The perivascular space as a path of hematopoietic progenitor cells and mature T cells between the blood circulation and the thymic parenchyma

Kazuya Mori[1],*, Manami Itoi[1],*, Noriyuki Tsukamoto[1], Hajime Kubo[2] and Takashi Amagai[1],*

[1]Department of Immunology and Microbiology, Meiji University of Oriental Medicine, Hiyoshi-cho, Nantan, Kyoto 629-0392, Japan
[2]Horizontal Medical Research Organization, Faculty of Medicine and Graduate School of Medicine, Kyoto 606-8501, Japan

Abstract

It is known that selected populations of lymphoid cells migrate into and from the adult thymus through blood vessels at the cortico-medullary junction and in the medulla. Here, we show that in the perivascular spaces (PVS) of mice surrounding large blood vessels, CD117-positive hematopoietic progenitor cells, CD4 single-positive (SP) and CD8SP T cells are located. However, developing thymocytes, CD25-positive cells and CD4 and CD8 double-positive cells, are not detectable in the PVS. After intravenous (i.v.) injection of CD117-positive bone marrow (BM) cells from C57BL/6 mice into non-irradiated RAG2 mutant mice i.v., donor-derived cells first preferentially migrate into the PVS within 30 min, and then the number of donor-derived cells in the thymic parenchyma increases. Likewise, newly developed mature T cells in the thymic parenchyma of RAG2 mutant mice transferred with wild-type BM cells migrate to the PVS, before leaving the thymus to the circulation. Accumulation of mature T cells was observed after treatment with sphingosine-1 phosphate receptor agonist FTY720 not only in the medulla but also in the thymic PVS. These results suggest that the PVS is a transit pathway for progenitor cells to immigrate into the thymus and for mature T cells to emigrate from the thymus.

Introduction

Cell migration into and out of lymphoid organs regulate homeostasis of the immune system (1, 2). During embryonic life, hematopoietic stem cells immigrate from the fetal liver into the bone marrow (BM) (3). Hematopoietic stem cells proliferate and differentiate to B cells and mature blood cells within stromal niches, located in between the venous sinuses (1, 4). After maturation, these cells enter the venous sinuses to leave the BM through the blood circulation (5). Arriving at the lymph node, lymphocytes migrate across high endothelial venules to enter the lymphoid parenchyma, while emigrating lymphocytes leave the lymph node via the medullary sinus through efferent lymphatics to the circulation (6). During organogenesis, hematopoietic progenitor cells enter the thymus anlage from the surrounding connective tissue (7, 8). However, in the post-natal thymus, progenitor cells migrate through blood vessels located in the medulla and at the cortico-medullary junction (9). Within the thymus, T cells develop in niches formed by stromal cells in the thymic pa-

renchyma (10–12). After full maturation in the parenchyma, T cells arrive in the medulla and at the cortico-medullary junction and leave the thymus to the periphery.

Only small populations of T lineage cells enter or leave the thymus through blood vessels (11). Therefore, to ensure selective migration of the cells into or from the thymus, the blood vessels located at the sites of cell migration in the thymus must harbor specific recognition structures and transition mechanisms.

Some vessels in the thymus are known to have the perivascular spaces (PVS) compartmentalized with a vascular basement membrane and a second basement membrane bordering the thymic parenchyma (13–19). Several authors claimed that the PVS are exclusively seen in the medulla and at the cortico-medullary junction of the thymus (13, 14, 16), while others described that the PVS are also found in the thymic cortex (19). So, the distribution of the PVS in the thymus still remains unclear. Additionally, the presence of

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lymphoid cells in the thymic PVS has been reported (15–17, 19). However, the phenotype of the lymphoid cells within the thymic PVS and the role of the PVS in cellular transit are still not clearly understood.

To clarify the function of the PVS in the thymus, we analyzed the anatomical distribution of the thymic PVS and the phenotype of the lymphoid cells enclosed in the PVS. We also examined sites where the lymphoid cells immigrate into the thymus and emigrate from the thymus. We show that the PVS located around vessels in the medulla and at the cortico-medullary junction of the mouse thymus contain hematopoietic progenitor cells and mature T cells, but not immature thymocytes. Intravenously (i.v.) injected progenitor cells first preferentially migrate into the PVS, and then the number of donor-derived cells in the thymic parenchyma increases, while newly developed mature T cells migrate into the PVS before exiting from the thymus to the circulation.

Methods

Mice

C57BL/6 (B6; CD45.2), RAG2 mutant (B6 background) and B6.CD45.1 mice were used at the ages of 7–8 weeks. Mice were maintained in our animal facility under Specific pathogen free conditions.

Immunofluorescence staining

Thymi were embedded in OCT compound and snap frozen. Five-micrometer thick cryostat sections were mounted onto 5% 3-amino propyltriethoxysilane (Sigma, St Louis, MO, USA)-coated microscope slides. Freshly cut frozen sections were fixed with acetone at room temperature for 2 min. Sections were blocked with 1% blocking reagent (Molecular Probes, Eugene, OR, USA) or 10% Immunoblock (Dainippon Pharmaceutical Co., Osaka, Japan) in PBS for 1 h. Primary antibodies used in these stainings were mAbs: anti-laminin α2 (4H8-2; Alexis, Lausen, Switzerland), anti-CD31 (PECAM-1, 390; BD PharMingen, San Diego, CA, USA), anti-platelet derived growth factor receptor (PDGFR)β (APB5), ER-TR7, anti-CD127 (IL-7Rα, A7R34; e-Bioscience, San Jose, CA, USA), anti-TCRαβ (H57-597), anti-CD3ε (145-2C11), anti-CD62L (Mel-14), digoxigenin-labeled anti-CD25 (PC61), anti-CD117 (c-kit, 2B8; BD PharMingen and ACK-2), anti-CD11b (Mac-1, M1/70), anti-Ly6G and Ly6C (Gr-1, RB6-8C5), anti-TER119 (Ly76), anti-CD45R (B220; RA3-6B2, BD PharMingen), digoxigenin-labeled anti-CD4 (GK1.5; e-Bioscience), anti-CD8 (53-6.7; e-Bioscience) and anti-type I collagen (AB765; Chemicon, Temecula, CA, USA) or polyclonal antibodies: anti-type IV collagen (LSL, Tokyo, Japan), anti-pan-laminin (α1β1γ1; Dako, Glostrup, Denmark), anti-laminin β1 (C-19; Santa Cruz, Delaware Avenue, CA, USA), anti-fibronectin (Cappel, West Chester, PA, USA) and anti-keratin (anti-human broad-spectrum cytokeratin, Binding Site, West Chester, PA, USA). Antibody mixture of anti-Gr1, anti-TER119, anti-CD11b and anti-CD45R were used as lineage markers. Secondary antibodies used were donkey anti-sheep IgG Alexa Fluor488, goat anti-rat IgG Alexa Fluor488, goat anti-rat Alexa Fluor594, goat anti-hamster IgG Alexa Fluor488, donkey anti-rabbit IgG Alexa Fluor594, goat anti-donkey IgG Alexa

Fluor350 (Molecular Probes), biotinylated rabbit anti-mouse IgG (Invitrogen, Karlsruhe, Germany), biotinylated goat anti-hamster IgG (Invitrogen), biotinylated goat anti-mouse IgG, sheep anti-digoxigenin IgG (Biogenesis, Poole, UK) and anti-sheep Alexa Fluor488 (Molecular Probes). Biotinylated secondary antibodies were visualized by streptavidin Alexa Fluor488 or 594 (Molecular Probes), HRP–streptavidin and Alexa Fluor488 tyramide (TSA kit no. 22, Molecular Probes) or HRP–streptavidin and Alexa Fluor594 tyramide (TSA kit no. 25, Molecular Probes). Antibodies and reagents were diluted in 1% blocking reagent or 10% Immunoblock in PBS. The thymus images were captured by Olympus Provis AX80 microscopy (Tokyo, Japan). The digital images were acquired by Coolsnap (Rooper Japan, Tokyo, Japan). Image procession was done by Photoshop (Adobe, San Jose, CA, USA).

Flow cytometry

Heparinized peripheral blood was harvested from the recipient mice, and after the cell counts, peripheral white blood cells were prepared by the treatment with 0.017 M Tris (pH 7.65) 0.144 M ammonium chloride solution. FITC-labeled anti-CD8α mAb (53-6.7) and R-PE-labeled anti-CD4 mAb (L3T4) were purchased from BD PharMingen and were used for staining. The cells were made in suspension in MEM (Nissui, Tokyo, Japan) supplemented with 1% FCS and 0.1% NaN$_3$. Next, cells were stained with the mAb for 30 min on ice. After washing by centrifugation, the cells were analyzed with a FACScan (Becton Dickinson, Mountain View, CA, USA) gated to exclude non-viable cells.

Cell transfer

To examine progenitor cell migration, BM cells from the femurs of B6 mice were made into suspension in MEM and incubated with anti-CD117 (c-kit) antibody for 30 min at 4°C. After washing twice, cells were incubated with Dynabeads M-450, coated with sheep anti-rat IgG (Dynal, Lake Success, NY, USA) for 60 min at 4°C and CD117-positive BM cells were recovered by magnetic sorting according to the manufacturer's instruction. Then, sorted CD117-positive cells were labeled with 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) using the Vybrant CFDA-SE Cell Tracer kit (CFSE: C-1157, Molecular Probes). After labeling, the cells were washed twice and CFSE-labeled CD117-positive BM cells (2×10^6) were transferred i.v. into non-irradiated RAG2 mutant mice. The numbers of donor-derived CFSE-labeled cells observed in the PVS and the parenchyma of the thymus were counted on every second serial sections (5-μm thick) throughout the whole thymi of RAG2 mutant recipient mice in various time after the cell transfer. The numbers of CFSE-labeled cells in the PVS were summed, doubled and mean values and SDs were calculated. To examine migration of developed thymocytes into the thymic PVS, BM cells were harvested from B6 mice and treated with anti-Thy1.2, anti-CD4 and anti-CD8 antibody plus complement to remove T cells. Then, these BM cells (2×10^7) or spleen cells (2×10^7) were transferred i.v. into non-irradiated RAG2 mutant mice.

Treatment with FTY720

FTY720 was kindly provided by Mitsubishi Pharma Corporation (Osaka, Japan). The drug was dissolved in physiological

saline. B6 mice received daily intra-peritoneal (i.p.) injection with 1 mg kg^{-1} FTY720, as described previously (20).

Results

Hematopoietic progenitor cells and mature T cells are localized in the PVS distributed in the cortico-medullary junction of the mouse thymus

To investigate whether the PVS exist in the medulla and/or the cortex of the adult mouse thymus, we stained frozen sections of thymi from 7-week-old B6 mice by immunohisto-chemistry. Antibodies to type IV collagen and ER-TR5 were used to detect basement membranes and medullary epithelial cells, respectively. The PVS are identified as regions separated by two basement membranes: one basement membrane is associated with a blood vessel, while the other is associated with the epithelial border. In the cortex of the adult thymus, only small blood vessels were seen (Fig. 1A). On the other hand, in the medulla and at the cortico-medullary junction, large venules were observed besides small vessels. The PVS were only present around the large venules which are distributed in the medulla and at the cortico-medullary junction of the thymus (Fig. 1B).

Other investigators (9) have previously reported that migration of the cells in and out of the thymus occurs in the medulla and at the cortico-medullary junction where the PVS are present. Therefore, we addressed the question of whether the thymic PVS are involved in migration of lymphoid cells. Phenotypes of the cells localized within the PVS of the adult thymus were examined by immunohistochem-istry. As shown in Fig. 1(C–H), a small number of CD117-positive cells were observed in the PVS (Fig. 1C). Large numbers of CD127-positive cells, CD3ε-positive cells and TCRαβ-positive cells were distributed in the parenchyma, and a few of them were also found in the PVS (Fig. 1D, F and G). CD4 single-positive (SP) cells and CD8SP cells were present predominantly in the medulla but also in the PVS (Fig. 1H). In contrast, a few CD4-positive CD8-positive double-positive (DP) cells and a few CD25-positive cells were observed in the medulla, but such cells were not observed in the PVS (Fig. 1E and H). These results suggest that both T cell progenitors, as well as mature T cells selectively localize within the PVS. However, developing thymocytes, from CD4-negative CD8-negative CD44-positive CD25-positive (double-negative (DN) 2) to DP cells, were not observed in the PVS. The PVS are found around large blood vessels distributed in the medulla and at the cortico-medullary junction, and this is where immigration of hematopoietic progenitor cells and emigration of mature T cells occurs. These results suggest that the PVS may serve as a path for cell trafficking between the thymic parenchyma and the blood stream.

Basement membrane components and cells compartmentalizing the thymic PVS

To analyze the molecular components of basement membranes and cellular components forming the PVS, immunofluorescent staining of the adult thymi was performed (Fig. 2). Both basement membranes, creating the borders of the PVS, reacted with anti-type IV collagen, anti-type I colla-
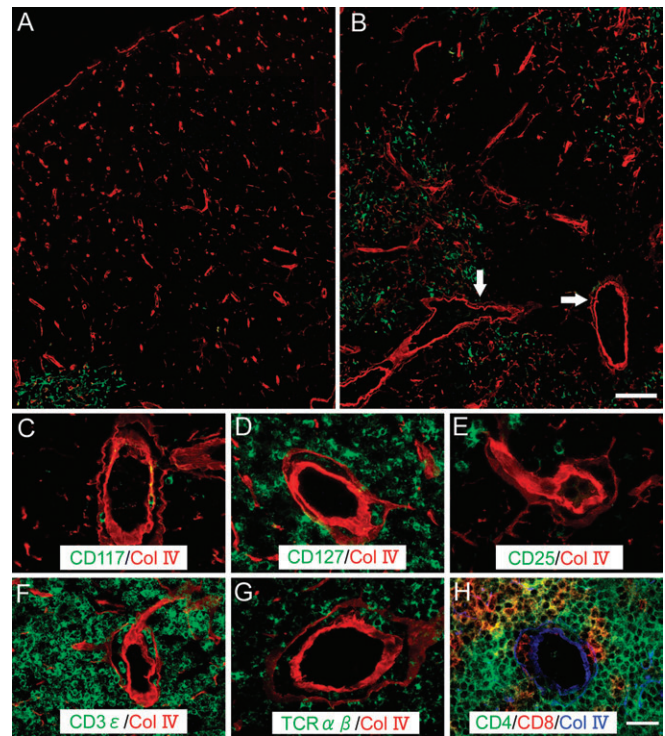

Fig. 1. Hematopoietic progenitor cells and mature T cells are present in the PVS located in the cortico-medullary junction of the adult mouse thymus. Immunofluorescent staining of frozen sections of the adult thymi was performed to detect type IV collagen (Col IV, A–G: red, H: blue), ER-TR5 (medullary epithelial marker; A and B: green), CD117 (C: green), CD127 (D: green), CD25 (E: green), CD3ε (F: green), TCRαβ (G: green), CD4 (H: green) and CD8 (H: red). (A and B) Basement membranes of blood vessels were shown by type IV collagen staining. The PVS, indicated in arrows in B, was found preferentially around large blood vessels, present only in the medulla and the cortico-medullary junction. (C–H) In the PVS of the thymus, CD117$^+$ cells, CD127$^+$ cells, CD3ε$^+$ cells, TCRαβ$^+$ cells, CD4SP cells and CD8SP cells were detected. CD25$^+$ cells and CD4$^+$CD8$^+$ DP cells were seen in the thymic parenchyma, but not in the PVS. Bars represent 125 µm (A and B) and 25 µm (C–H).

gen, anti-laminin α2, anti-pan-laminin and anti-fibronectin antibodies. As far as we examined, both basement membranes of the PVS are composed with same extracellular matrix (ECM) proteins. The reactivity to pan-laminin indicates the presence of α1, β1 or γ1 chains of laminin. It has been reported that laminin α1 is not expressed in the murine thymus (21). Moreover, laminin β1 was not detected in the basement membranes of the thymus (data not shown). Accordingly, positive staining with anti-pan-laminin antibody is most likely caused by the expression of laminin γ1 in the basement membranes, and the laminin which we detected in the PVS could be laminin 4 [α2β2γ1 (22)].

Concerning the cellular composition of the layer associating the double basement membranes of the PVS, one basement membrane is found between CD31-positive endothelial cells and PDGFRβ-positive and ER-TR7-positive pericytes (Fig. 2F–H) while the other is detected between PDGFRβ-negative and ER-TR7-positive fibroblasts and thymic epithelial cells (Fig. 2H and I). These results indicate that the individual basement membranes compartmentalize the PVS as
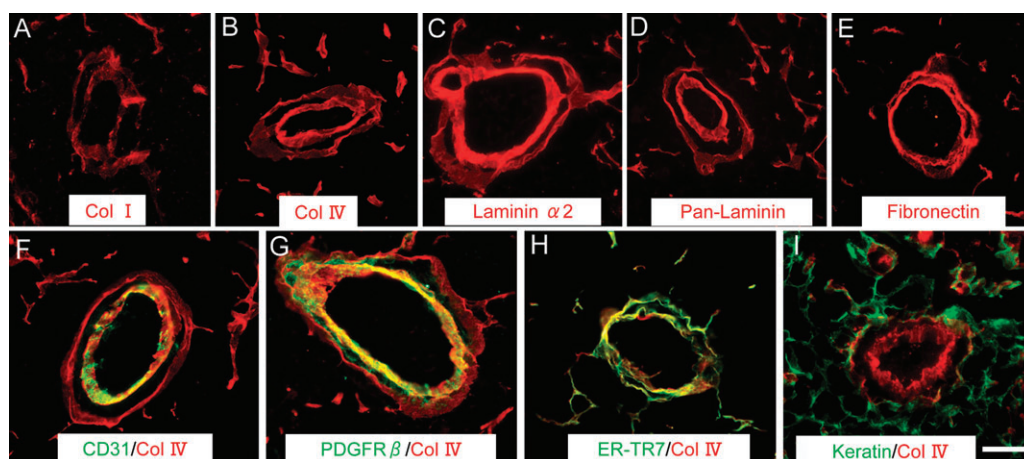
Fig. 2. The components of basement membranes and the cells compartmentalizing the thymic PVS. Immunofluorescent staining of the sections of the adult thymi was performed to detect type I collagen (Col I, A: red), type IV collagen (Col IV, B and F–I: red), laminin α2 (C: red), pan-laminin (D: red), fibronectin (E: red), CD31 (F: green), PDGFRβ (G: green), ER-TR7 (H: green) and keratin (I: green). Double basement membranes contain ECM molecules: type IV collagen, type I collagen, laminin and fibronectin. One basement membrane of the thymic PVS is located between CD31⁺ vascular endothelial cells and PDGFRβ⁺ ER-TR7⁺ pericytes and the other basement membrane is located between ER-TR7⁺ fibroblastic cells and keratin⁺ epithelial cells. Bar represents 25 µm.

previously reported (13–19), and distinct types of cell layers are lining the insides of the PVS.

Hematopoietic progenitor cells migrate to the thymic PVS before entering the thymic parenchyma

To clarify whether progenitor cells in the circulation directly migrate into the thymic PVS, we transferred progenitor cells and analyzed distributions of donor-derived cells in the recipient thymi at various time after cell transfer. CD117-positive BM cells from B6 mice were purified by magnetic sorting, labeled with CFSE and transferred i.v. into non-irradiated RAG2 mutant mice. The thymi of recipient mice were excised 0.5, 5 and 24 h after the cell transfer. Sections of the thymi from control RAG2 mutant mice and the recipient mice were analyzed by immunohistochemistry. Since CD117-positive BM cells contain a few lineage positive cells, we also examined the expression of CD127, as another progenitor marker, and lineage markers on donor-derived CFSE-labeled cells in the PVS. In the thymi of RAG2 mutant mice, organized medullary regions are not found (10), however, the PVS were detected around large blood vessels localized in deep cortex (Fig. 3A–C). A small number of CD117-positive cells was found in the thymic PVS of RAG2 mutant mice (Fig. 3A), comparable to those in the normal mouse thymus (Fig. 1C). Although many CD25-positive cells (Fig. 3B) and CD3ε-positive cells (Fig. 3C) were observed in the thymic epithelial cell region, none of them were detected in the thymic PVS. As expected, TCRαβ-positive cells, CD4SP cells or CD8SP cells were not detected in the thymi of RAG2 mutant mice (data not shown). As early as 0.5 h after the transfer with CFSE-labeled CD117-positive BM cells, CFSE-labeled cells were observed in the thymic PVS (Fig. 3D–I). The donor-derived CFSE-labeled CD117-positive cells in the PVS of RAG2 mutant mice express CD127 (Fig. 3D and E) but not lineage markers (Fig. 3G and H), though recipient-derived lineage positive cells were observed both in

the PVS and the parenchyma of the thymus (Fig. 3I). Distribution of non-T lineage cells in the thymic PVS of B6 mice is shown in supplementary Fig. 1 (available at *International Immunology* Online).

By the analysis of every second serial section throughout the recipient thymi ($N = 4$), we counted the number of donor-derived CFSE-labeled CD117-positive cells in the PVS and in the parenchyma of the thymus at 0.5, 5 and 24 h after the transfer. As shown in Fig. 3(J), donor-derived CFSE-labeled cells were first observed preferentially in the thymic PVS, and the number of donor-derived CFSE-labeled cells in the PVS increased gradually during 0.5–24 h after the transfer. Following the increase of CFSE-labeled cells in the PVS, the numbers of CFSE-labeled cells in the parenchyma were getting larger during 24 h after the transfer. These results seem to show that progenitor cells in the circulation first enter the thymic PVS, and then these cells move into the thymic parenchyma in the course of their migration into the thymus.

Newly developed mature T cells in the thymic parenchyma enter the thymic PVS before leaving the thymus

Next, we investigated whether mature T cells, developed in the thymic parenchyma, directly migrate into the thymic PVS. We transferred T cell depleted BM cells from B6 mice into RAG2 mutant mice i.v. and examined the distribution of mature T cells in the recipient thymus at weekly intervals after cell transfer. The thymi of the recipient RAG2 mutant mice were analyzed for the expression of type IV collagen and T cell markers by immunohistochemistry (Fig. 4A–O). Simultaneously, peripheral blood mononuclear cells of these mice were analyzed by a flow cytometry to determine percentages of CD4SP and CD8SP cells (Fig. 4P) and their cell numbers (Fig. 4Q). Two weeks after cell transfer, a small number of TCRαβ-positive cells and DP cells were found in the epithelial region, but not inside the PVS of the recipient
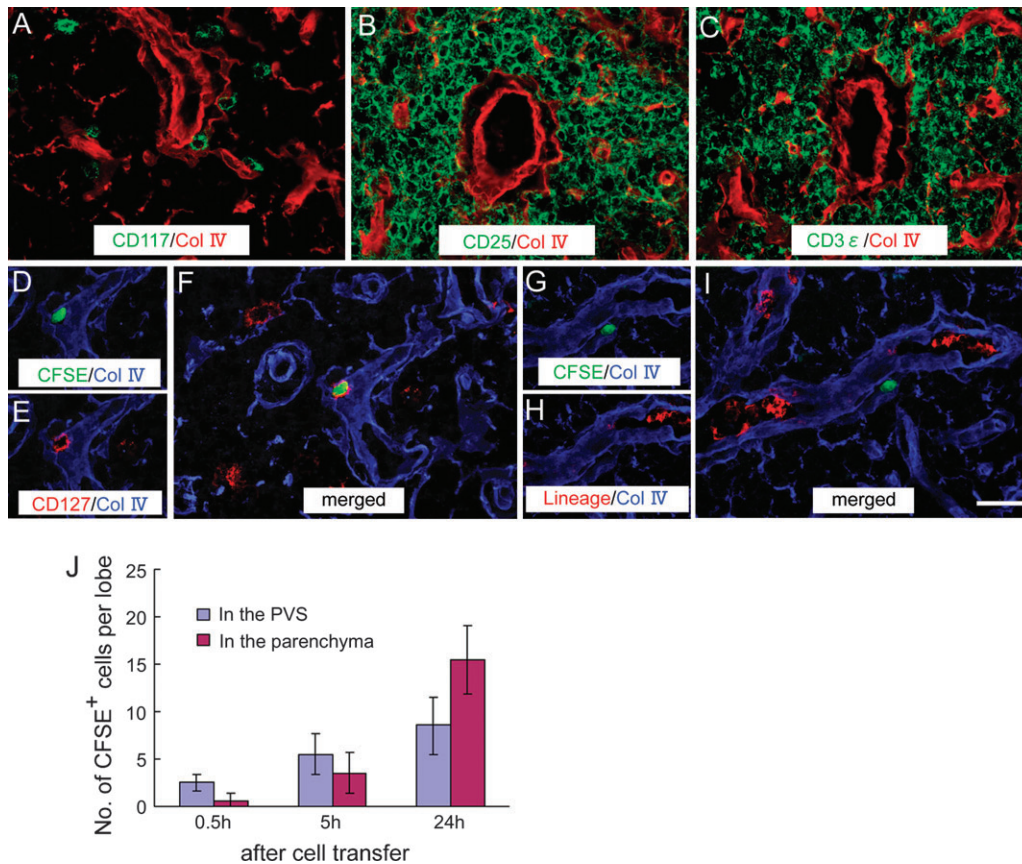
Fig. 3. BM-derived hematopoietic progenitor cells in the circulation migrate into the thymic PVS. Immunofluorescent staining was performed on the thymic sections of control RAG2 mutant mice (A–C) and RAG2 mutant mice transferred with CFSE-labeled CD117$^+$ progenitor cells of B6 mice (D–I). Type IV collagen (Col IV, A–C: red, D–I: blue), CD117 (A: green), CD25 (B: green), CD3ε (C: green), CFSE (D, F, G and I: green), CD127 (E and F: red) and lineage markers (H and I: red). (A–C) In the thymic PVS of RAG2 mutant mice CD117$^+$ cells were detected but not CD25$^+$ cells or CD3ε$^+$ cells. (D–I) Donor-derived CFSE-labeled CD117$^+$ cells were observed in the PVS, and those cells were positive for CD127 and negative for lineage markers. (F) Merged image of D and E. (I) Merged image of G and H. Bar represents 25 μm. (J) The numbers of donor-derived CFSE-labeled CD117$^+$ cells in the PVS and the parenchyma of the thymus of RAG2 mutant recipient mice in various time after the cell transfer. The numbers of donor-derived CFSE-labeled cells observed in the PVS and the parenchyma of the thymus were counted on every second serial sections (5-μm thick) throughout the whole thymus of RAG2 mutant recipient mice in various time after the cell transfer. The numbers of CFSE-labeled cells in the PVS were summed, doubled and mean values and SDs were calculated ($N = 4$). Bars represent means ± SDs. Donor-derived CFSE-labeled cells were detected in the thymic PVS within 0.5 h after cell transfer, and then the numbers of CFSE-labeled cells in the PVS and the parenchyma increased 5 and 24 h after cell transfer.

thymus (Fig. 4B, G and L). Three weeks after the transfer, many TCRαβ-positive cells and DP cells and a substantial number of CD4SP and CD8SP cells were found in the recipient thymus (Fig. 4C, H and M). Accordingly, induction of organized ER-TR5-expressing medullary epithelial regions was observed, where many TCRαβ-positive cells, CD4SP cells and CD8SP cells accumulated (data not shown). Moreover, a small number of TCRαβ-positive cells and CD4SP and CD8SP cells were also located within the PVS. Then, 4 weeks after the cell transfer, many TCRαβ-positive cells, CD4SP cells and CD8SP cells accumulated in the thymic PVS (Fig. 4D, I and N). The number of TCRαβ-positive and CD4SP and CD8SP mature T cells in the PVS seemed to decrease gradually 6–8 weeks after the transfer (Fig. 4E, J and O and data not shown). In peripheral blood of the recipient RAG2 mutant mice, only a small numbers of CD4SP and CD8SP cells were first observed 3 weeks after the transfer when CD4SP and CD8SP cells appeared both in the thymic

parenchyma and in the PVS ($N = 3$, Fig. 4P and Q). Four weeks after cell transfer, a significant number of CD4SP and CD8SP cells in peripheral blood cells were detected. Then, the numbers of CD4SP and CD8SP cells gradually increased during 8 weeks after the transfer. It is likely that mature T cells, newly developed in the thymic parenchyma, migrate into the PVS before their distribution in the periphery.

Next, we examined the possibility that mature T cells, detected in the thymic PVS, are from peripheral T cells which had re-entered the thymus. To this purpose, RAG2 mutant mice were transferred with spleen cells from B6.CD45.1 mice. Twenty-four hours after cell transfer, the thymi of recipient mice were examined for the presence of donor-derived CD45.1-positive cells in the PVS by immunohistochemistry. However, we were unable to detect CD45.1-positive cells in the PVS of the recipient thymus (data not shown). These results may indicate that mature T cells in the thymic PVS are not from the periphery but from the thymic parenchyma.
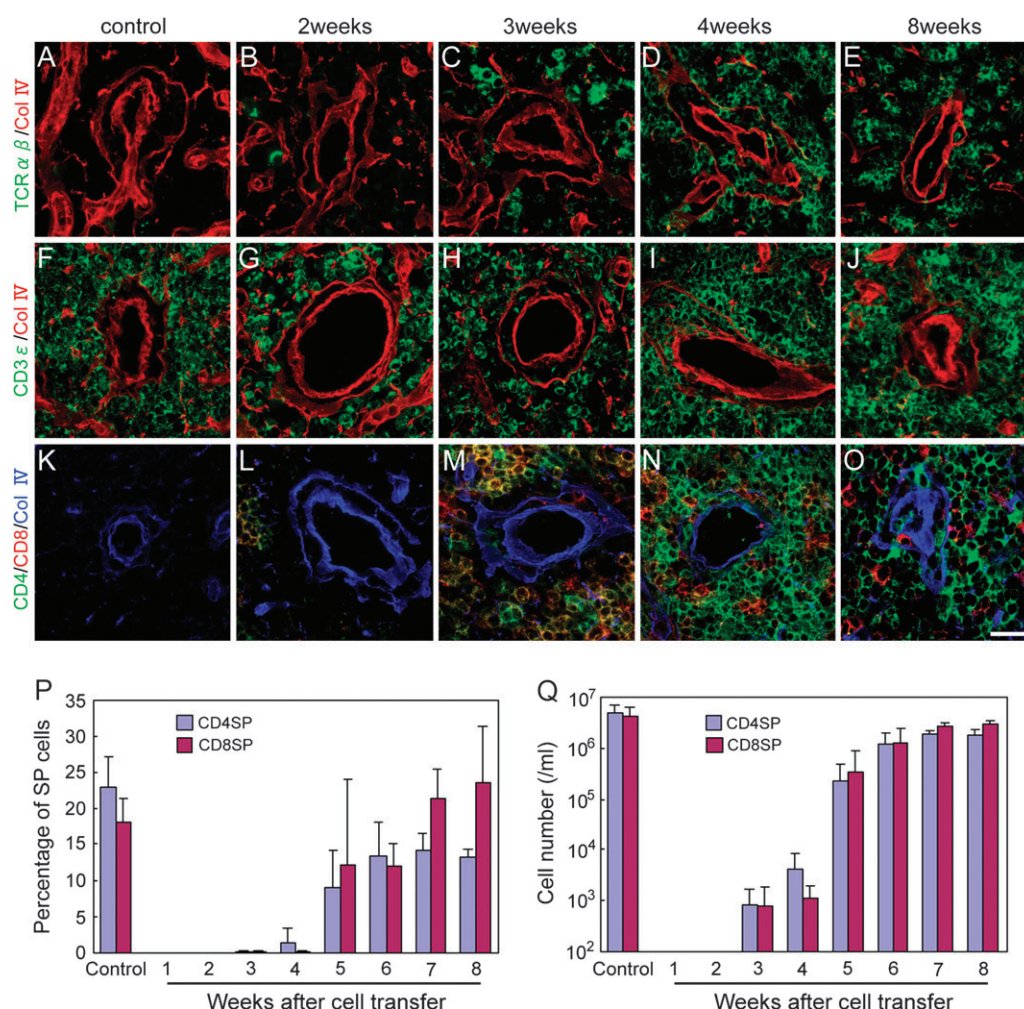
Fig. 4. Newly developed mature T cells in the thymus migrate into the PVS before leaving the thymus, and then appear in the circulation. Sections of control thymi of RAG2 mutant mice and thymi of RAG2 mutant mice transferred with BM cells from normal B6 mice 2, 3, 4 and 8 weeks before were stained with antibodies to detect type IV collagen (A–J: red, K–O: blue), TCRαβ (A–E: green), CD3ε (F–J: green), CD4 (K–O: green) and CD8 (K–O: red). In the thymic PVS of control RAG2 mutant mice, TCRαβ+, CD3ε+, CD4+ or CD8+ T cells were not detectable. Two weeks after cell transfer, a few TCRαβ+ cells and CD4+CD8+ DP cells were observed in the parenchyma but not in the PVS of the recipient thymus. A large number of TCRαβ+ cells, CD3ε+ cells, CD4SP cells and CD8SP cells was clearly present not only in the parenchyma but also in the thymic PVS 3 weeks after the transfer. The number of mature T cells in the PVS seemed to increase at 4 weeks and then decrease at 8 weeks after cell transfer. Bar represents 25 μm. (P and Q) Peripheral white blood cells were prepared from RAG2 mutant mice transferred with T cell-depleted BM cells of normal B6 mice weekly, from 1 to 8 weeks after the transfer. The cells were stained with antibodies to CD4 and CD8 and analyzed by FACScan. The percentages (P) and the numbers (Q) of CD4SP and CD8SP cells in peripheral blood are shown. A few CD4SP and CD8SP cells were detectable 3 weeks after cell transfer, and the numbers of these cells significantly increased 4 weeks after transfer. Thereafter the percentages of SP cells increased gradually during 8 weeks after transfer. Bars represent means ± SDs ($n = 3$).

Treatment with FTY720 causes accumulation of mature T cells not only in the medulla but also in the PVS

It has been shown previously that a lack of sphingosine-1 phosphate receptor 1 (S1P$_1$) results in an absence of T cells in the periphery, because mature T cells are unable to emigrate from the thymus (23, 24). Treatment of a S1P$_1$ agonist FTY720 accelerates differentiation of CD69-negative SP cells and down-regulates S1P$_1$, creating a temporally S1P$_1$-null state (25). Under these conditions, emigration of T cells from the thymus is blocked (20, 25). To examine whether FTY720 treatment causes accumulation of mature T cells exclusively in the medulla or in the PVS, or both in the medulla and the PVS, B6 mice were injected daily with FTY720 i.p. and the thymi were excised 10 and 20 days after the first injection.

The sections of the thymi were examined immunohistochemically. As shown in Fig. 5, accumulation of both CD4SP and CD8SP T cells, which was determined density of 4′6-diamidino-2-phenylindole-stained nuclei, was first detected in the medulla 10 days after the first injection. Twenty days after the first injection, accumulation of these cells was also seen in the PVS of the thymus. These results indicate that S1P$_1$ signaling is involved in the migration of mature T cells into the medulla as well as into the PVS of the thymus.

Discussion

Hematopoietic progenitor cells are selectively recruited to the thymus, and exclusively mature thymocytes leave the
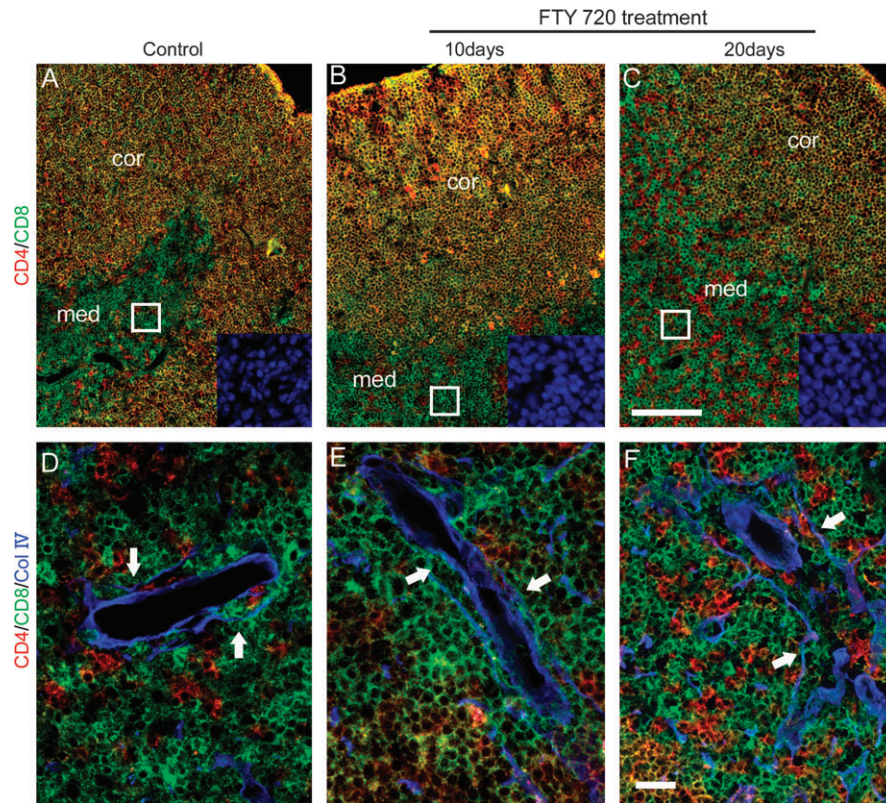
Fig. 5. Accumulation of mature T cells both in the medulla and the PVS of the adult mouse thymus after treatment with FTY720. Immunofluorescent staining of frozen sections of the adult thymi of mice i.p. injected either with physiological saline or FTY720 (1 mg kg^{-1}), daily for 10 or 20 days, was performed. CD4 (A–F: green), CD8 (A–F: red), 4′6-diamidino-2-phenylindole (inset of A–C: blue) and type IV collagen (Col IV, D–F: blue). As compared with control mice, large number of CD4SP and CD8SP cells were detected in the medulla 10 and 20 days after the first injection (A–C). The insets in A–C show the magnified views of nuclei in the boxed areas stained by 4′6-diamidino-2-phenylindole. CD4SP and CD8SP cells accumulate in the PVS of the thymus of FTY720-treated mice for 20 days (D–F). Arrows indicate the PVS. Cor, cortex and Med, medulla. Bars represent 125 μm (A–C) and 75 μm (D–F).

thymus to the circulating blood (11). Moreover, the migration of these cells in or out of the thymus occurs in the medulla and at the cortico-medullary junction (9). However, the mechanisms underlying the migration of these cells are not fully understood. Our present study shows that in the PVS of the mouse thymus, which form separated compartments around the venules in the medulla and at the cortico-medullary junction, CD117-positive progenitor cells and CD4SP and CD8SP mature T cells are present. On the other hand, another stages of developing T cells, such as CD25-positive cells and DP cells, are not seen in the thymic PVS. We also show that donor-derived CD117-positive cells preferentially migrate to the thymic PVS of non-irradiated RAG2 mutant mice within 0.5 h after the cell transfer. Furthermore, donor-derived CD117-positive cells in the thymic PVS expressed CD127 but did not express lineage markers. These results suggest that donor-derived CD117-positive cells in the recipient thymic PVS are progenitor cells for T cells. The numbers of donor-derived CD117-positive cells in the parenchyma of the thymus increased during 5–24 h after the transfer. This suggests that progenitor cells in the circulation rapidly migrate to the PVS across the walls of the blood vessels in the medulla and at the cortico-medullary junction, before entering the thymic parenchyma. At present, our results do not exclude the possibility that some progenitor cells directly mi-

grate into the thymic parenchyma from post-capillary venule distributed in the medulla and at the cortico-medullary junction. On the other hand, donor-derived mature T cells, newly developed in the thymic parenchyma of RAG2 mutant mice transplanted with wild-type BM cells, enter the thymic PVS before leaving the thymus. Using morphological methods the previous reports showed that lymphatics were present in the rodent thymus (16, 26). We have extensively investigated whether adult mouse thymus contain lymphatics, using various lymphatic vessel markers, VEGFR3, Prox1, podoplanin and LYVE-1 (27). None of the lymphatic markers, however, were detectable in the young adult mouse thymus (our unpublished data). The discrepancy may attributable to genetic background, age or breeding conditions of mice. Furthermore, we showed that mature T cells i.v. injected into RAG2 mutant mice do not re-enter the thymic PVS. Taken together, our present results suggest that the thymic PVS function as a transit path for progenitor cells and mature T cells between the thymic parenchyma and the blood stream. However, the possibility that lymphatic vessel in the mouse thymus serves as a passageway of mature T cells to periphery still remains.

We have shown that the thymic PVS are compartmentalized by double basement membranes. One of these is beneath the endothelial cells and is associated with

PDGFRβ-positive pericytes, while the other borders the epithelial cells, and is associated with PDGFRβ-negative fibroblasts. These membranes are composed of ECM molecules including type I and type IV collagens, fibronectin and laminin. It is well known that ECM proteins bind cytokines and regulate their activity, and cytokines also attach to the cells via integrins expressed on the surface of lymphoid cells (28). Therefore, the lymphoid cells present in the thymic PVS may be supported by some cytokines in the basement membrane and/or adhere to basement membrane through integrin molecules which are expressed on progenitor cells (29) and mature T cells (30). The cells retained in the PVS may be anchoring to ECM molecules (17) and/or to the cell layers (31) in the PVS. In order to enter the PVS and to leave the PVS, lymphoid cells should be able to digest ECM by an enzyme, like metalloproteinase (32). Selective migration of the cells via the PVS into and out of the thymus may be limited to the cells secreting such an enzyme.

Among various hemato-lymphoid cells, only selected progenitor cells in the blood have the potential to migrate into the thymus not only in embryonic life (7, 8) but also in post-natal life (9). A small number of CD117-positive progenitor cells can migrate to the thymus after the transfer of BM cells (33, 34). Migration of progenitor cells into the thymus was observed in the cortico-medullary junction (9). Our present results show that CD117-positive progenitor cells in blood first migrate into the thymic PVS around large venules localized in the medulla and at the cortico-medullary junction. Specific mechanisms, which have not been clarified yet, must be operating for migration of progenitor cells into the thymic PVS. Recent report (35) shows that P-selectin expressed on thymic endothelial cells and p-selectin glycoprotein ligand-1 expressed on T progenitor cells have crucial role in migration of progenitor cells into the thymus and that P-selectin is ubiquitously expressed on blood vessels in the thymus. Therefore, P-selectin expression on thymic endothelial cells do not specify entry site of progenitor cells on the endothelial cells. Thus, progenitor cells seem to require specific adhesion molecules and/or chemokine receptors for migration into the thymus (36–38).

Several reports have shown that even after entry to the thymus, DN1 cells still maintain progenitor cell activity which can generate T/NK cells (30, 39). Development of progenitor cells in the thymus depends on stromal niches in the thymic parenchyma (40). It has been reported that DN1 cells expressing Notch1 can be activated by Notch ligands, expressed on thymic epithelial cells and proceed differentiation (41). Therefore, recent immigrants in the thymic PVS, where Notch ligands are absent (our unpublished observation), might maintain their progenitor activity before making cell-fate decisions in the thymic epithelial parenchyma.

Our cell transfer experiments indicate that newly developed mature T cells in the thymic parenchyma migrate to the PVS before leaving the thymus to the circulation. Moreover, we have shown that peripheral mature T cells do not re-enter the thymic PVS. On the basis of these results, we hypothesize that there are three steps in the emigration of mature T cells from the thymus. First, developing T cells migrate from the cortex to the medulla. Second, mature T cells migrate from the medulla to the thymic PVS. Third, they migrate from the thymic PVS to the circulating blood. The PVS in the thymus

are already present in newborn mice (our unpublished data). These results correspond to the data that emigration of T cells initiates from 2 days after birth in mice (42). Therefore, from the initial stage, T cell export from the thymus may occur through the PVS. Emigration of mature T cells from the thymus is inhibited by the treatment with pertussis toxin, an inhibitor of G protein-mediated signaling (43). Therefore, some of the migration steps could be controlled by G protein-coupled receptor signaling. The first step, migration of mature T cells from the cortex to the medulla, is regulated by CCL19, produced by medullary epithelial cells and dendritic cells (44, 45). CCL19 activates CCR7, one of the G protein-coupled receptors on mature T cells. Ueno *et al.* (42) reported that emigration of mature T cells in the thymus of neonatal mice depends on CCL19 which stimulates CCR9 on mature T cells. However, the nature of chemokines inducing emigration of mature T cells from the adult thymus is still unclear. Moreover, we could detect neither CCL19 nor CCL21 expression on the walls of the thymic PVS of adult mice, consisting of basement membranes and cell layers (our unpublished observation). Sphingosine-1 phosphate (S1P) receptor, another G protein-coupled receptor, is expressed on mature T cells (23). Thymic mature T cells lacking $S1P_1$, one of the S1P receptors, accumulate in the thymic medulla and are unable to emigrate from the thymus (23–25). Our present results using FTY720, an agonist of $S1P_1$, indicate that mature T cells first accumulate in the medulla and later in the PVS of the thymus. These results suggest that accumulation of intra-thymically differentiated mature T cells occurs where the barriers of the double basement membranes of the PVS are present. We therefore suggest that S1P signaling regulates mainly the second step (from medulla to the thymic PVS) and also the third step (from the thymic PVS to circulation) of mature T cell emigration from the thymus. For this stepwise migration, mature T cells should move across two basement membranes compartmentalizing the thymic PVS. As a possible role for the PVS in the regulation of cell traffic, we hypothesize that the PVS function to limit the migrating cells into the thymic parenchyma and regulate the number of mature T cells leaving the thymus to the circulation.

Supplementary data

Supplementary Figure 1 is available at *International Immunology* Online.

Abbreviations

B6	C57BL/6
BM	bone marrow
CFSE	5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester

DP	double positive
DN	double-negative
ECM	extracellular matrix
i.p.	intra-peritoneal
i.v.	intravenous
PDGFR	platelet derived growth factor receptor
PVS	perivascular spaces
SP	single positive
S1P	sphingosine-1 phosphate
$S1P_1$	sphingosine-1 phosphate receptor 1

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