for epitope retrieval in a citrate buffer solution (pH 6.0) (Dako, Glostrup, Denmark) for 10 min. Then, samples were incubated with a primary antibody against CD44 (1:200; rat, Thermo Fisher Scientific, Waltham, MA, USA), followed by reaction with a secondary antibody harboring a universal immunoperoxidase (N-Histofine Simple Stain Mouse MAX PO, Nichirei Corporation, Japan). Sections were stained by 3,3'-diaminobenzide tetrahydrochloride (DAB; Invitrogen, California, USA) and counterstained with hematoxylin. Images were captured using a microscope (Olympus, Tokyo, Japan).

In separate experiments, brain sections (16-μm thick) were subjected to double-stained immunohistochemistry as previously described (23-26,40-43). In brief, sections were stained using primary antibodies against CD44 (1:200; rat, Thermo Fisher Scientific, 1:200; sheep, R&D systems, Minneapolis, MN, USA), microtubule-associated protein 2 (MAP2) (1:500; mouse, Sigma-Aldrich, St. Louis, MO, USA), MBP (1:100; mouse, R&D systems), glial fibrillary acidic protein (GFAP) (1:500; mouse, Millipore, Temecula, CA, USA), GFP (1:1,000; rabbit, Abcam), CD31 (1:100, rat, BD Pharmingen, San Diego, CA, USA), PDGFRβ (1:500, goat, R&D systems), CD45 (1:200; rat, R&D systems), Iba1 (1:500; rabbit, Wako, Osaka, Japan), CD86 (1:100; rat, Thermo Fisher Scientific), and CD206 (1:50; goat, R&D systems). After washing in PBS, sections were stained using Alexa Fluor 488- or 555-conjugated secondary antibodies (1:500; Molecular Probes, Eugene, OR, USA). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (1:500; DAPI, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA). Images were captured using a confocal laser microscope (LSM780; Carl Zeiss AG, Oberkochen, Germany).

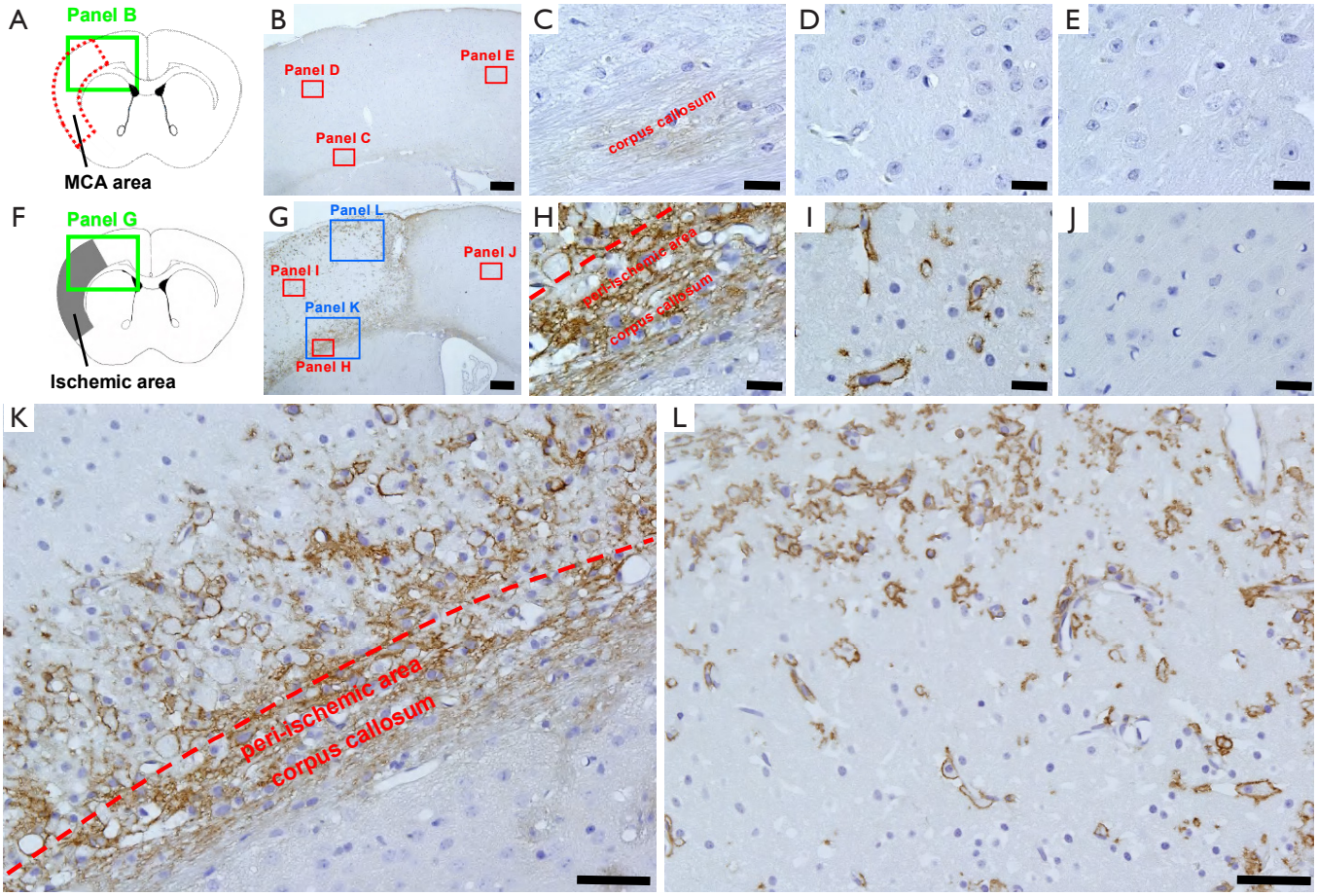

Figure 1 CD44$^+$ cells emerge within ischemic areas during acute phase following stroke. Immunohistochemistry at post-stroke day 7 (A-E) displays weak CD44 staining in the corpus callosum in sham-operated mice (B,C). CD44 was rarely observed in cells of MCA (B and D) and ACA regions (B and E). On day 7 after ischemic stroke (F-I), in addition to the increased expression of CD44$^+$ cells in the corpus callosum (G and H), many CD44$^+$ cells also emerged in peri-ischemic areas (G, H, and K) and ischemic cores (G, I, K, and L). In contrast, CD44$^+$ cells were rarely observed in ipsilateral non-ischemic areas (G and J). Results displayed are representative of three replicates (N=3). Scale bars =200 μm (B and G), 20 μm (C, D, E, H, I, and J), and 50 μm (K and L). MCA, middle cerebral artery; ACA, anterior cerebral artery.

Results

Expression patterns of CD44$^+$ cells during the acute phase following ischemic stroke

We first examined the localization of CD44$^+$ cells following ischemic stroke (*Figure 1*). In sham-operated mice (*Figure 1A,B,C,D,E*), we observed weak staining for CD44$^+$ cells in the corpus callosum (*Figure 1B,C*). However, CD44$^+$ cells were rarely observed in the cells of cortex, which is fed by the MCA (*Figure 1B,D*) and the anterior cerebral artery (ACA) (*Figure 1B,E*). However, on day 7 after ischemic stroke (*Figure 1F,G,H,I*), in addition to enhanced expression of CD44$^+$ cells at the corpus callosum (*Figure 1G,H*), many CD44$^+$ cells appeared in peri-ischemic areas (*Figure 1G,H,K*) and in ischemic cores (*Figure 1G,I,K,L*), while they were rarely observed in ipsilateral non-ischemic areas (*Figure 1G,J*).

CD44 expression by neural lineages including NSPCs following ischemic stroke

Thus far, our data indicates that CD44$^+$ cells are dramatically increased within ischemic areas following stroke. Previous studies demonstrate that CD44 is expressed in various types of neural cells, including neurons (4), oligodendrocytes (6,7), and astrocytes (4-6). Thus, using brain sections at post-stroke day 7, we examined

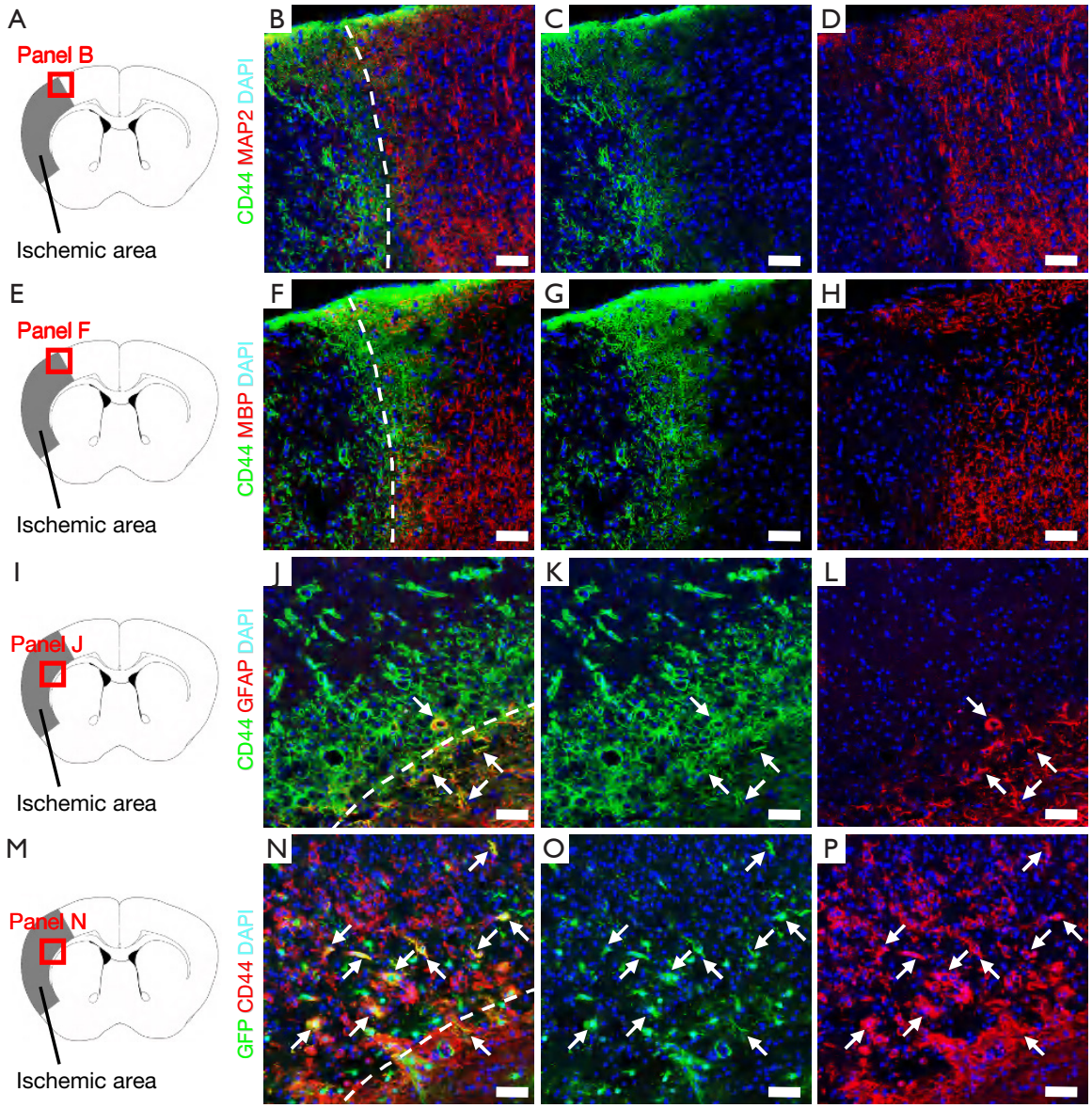

Figure 2 CD44⁺ cells within ischemic areas are observed in neural lineage cells, including NSPCs. Immunohistochemistry at post-stroke day 7 (A-H) displays CD44⁺ cells within ischemic areas do not express MAP2 [CD44 (B, C: green), MAP2 (B, D: red), and DAPI (B-D: blue)] or MBP [CD44 (F, G: green), MBP (F, H: red), and DAPI (F-H: blue)]. Immunohistochemistry at post-stroke day 7 (I-L) demonstrates that CD44 is expressed in GFAP⁺ astrocytes primarily localized in peri-ischemic areas [CD44 (J, K: green), GFAP (J, L: red), and DAPI (J-L: blue)] (arrows). Immunohistochemistry at post-stroke day 7 (M-P) display CD44 expression in GFP⁺ NSPCs which localize in ischemic cores and peri-ischemic areas [GFP (N, O: green), CD44 (N, P: red), and DAPI (N-P: blue)] (arrows). Results displayed are representative of three replicates (N=3). Scale bars =50 μm (B, C, D, F, G, H, J, K, L, N, O, and P).

whether CD44 is expressed in our cerebral infarctions. Immunohistochemistry showed that CD44⁺ cells within ischemic areas did not express the neuronal marker MAP2 (*Figure 2A,B,C,D*) or then oligodendrocytic marker MBP (*Figure 2E,F,G,H*). In addition, CD44⁺ cells in peri-ischemic areas including the corpus callosum did not express MAP2 or MBP (data not shown). However, some CD44⁺ cells in peri-ischemic areas expressed the astrocytic marker GFAP (*Figure 2I,J,K,L*).

NSPCs produce various types of neural cells, including

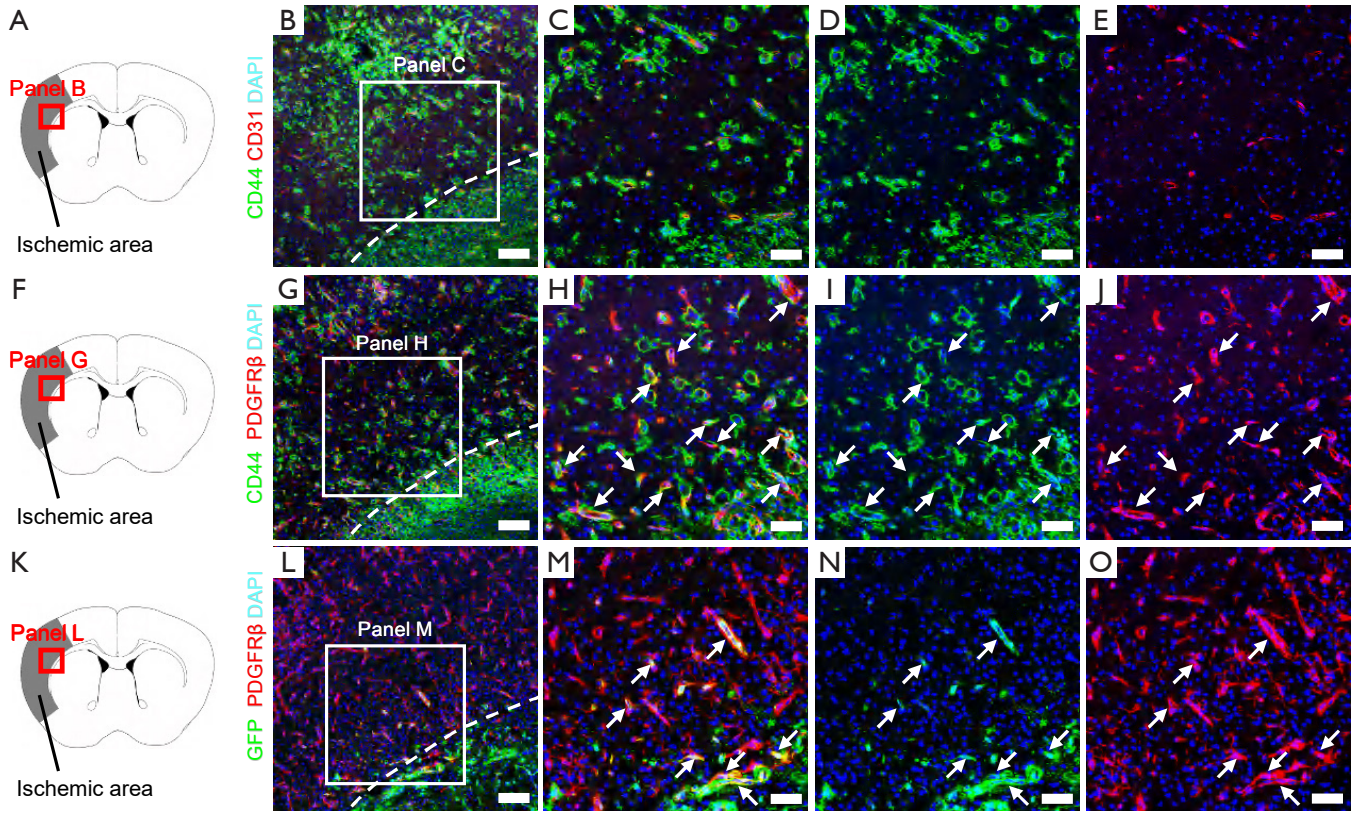

Figure 3 Ischemia induces CD44 expression within some stem cells in stroke-affected areas. Immunohistochemistry at post-stroke day 7 (A-O) demonstrates that CD44$^+$ cells do not express CD31 [CD44 (B, C, and D: green), CD31 (B, C, and E: red), and DAPI (B-E: blue)] but do express PDGFRβ [CD44 (G, H, and I: green), PDGFRβ (G, H, and J: red), and DAPI (G-J: blue)] (arrows). Immunohistochemistry at post-stroke day 7 (K-O) displays that some GFP$^+$ cells co-express PDGFRβ [GFP (L M, and N: green), PDGFRβ (L, M, and O: red), and DAPI (L-O: blue)] (arrows). Results displayed are representative of three replicates (N=3). Scale bars =100 µm (B, G, and L) and 50 µm (C, D, E, H, I, J, M, N, and O).

neurons, astrocytes, and oligodendrocytes (11). In addition, previous studies showed that CD44 is expressed in NSPCs (8,9). Furthermore, we previously demonstrated that NSPCs develop in ischemic cores and in peri-ischemic areas following stroke (26,40). Thus, using nestin-GFP transgenic mice, we investigated whether GFP$^+$ cells at the site of ischemic areas express CD44. Using immunohistochemistry, we observed that GFP, under the control of the nestin (a NSPC marker) promoter, was present in ischemic cores and peri-ischemic areas and that some GFP$^+$ cells co-expressed CD44 (*Figure 2M,N,O,P*).

CD44 expression by ischemia-induced stem cells

The above findings demonstrate that CD44 is expressed in neural lineages, including NSPCs, after ischemic stroke. However, evidence exists for CD44 expression in various types of stem cells, including MSCs (12,13) and ADSCs (14,15), as well as NSPCs (8,9). Although the precise origin of MSCs and ADSCs is unclear, increasing evidence indicates that they have similar traits to those of pericytes (44-48). In addition, we demonstrated that brain pericytes following ischemia develop into iSCs (20,24,43) and that CD44 is expressed in iSCs that co-express nestin as well as PDGFRβ (21,35,36). Thus, we investigated whether CD44 is expressed in iSCs. Immunohistochemistry identified CD44$^+$ cells at the site of ischemic areas localizing around CD31$^+$ endothelial cells (*Figure 3A,B,C,D,E*) and that they also expressed PDGFRβ (*Figure 3F,G,H,I,J*). Furthermore, some GFP$^+$ cells in ischemic cores and peri-ischemic areas co-expressed PDGFRβ (*Figure 3K,L,M,N,O*). These results indicate that CD44 is, in part, expressed in iSCs; which

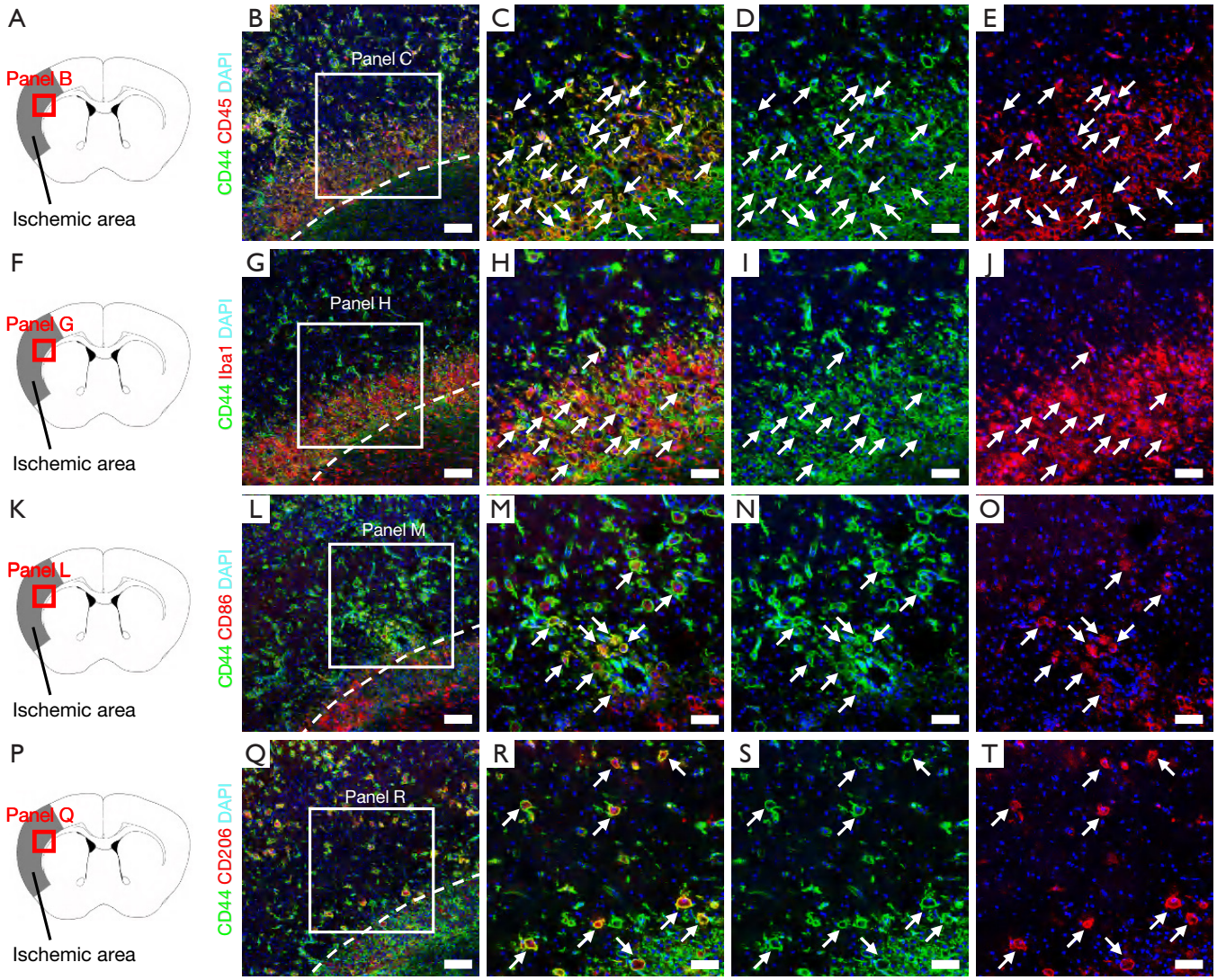

Figure 4 CD44 is expressed in hematopoietic cells including microglia/macrophages within ischemic areas. Immunohistochemistry at post-stroke day 7 (A-L) demonstrates that some CD44$^+$ cells within ischemic areas express CD45 [CD44 (B, C, and D: green), CD45 (B, C, and E: red), and DAPI (B-E: blue)] (arrows) and other various microglial/macrophage markers, including Iba1 [CD44 (G, H, and I: green), Iba1 (G, H, and J: red), and DAPI (G-J: blue)] (arrows), CD86 [CD44 (L, M, and N: green), CD86 (L, M, and O: red), and DAPI (L-O: blue)] (arrows), and CD206 [CD44 (Q, R, and S: green), CD206 (Q, R, and T: red), and DAPI (Q-T: blue)] (arrows). Results displayed are representative of three replicates (N=3). Scale bars =100 μm (B, G, L, and Q) and 50 μm (C, D, E, H, I, J, M, N, O, R, S, and T).

likely originate from brain pericytes.

CD44 expression by hematopoietic lineages including microglia/macrophages

Given the variety of CD44 expression we observe in our infarctions, we investigated whether CD44 is expressed in stem cell niches (e.g., hematopoietic cells including inflammatory cells) surrounding NSPCs and/or iSCs. Immunohistochemistry at post-stroke day 7 demonstrated that many CD44$^+$ cells, in particularly those with round shapes, expressed the hematopoietic marker CD45 (*Figure 4A,B,C,D,E*). As CD45 is known to be expressed in microglial lineages during brain pathologies (5,49), we further examined whether CD44 is expressed in microglia/marophages following ischemia. Immunohistochemistry

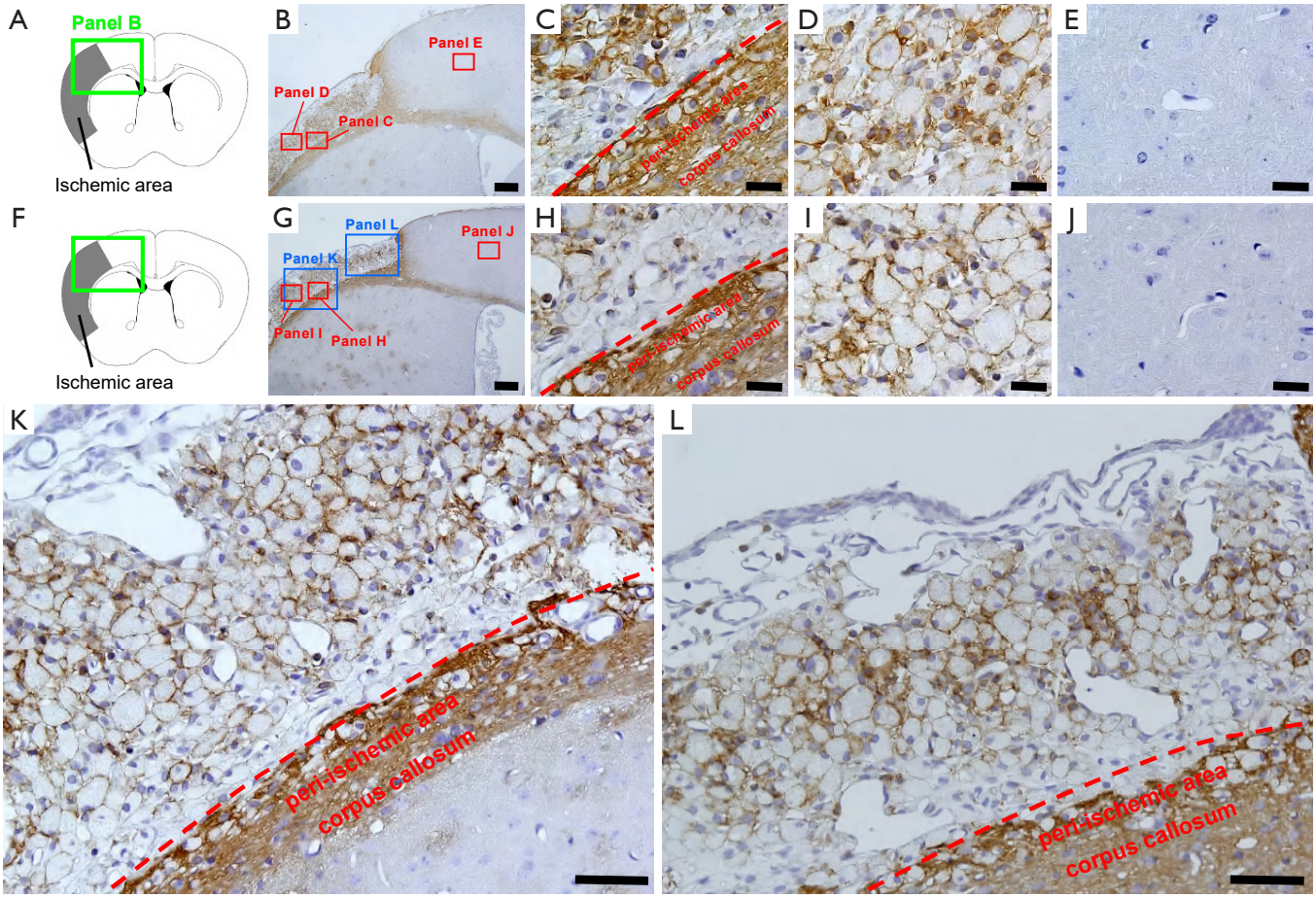

Figure 5 CD44 expression by microglia/macrophages within ischemic areas persists through chronic phases. Immunohistochemistry at post-stroke 2 (A-E) and 4 weeks (F-L) demonstrates that CD44 expression by microglia/macrophages within ischemic areas lasts during subacute (B-E) and chronic periods post-stroke (G-L). Results displayed are representative of three replicates (N=3). Scale bars =200 µm (B, G), 20 µm (C, D, E, H, I, J), and 50 µm (K, L).

at post-stroke day 7 indicated that some CD44$^+$ cells in ischemic cores and peri-ischemic areas expressed the microglia/macrophage cell marker Iba1 (*Figure 4F,G,H,I,J*). These results indicate that, under ischemic stroke, CD44 is expressed in stem cell niches including activated microglia/macrophages as well as stem cell populations.

As activated microglia/macrophages are known to polarize into two distinct subtypes (pro-inflammatory M1 and anti-inflammatory M2 types) (50-52), we next investigated whether CD44$^+$ cells in ischemic areas expressing the M1 marker CD86 and/or M2 marker CD206. Immunohistochemistry demonstrated that some CD44$^+$ cells at these areas co-expressed CD86 (*Figure 4K,L,M,N,O*) and CD206 (*Figure 4P,Q,R,S,T*).

Long-lasting expression of CD44 by microglia/macrophages within and around peri-ischemic areas

Our final experiments in this study investigated the fate of CD44$^+$ cells at later time points following ischemia. Immunohistochemistry was performed at 2 (*Figure 5A,B,C,D,E*) and 4 weeks after ischemic stroke (*Figure 5F,G,H,I,J,K,L*). Immunohistochemistry displayed CD44$^+$ cells in the corpus callosum (2 weeks after ischemic stroke, *Figure 5C*; 4 weeks after ischemic stroke, *Figure 5H,K,L*). In addition, the majority of the ischemic cores and the peri-ischemic areas contained CD44$^+$ foam cells which were morphologically microglia/macrophages (2 weeks after ischemic stroke, *Figure 5C,D*; 4 weeks after ischemic stroke, *Figure 5H,I,K,L*). These findings indicate

that CD44 in ischemic areas is primarily expressed in microglia/macrophages at later time points, including both subacute and chronic phases. These findings also demonstrate that CD44 expression by microglia/macrophages at these areas is persistent, indicating that CD44 can be used as a marker of activated microglia/macrophages following brain injuries, such as ischemic stroke.

Discussion

This study demonstrates that CD44 is expressed in not only stem cells but also stem cell niches such as microglia/macrophages following stroke. We found that CD44 is expressed in neural lineages, including astrocytes as well as NSPCs, in ischemic areas. Although the precise origin of $CD44^+$ astrocytes remains unclear, we previously demonstrated that NSCPs isolated from ischemic areas differentiate into various neural cells, including glial cells (40). Thus, it is possible that $CD44^+$ NSPCs are origin of $CD44^+$ glial cells. However, the precise relationships between $CD44^+$ NSPCs and $CD44^+$ glia need clarification; this will be a focus of our future studies.

Under neuroinflammatory conditions, such as after ischemic stroke, it is well-documented that microglia/macrophages accumulate in the brain (50,52). It has also been reported that brain microglia/macrophages originate from progenitors in the embryonic yolk sac and that they reside in the brain until adulthood under normal conditions (53). Although the precise origin of $CD44^+$ microglia/macrophages under pathological conditions remains unclear, our previous studies (22) and those of others (54,55) demonstrate that activated microglia are, in part, derived from brain pericytes. However, contrary to these finding, it has also been reported that a subset of pericytes originate from hematopoietic lineages, including microglia (56,57). In this study, we found CD44 in ischemic areas was expressed in some $CD45^+$ hematopoietic lineages. As CD44 is also expressed in hematopoietic cells during early CNS development (58), some $CD44^+$ cells observed in ischemic areas may have the features of immature hematopoietic cells. However, the precise relationship between $CD44^+/CD45^-$ pericytes and $CD44^+/CD45^+$ hematopoietic lineage cells in ischemic areas needs to be clarified in further studies.

In this study, we found that many $CD44^+$ cells emerge in ischemic areas, while $CD44^+$ cells are rarely observed in the MCA cells of sham-operated mice. The precise mechanism by which CD44 is selectively enhanced in cells at these areas remains unclear. However, previous studies demonstrate that CD44 expression is up-regulated in microglia/macrophages after brain injuries, including ischemic stroke (3,59,60), and that CD44 expression is regulated by various cytokines and/or molecules (61,62). This suggests that CD44 can be induced in cells stimulated by several mechanisms, including hypoxia and cytokine production.

Although the precise role of CD44 under ischemic conditions remains unclear, a previous study indicates that CD44-deficiencient mice have lower levels of pro-inflammatory cytokines after ischemic stroke, resulting in a reduction of ischemic areas, and improved neurological deficits when compared with wild-type control mice (3). These findings suggest that CD44 may function as a negative regulator during ischemic stroke; presumably through an increase of pro-inflammatory cytokines. In contrast to these findings, CD44 deficient mice displayed increased inflammatory responses during experimental autoimmune encephalomyelitis (EAE) when compared to wild-type control mice (63), suggesting that CD44 exerts anti-inflammatory effects. We do not understand the discrepancy of why CD44 displays diverse roles in CD44-deficient mice under neuroinflammatory conditions (e.g., ischemic stroke and EAE). However, our current study indicates that CD44 is expressed in activated microglia/macrophages with two different phenotypes, known as pro-inflammatory M1 and anti-inflammatory M2 types. Thus, the different results obtained in previous studies regarding CD44-deficient mice may be attributable to the diverse effects of CD44 in microglia/macrophages.

In addition to CD44, the ligands of CD44, such as hyaluronic acid (HA) (64) and osteopontin (OPN) (65), play important roles in the reparative process after injury. Although the precise function of CD44 as a receptor for HA remains unclear, multiple reports demonstrate that implantation of HA scaffolds has various positive effects on CNS regeneration, in part through inhibition of glial scar formation (66-68). OPN is a glycoprotein that was originally purified from bone. However, OPN is expressed in various types of cells, including macrophages (69,70). Thus, it is possible that $CD44^+$ microglia/macrophages also secrete OPN following ischemic stroke. Furthermore, it has been reported that CD44-OPN signaling promotes the proliferation of CNS stem cells, including NSPCs (71,72), neuronal differentiation (72), and axon growth (73). Although the precious roles of microglia/macrophages in during brain pathologies are still unclear, microglia/macrophages are known to have important roles in stem cell

niches (37,38). Thus, CD44$^+$ microglia/macrophages may alter the fates of CD44$^+$ stem cells, such as NSPCs and/or iSCs, following ischemic stroke; thereby regulating the neural reparative processes during brain injuries. However, the precise relationship between CD44$^+$ stem cells and CD44$^+$ microglia/macrophages in stem cell niches needs to be elucidated by future studies.

In conclusion, we demonstrated that CD44 is expressed, not only stem cells, but also in niche microglia/macrophages following ischemic stroke, suggesting that CD44 plays important roles during reparative processes under neuroinflammatory conditions. However, the precise traits and roles of CD44$^+$ cells in during brain pathologies require further studies.

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Footnote

Conflicts of Interest: Takayuki Nakagomi serves as the unpaid Editorial Board Member of Stem Cell Investigation. Department of Therapeutic Progress in Brain Diseases is financially supported by Daiichi Sankyo Co., Ltd., Nippon Zoki Pharmaceutical Co., Ltd., and CLEA Japan, Inc. The sponsors had no roles in this study, including those of study design, data collection, data analysis, data interpretation, and manuscript writing. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All experimental procedures were approved by the Animal Care Committee of the Hyogo College of Medicine (License number: 17-051, 18-061). All procedure performed in this study involving animal experiments were in accordance with the ethical standards as described in the methods sections.

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