Review

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The microstructure of secondary lymphoid organs that support immune cell trafficking

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Summary. Immune cell trafficking in the secondary lymphoid organs is crucial for an effective immune response. Recirculating T cells constantly patrol not only secondary lymphoid organs but also the whole peripheral organs. Thoracic duct lymphocytes represent an ideal cell source for analyzing T cell trafficking: high endothelial venules (HEVs) allow recirculating lymphocytes to transmigrate from the blood directly, and recirculating T cells form a cluster with dendritic cells (DCs) to survey antigen invasions even in a steady state. This cluster becomes an actual site for the antigen presentation when DCs have captured antigens. On activation, effector and memory T cells differentiate into several subsets that have different trafficking molecules and patterns. DCs also migrate actively in a manner depending upon their maturational stages. Danger signals induce the recruitment of several DC precursor subsets with different trafficking patterns and functions. In this review, we describe general and specialized structures of the secondary lymphoid organs for the trafficking of T cells and DCs by a multicolor immunoenzyme staining technique. The lymph nodes, spleen, and Peyer's patches of rats were selected as the major representatives. *In vivo* trafficking of subsets of T cells and DCs within these organs under steady or emergency states are shown and discussed, and unsolved questions and future prospects are also considered.

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Introduction

For an effective immune response, intricate cellular events must occur sequentially. *In vivo*, the integration of the complex cellular interactions takes place most efficiently within the organized architecture of secondary lymphoid organs that include the lymph nodes, spleen, and mucosa-associated lymphoid tissues. Because the immune response is associated with dynamic cellular movements, a study of immune cell trafficking in the secondary lymphoid organs should provide crucial information for understanding the host defense system and the pathogenesis of inflammatory diseases. However, structures and molecules that guide immune cell trafficking remain poorly defined. These molecules, hereafter called trafficking molecules, include adhesion molecules known as vascular addressins, homing receptors, and chemotactic cytokines—including chemokines and chemokine receptors (Miyasaka and Tanaka, 2004; Mora and von Andrian, 2006).

The aim of this review is to outline both the structures and molecules of rat secondary lymphoid organs that support the trafficking of T cells and dendritic cells (DCs) by a multicolor immunoenzyme staining technique (Matsuno, Ezaki, Kotani, 1989; Saiki *et al*., 2001a, b; Ueta *et al*., 2008) using a panel of antibodies to rat cell and tissue markers (Table 1). The lymph nodes, spleen, and Peyer's patches were selected as representatives of the secondary lymphoid organs. Vascular specializations in target organs are also described and several experimental models are reviewed and discussed.

General Structures of the secondary lymphoid organs

Strategic organization

Essentially, lymph nodes receive antigen either drained in a free form from tissues or carried by class II major histocompatibility complex antigen-positive ($MHCII^+$) dendritic cells (DCs). On the other hand, the spleen monitors the blood, and mucosa-associated lymphoid tissues are strategically integrated into mucosal surfaces of the body as a forward defensive system. Lymphatics and their associated lymph nodes form an intensive network for draining the viscera and the superficial body structures, and return the lymph to the blood by way of the central lymphatic trunks such as the thoracic duct (Fig.1). Macrophages are abundant in the secondary lymphoid organs so as to filter the lymph, blood, or foreign substances from the skin and mucosal surface. Communication between these tissues and the rest of the body is maintained by a pool of recirculating lymphocytes.

One striking feature of the organization of all the secondary lymphoid organs is that the T and B cells are largely segregated into different anatomical compartments, a process directed to a large extent by chemokines and adhesion molecules. This segregation may have developed for creating a microenvironment where only participating cells in various immune responses can meet each other appropriately.

Structural components and a minimal basic unit

T cells are mainly confined to a region referred to as the T cell area, or the thymus-dependent area. This region is characterized by the presence of DCs, which are

Table 1. Antibodies used in this study

1st Ab

Antigen	Clone	Antigen	Clone
CD45.2 ($RT7^b$)	HIS41[A]	Ig μ chain	MARM4 [A]
CD45R (B220)	HIS24[A]	TCR $\alpha\ \beta$	R73[C]
CD54 (ICAM-1)	1A29[A]	BrdU	BU1/75[D]
CD62E (E-selectin)	(polyclonal)[B]	Type IV collagen	(polyclonal)[E]
CD68?	ED1[A]	LYVE1	(polyclonal)[F]
CD163	ED2[A]	Reticular cell subset	ED14[G]
CD169 (sialoadhesin)	ED3[A]	MAdCAM-1	OST2[H]
$RT1.B^{a/c}$ (donor MHCII)	OX76[C]	Selectin ligands	2H5[I]
$RT1.B^l$ (recipient MHCII)	OX3[C]		

2nd Ab

Product	Conjugate	Source
Goat Ig to mouse Ig	Alkaline phosphatase	Sigma, A9316
	Alexa flour 594	Invitrogen, A11032
Rabbit Ig to mouse Ig	Horseradish peroxidase	Dako, P161
Sheep $F(ab')_2$ to rat Ig	Alkaline phosphatase	Sigma, A4812
Goat $F(ab')_2$ to rabbit Ig	Horseradish peroxidase	Cappel, 55693
Donkey Ig to rabbit Ig	7-amino-4-methylcoumarin-3-acetic acid	Jackson 711-155-152

[A] Serotec, [B] Biovision, [C] ECACC, [D] Oxford Biotech, [E] donated by Dr. Y. Sado (Shigei Med. Res. Inst., Okayama), also available from LSL, [F] abcam, [G] donated by Dr. T.K. van den Berg (Sanquin Res. Landsteiner Lab. Amsterdam, The Netherlands), [H] donated by Dr. M. Miyasaka (Osaka Univ., Osaka), [I] donated by Dr. R. Kannagi (Aichi Cancer Center, Aichi)

scattered in a reticular meshwork formed by fibroblastic reticular cells in the stroma. These DCs are called interdigitating cells or DCs because they possess many cytoplasmic projections that interdigitate each other with neighboring DCs: in this review we call them interdigitating DCs. In the T cell area of the lymph nodes and mucosa-associated lymphoid tissues but not of the spleen, high endothelial venules (HEVs) are almost exclusively distributed. HEVs are a specialized structure differentiated from the postcapillary venules in the T cell area. They allow recirculating lymphocytes to transmigrate directly through the vascular wall from the blood circulation.

The follicular aggregations of B cells are a prominent feature of the B cell area in the secondary lymphoid organs. In the unstimulated state, they are present as spherical collections of small lymphocytes, and are termed primary lymph follicles or nodules that consist of resting, small B cells. Although reticular fibers are rather scarce in the stroma, it contains follicular dendritic cells (FDCs), which are distinct from the ordinary DC lineage (Liu *et al*., 1996). After antigenic challenge, they form secondary lymph follicles; they consist of a germinal center and a surrounding follicle corona (also called the lymphocyte corona, mantle zone, or dark shell), the latter being the remainder of the primary follicle. The germinal center contains large—usually proliferating—B lymphoblasts, a minority of follicular T cells ($CD4^+$) and macrophages, and a tight network of FDCs.

In inflamed conditions in the stroma of non-lymphoid organs, a solitary lymph follicle with its surrounding T cell area and the HEV appears *de novo* (Yoneyama *et al*.,

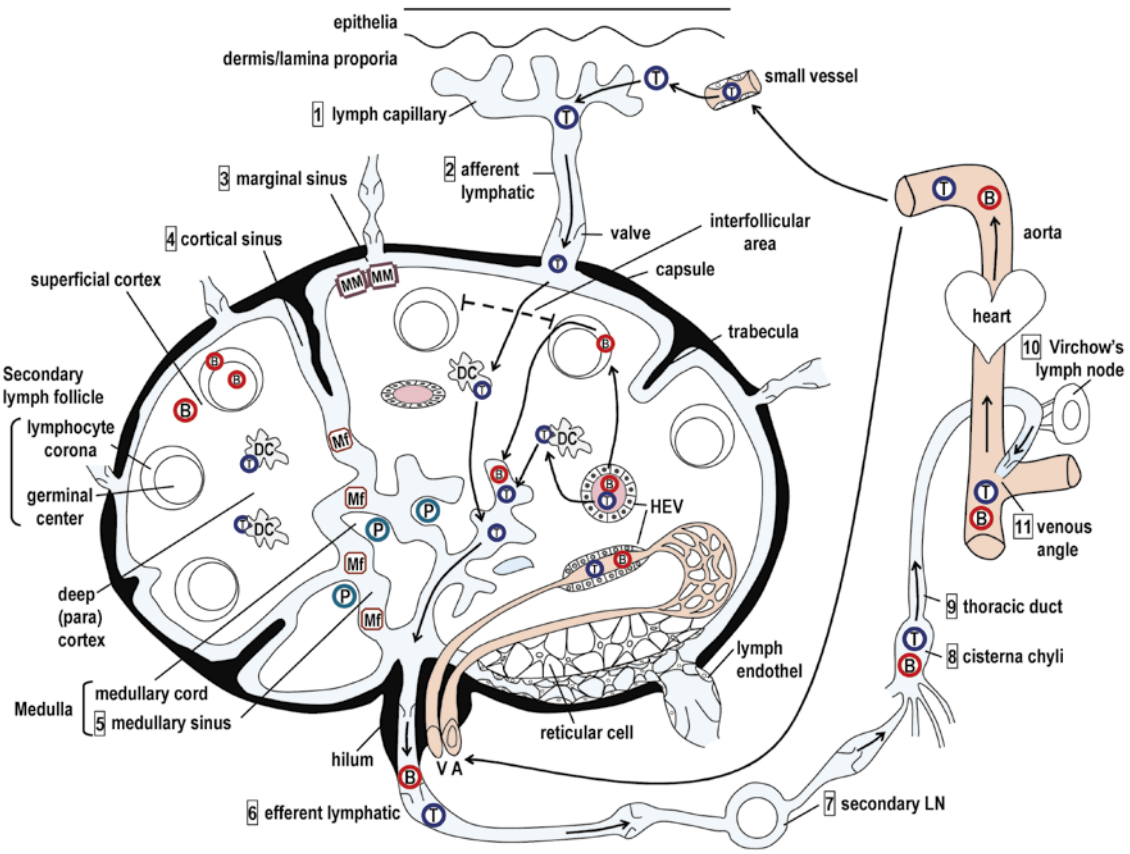


Fig. 1. A schematic drawing of the structure of the lymph node and trafficking routes for T cells (T) and B cells (B). Numbers in the square indicate the direction of lymph flow from the initial lymphatic capillary in peripheral organs to the draining lymph node and then to the blood circulation via the central thoracic duct. A: artery, DC: interdigitating dendritic cell, HEV: high endothelial venule, Mf: sinus macrophage, MM: marginal metallophilic macrophage, P: plasma cell, V: vein.

2001). Because all essential components for the immune response are present, we consider this structure a minimal basic unit of the secondary lymphoid organs.

Lymph nodes

Lymph nodes consist of the superficial (outer) cortex, deep (inner or para-) cortex, medullary cord, and marginal, cortical, and medullary lymphatic sinuses (Fig.1, 4). The superficial cortex constitutes B cell area with the lymph follicle and germinal center and the interfollicular area; the deep cortex is the T cell area with DCs and HEVs; the medullary cord is the plasma cell area with some B cells, while the lymphatic sinuses, especially medullary sinuses, are intraluminally populated by numerous ED1$^+$ (CD68-like antigen) sialoadhesin$^+$ macrophages (Fig. 4, 7f).

Spleen

The spleen has three components: the white pulp, the red pulp, and the interposing marginal zone (Fig. 2, 4). The central artery sends branches to the marginal zone and the red pulp cord where the blood empties directly into the reticular stroma. The outer margin of the white pulp is lined by sialoadhesin$^+$ marginal metallophilic macrophages. Just outside of these cells in the marginal zone, branches of the central artery form the marginal sinus and terminate with funnel-shaped open ends (Fig. 2) (Matsuno *et al*., 1986, 1989). The red pulp cord is populated by a large number of ED1$^+$CD163$^+$sialoadhesin$^-$ scavenger type macrophages. The spleen constitutes so-called open circulation, by which this organ acts as a very effective blood filter for removing effete blood cells and responding actively to

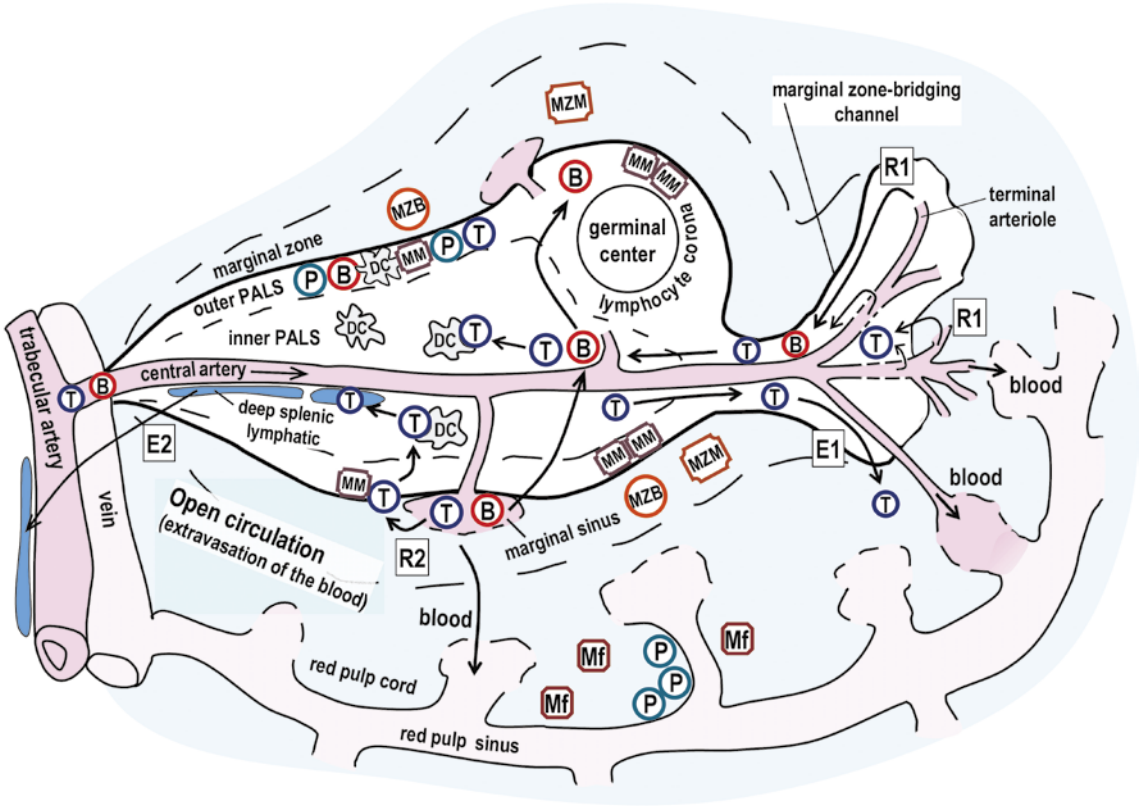


Fig. 2. A schematic drawing of the structure of the spleen and trafficking routes of T cells (T) and B cells (B). R1 and R2 are two possible entrance gates for migrating immune cells. R1: marginal zone bridging channel, R2: marginal sinus and marginal metallophilic macrophages (MM) in the outer rim of the PALS. E1 and E2 indicate two possible exit gates, the bridging channel and the deep splenic lymphatics, respectively. DC: dendritic cell, MZB: marginal zone B cell, MZM: marginal zone macrophage, P: plasma cell.

blood-borne antigens before returning to the circulation *via* the splenic sinuses. The white pulp consists of the periarterial lymphoid sheath (PALS, T cell area with DCs but no HEV) and the lymph follicle with/without the germinal centers (B cell area). In addition, the marginal zone is populated by a unique B cell subset, marginal zone B cells (Mebius and Kraal, 2005) and sialoadhesin$^+$ macrophages. The outer rim of the PALS is called the outer PALS. This area is populated by not only T cells and DCs but also B cells, plasmablasts, and CD163$^+$ macrophages and considered to be a site for the antibody forming cell response (Matsuno *et al*., 1989).

It should be noted that a strong species difference exists in the structure of the splenic marginal zone (Steiniger *et al*., 2006). Distinct from rats and mice, no marginal sinus or marginal metallophilic macrophages exist in humans. Furthermore, a strong expression of sialoadhesin only occurs in human macrophages forming perifollicular capillary sheaths outside the marginal zone, and marginal zone B cells appear to reside only in a superficial follicular compartment.

Peyer's patches

Peyer's patches are clusters of the lymph follicle (B cell area with a frequent association of germinal centers) and the interfollicular area (T cell area) in the wall of small intestine (Fig. 3, 4). Gut antigens enter this organ via the specialized follicle-associated epithelium (Nicoletti, 2000; Hase *et al*., 2009). In fact, numerous DCs are present within this epithelial layer and the dome area beneath the epithelium (Fig. 4I)(Wilders *et al*., 1983). The dome area is a specialized region where the capillary network is developed—though B and T cells are rather scarce in this area. In general, the tonsils, appendix, and the bronchus-associated lymphoid tissues as well as the Peyer's patches are the aggregation of the minimal basic units stated above, having the associated epithelia.

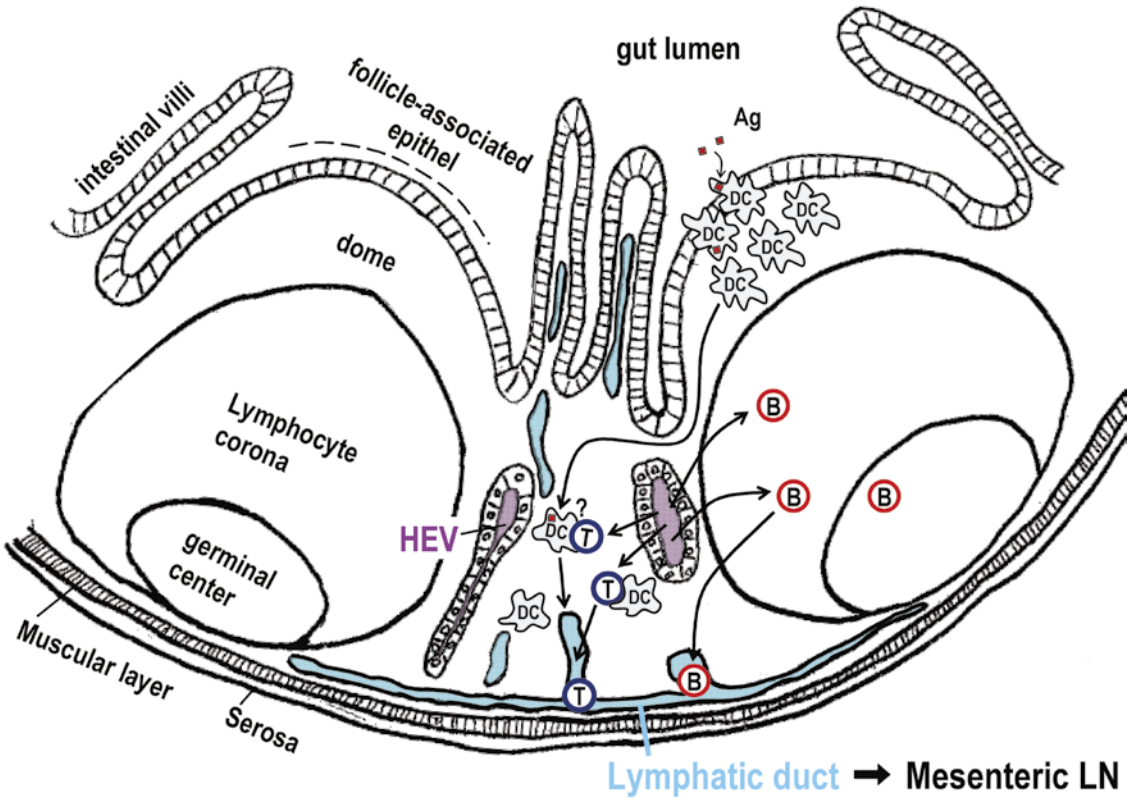


Fig. 3. A schematic drawing of the structure of Peyer's patches and trafficking route of T cells (T), B cells (B), and dendritic cells (DC). The exact trafficking of DCs in the dome area is still unsettled. HEV: high endothelial venule.

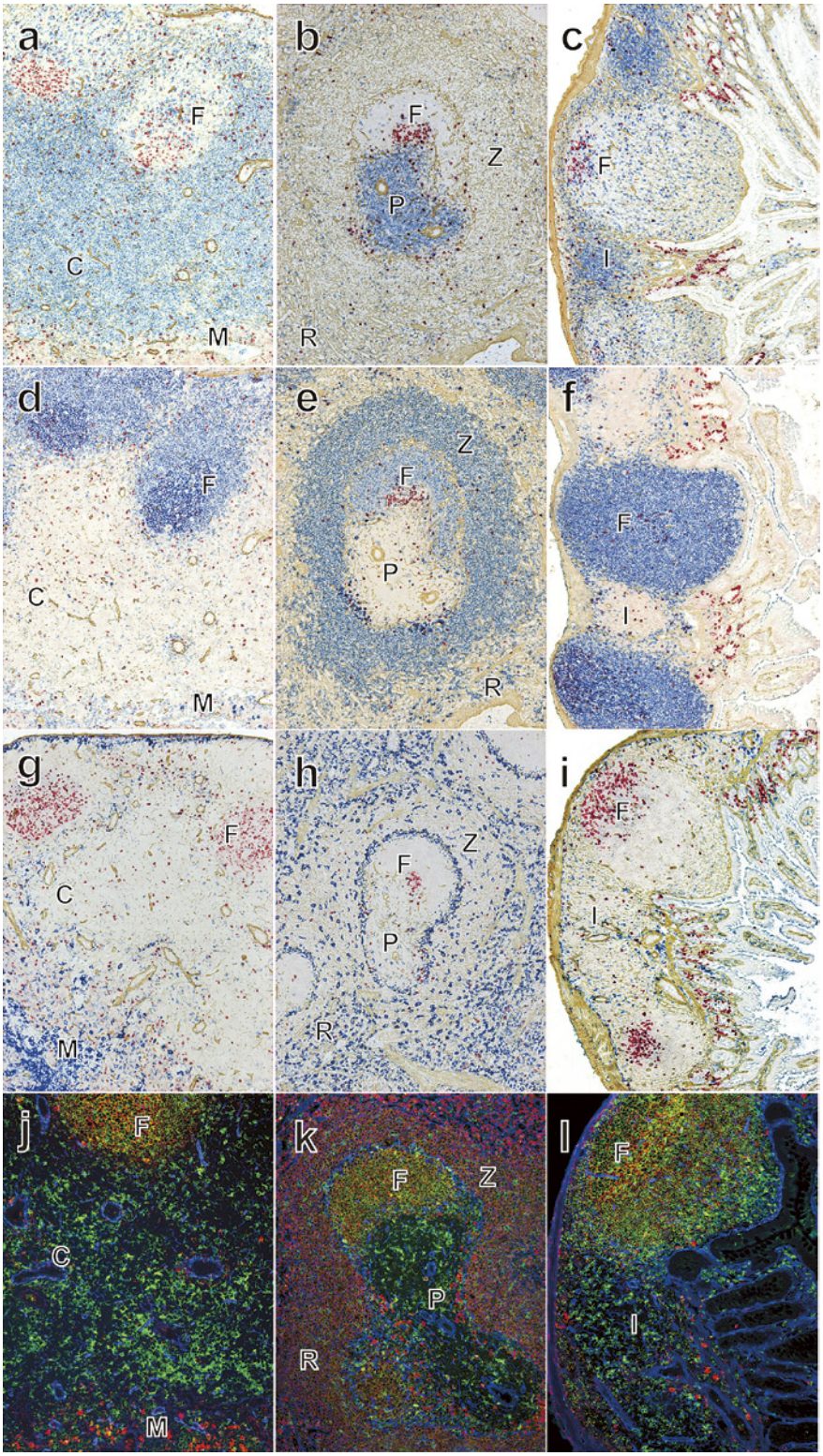


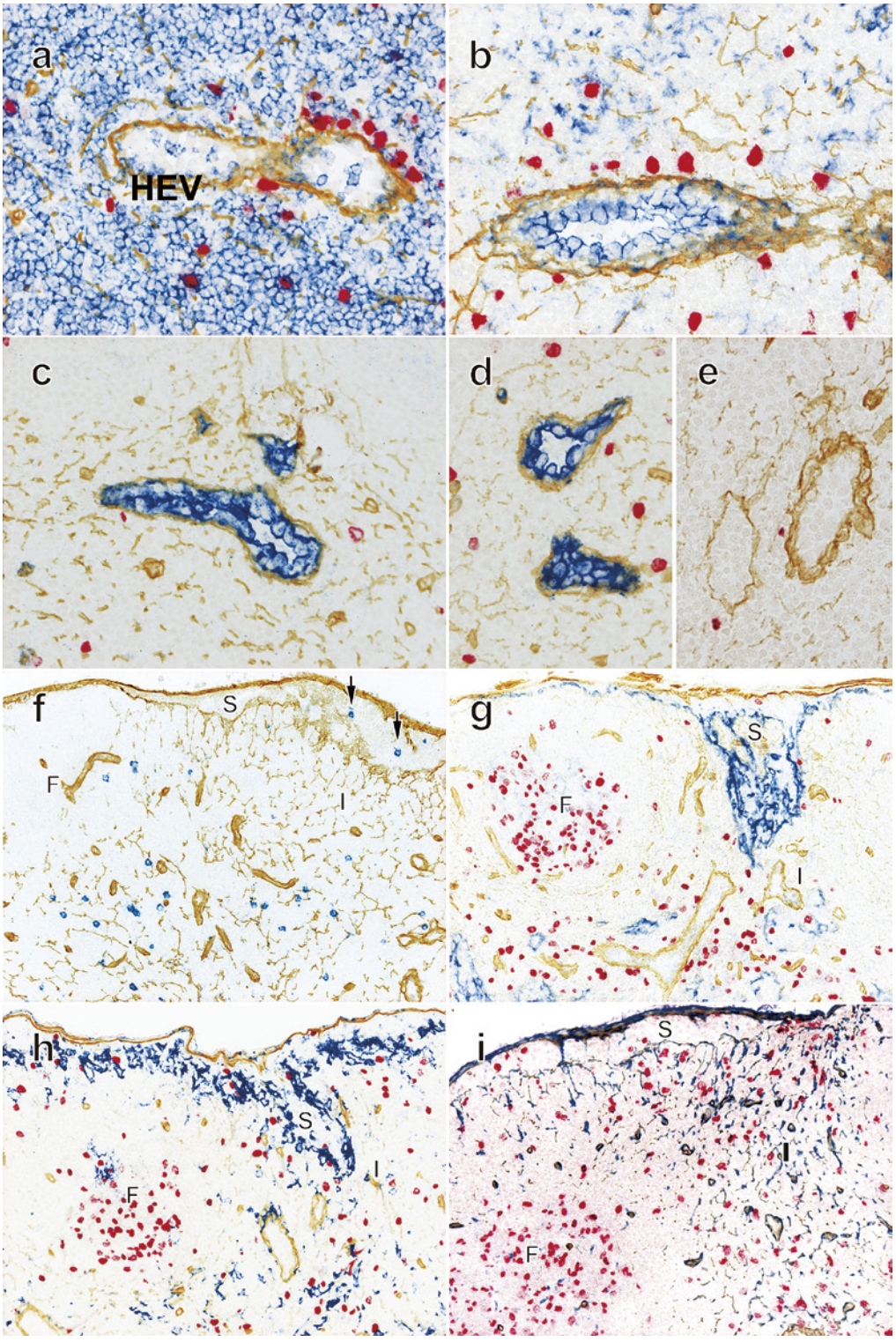
Fig. 4. Structural compartments and the segregation of immune cells (blue color) in the lymph node (**a, d, g, j**), spleen (**b, e, h, k**), and Peyer's patches (**c, f, i, l**). The nuclei of proliferating cells ($BrdU^+$) are colored red and the basement membrane or tissue frameworks (type IV $collagen^+$) are colored brown. T cells (TCR^+, **a, b, c**) are confined to the paracortex (C) in the lymph node, the periarterial lymphoid sheath (PALS, P) in the spleen, and the interfollicular area (I) in the Peyer's patches, respectively. B cells (IgM^+ or $CD45R^+$, **d, e, f**) are localized in the lymph follicle (F) in all 3 organs. In addition, marginal zone B cells are confined to the splenic marginal zone (Z). Macrophages ($ED1^+ED2^+ED3^+$ **g, h, i**) are localized in and around the lymph sinus in the lymph node. In the spleen, marginal metallophilic macrophages surrounding the outer rim of the white pulp, marginal zone macrophages, and red pulp macrophages are three major populations. The Peyer's patches contain a few macrophages in the submucosa above the muscular layer and in the interfollicular area. **j, k, l:** Immunofluorescence staining for MHCII (green), IgM and macrophage markers (red), and type IV collagen (blue). Dendritic cells (DCs) are depicted as MHCII-single positive cells (green) in the paracortex (C), the PALS (P), and the interfollicular area (I). B cells (IgM^+MHCII^+) stain yellow, IgM^+ plasma cells stain red ($MHCII^-$), and macrophages are either yellow ($MHCII^+$) or red ($MHCII^-$), respectively. M: medulla of the lymph node, R: splenic red pulp.

Table 2. Phenotype of rat T cell subsets

Subset	Residence	CD45RC	CD62L(L Selectin)	α4 integrin (CD49d) / αE integrin (CD103)	Chemokine receptor	Other molecules	References
Recent thymic emigrant (RTE)	Secondary lymphoid organs	–	+	–	CCR7 + ?	CD90 (Thy1) +	Yang and Bell, 1992
Naive T cell	Recirculating Thoracic duct lymph	+	+	α4 (~5%)	CCR7 + CXCR4 +	ICAM-1(~6%)	Westermann *et al*, 1997, 2005
Memory T cell	Recirculating Thoracic duct lymph	↓	↓	α4 ↑ (~40%)		ICAM-1 ↑ (~60%)	Westermann *et al*, 1997; 2005
Effector memory T cell (T_{EM})	Target organs	↓	↓	α4 ↓ (skin inflammation model)	CCR7 –	Kv1.3 channels ↑ ICAM-1 ↑	Azam *et al*., 2007 Matheu *et al*., 2008
Central memory T cell (T_{CM})	Recirculating	↓	+	?	CCR7 +	KCa3.1 channels ↑ ICAM-1 ↑	Azam *et al*., 2007 Matheu *et al*., 2008
Intraepithelial T cell	Gut epithelia Epidermis	?	?	CD103 +	?	Mostly TCR α β + CD8 + (~75%)	Vaage *et al*., 1990 Zhou *et al*., 2008
Gut-homing GvHD effector	Gut epithelia	↓ ?	↓ ?	α4 ↓ CD103 ↑	CCR9 + ?	CD8 + (~80%)	Zhou *et al*., 2008
Skin-homing GvHD effector	Epidermis	↓ ?	↓ ?	CD103 + CD8 + (~50%) CD103- CD4 + (~50%)			Zhou *et al*., 2008

Table 3. DC subsets in rats and mice

Cell (abbreviations)	Origin	Phenotype	Chemokine receptor	Function	References
Langerhans cell (LC) (mouse)	Hair region?	Langerin + CD103 + Macrophage galactose C-type lectin 2 –		Self tolerance? Migrate to regional lymph nodes	Kumamoto *et al*., 2009
Interstitial DC Dermal DC Lamina propria DC	Bone marrow	CD11c + CD103 + / – DEC205 + Macrophage galactose C-type lectin 2 + (mouse dermal DC)		Immunogenic Migrate to subfollicular area of the regional lymph nodes	Saiki *et al*., 2001 Kumamoto *et al*., 2009
Interdigitating DC in the secondary lymphoid organs	Peripheral organs Bone marrow	ED1 + / – CD11b + CD11c + ICAM1 + CD103 + / – DEC205 +		Immunogenic?	Matsuno and Ezaki, 2000
Conventional DCprecursor	Bone marrow	ED1 + CD11b/c + CD103 + / – (rat) CD11c + Lineage – (mouse)	CCR1/5 + (mouse)	Immunogenic Migrate to inflamed peripheral organs	Matsuno *et al*., 1996 Uwatoku *et al*., 2001 Yoneyama *et al*., 2001
Plasmacytoid DC precursor (mouse)	Bone marrow	CD11c + E-selectin ligand + B220 + (mouse)	CCR1/5 + CXCR3 + (mouse)	TransHEV migration to inflamed lymph nodes; Help conventional DCs (LFA2 ↑ CD40L ↑)	Yoneyama *et al*., 2004, 2005
Transmigrating DC (rat)	Liver Bone marrow	CD11c + α4 integrin + CD103 +		Transmigration to spleen, lymph nodes and Peyer's patches after liver graft/ bone marrow cell transfer; Allostimulation Precursor of interdigitating DCs?	Ueta *et al*., 2008



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Subsets of immune cells

Subsets of T cells

Phenotype and trafficking molecules of rat T cell subsets are shown in Table 2. According to the differentiation pathway, T cell subsets are divided into recent thymic emigrants (Yang and Bell, 1992), naive T cells, effector T cells (T_{EFF}) of either gut-homing T_{EFF} or skin-homing T_{EFF}, central memory T cells (T_{CM}), effector memory T cells (T_{EM}), and so on (Mora and von Andrian, 2006). Intestinal intraepithelial lymphocytes belong to a unique subset of T cells. While ~50% of mouse intraepithelial lymphocytes express T cell receptor $\gamma\delta$ and are derived from the cryptopatches (Ishikawa *et al*., 2007), the majority of the rat counterpart is shown to express the conventional T cell receptor $\alpha\beta$ and to be thymus-dependent (Vaage *et al*., 1990).

Among memory T cells, the protective immune memory is mediated by T_{EM} that migrate to peripheral tissues and display the immediate effector function. On the other hand, the reactive memory is mediated by T_{CM} that home to T cell areas of secondary lymphoid organs and, have little or no effector function but readily proliferate and differentiate to effector cells in response to antigenic stimulation (Sallusto *et al*., 2004). In rat allergic contact dermatitis (Azam *et al*., 2007) and delayed type hypersensitivity (Matheu *et al*., 2008) , T_{EM} selectively upregulate voltage-gated Kv1.3 potassium channels, whereas T_{CM} upregulate KCa3.1 channels. These channels can be specific markers for both subsets.

Dendritic cell (DC) subsets

The function and kinetics of DCs are dependent upon their five stages of maturation (Matsuno and Ezaki, 2000): 1) DC progenitors, the cells that replicate mostly in the bone marrow; 2) DC precursors, the cells that enter the blood and seed peripheral tissues; 3) sentinel DCs (Langerhans cells or interstitial DCs), the cells that can endocytose antigens; 4) antigen-transporting DCs (lymph DCs or veiled cells), the cells that leave peripheral tissues and migrate to regional lymph nodes *via* draining lymphatics, or possibly to the spleen *via* blood; and 5) mature DCs (interdigitating DCs) for antigen presentation, the cells that acquire a unique capacity for the potent activation of T and B cells in the lymphoid tissues.

In addition to these five stages, we have reported several distinct subsets of DCs that are recruited from the bone marrow or other places in response to danger signals. These are conventional DC precursors (Matsuno *et al*., 1996; Uwatoku *et al*., 2001a; Yoneyama *et al*., 2001), plasmacytoid DC precursors (Yoneyama *et al*., 2004; 2005), and semimature DCs transmigrating to the secondary lymphoid organs (Ueta *et al*., 2008) in rats and/or mice. Phenotype and trafficking molecules of rat or mouse DC subsets are shown in Table 3.

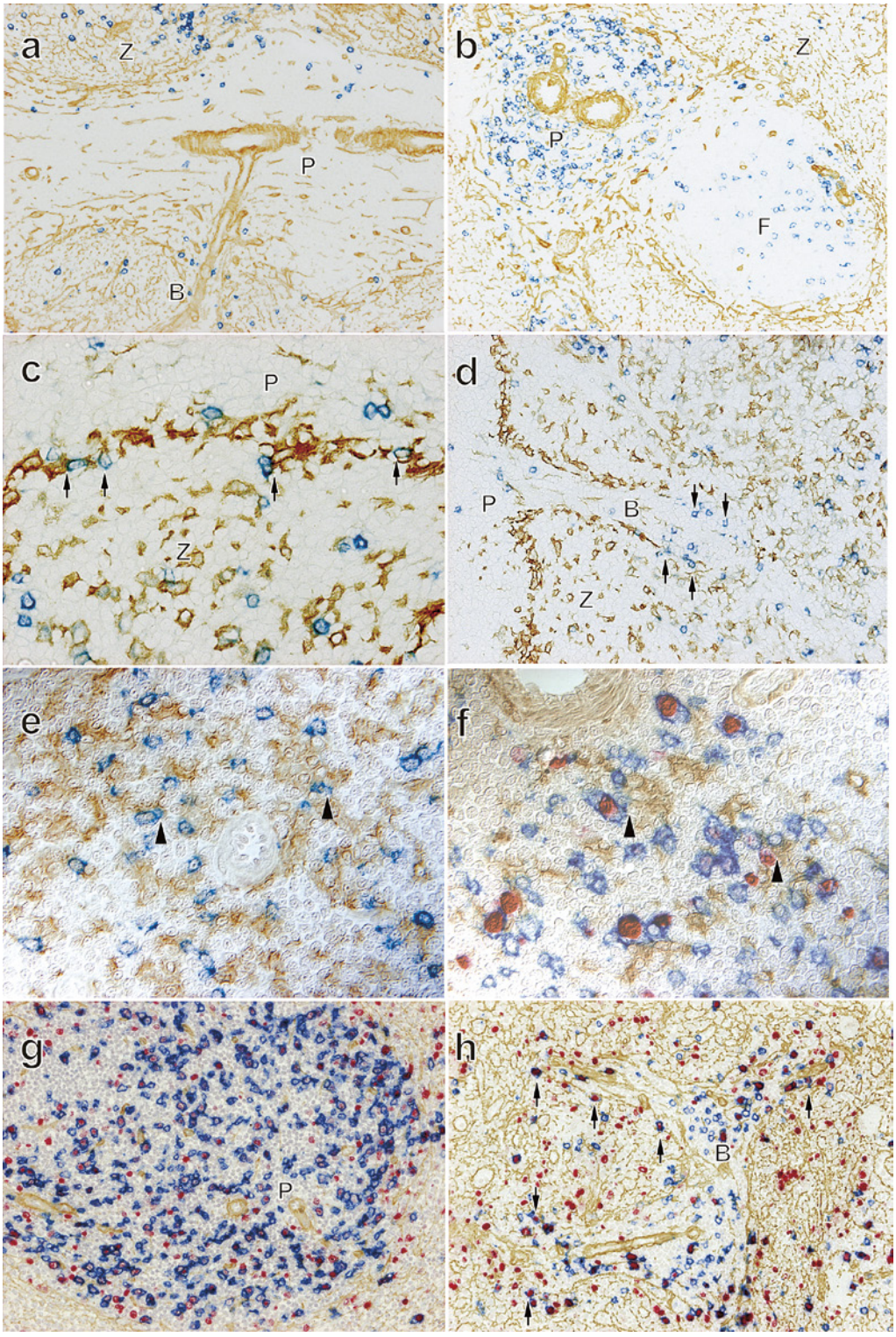
Specialized structures for immune cell migration

General view

The destination of migrating immune cells is determined by a series of trafficking molecules (i.e., adhesion molecules and chemokine receptors) that include members of the integrin superfamily, chemokine receptors and selectins. In the T cell area, stromal cells secrete chemokines CCL19 and CCL21 and bear ICAM-1 that attract and bind the chemokine receptor $CCR7^+$and integrin $LFA1^+$ T cells. In the B cell area, on the other hand, FDCs and stromal cells produce chemokine CXCL13 and possess ICAM-1 and VCAM-1 by which chemokine receptor $CXCR5^+$ and integrin $LFA1^+$ B cells and follicular T cells are recruited (Mora and von Andrian, 2006).

Each secondary lymphoid organ possesses both entrance and exit gates for migrating cells. In the lymph nodes, there are two entrance gates (Fig. 1, 5): one is the HEV from the blood, and the other is the marginal sinus from the afferent lymph. In contrast, Peyer's patches lack afferent lymphatics (Fig. 3) like the other mucosa-associated lymphoid tissues. Thus, they have only one

Fig. 5. Specialized structures of the lymph nodes for immune cell migration [1]. **a–d:** Entrance route via the high endothelial venules (HEVs). Transmigrating T cells are seen within the wall of HEVs (**a**). Adhesion molecules such as ICAM-1 (**b**) and selectin ligands (**c**) are ubiquitously expressed on HEVs in the lymph nodes and Peyer's patches, but MAdCAM-1 is selectively expressed on HEVs in the mesenteric lymph nodes (**d**) and Peyer's patches but not those in the skin lymph nodes (**e**). **f–i:** Entrance route via the marginal sinus (S). Lymphocytes migrating *via* the afferent lymph are seen in the marginal sinus (arrows, **f**). Lymph sinus endothelia ($LYVE\text{-}1^+$, **g**), marginal metallophilic macrophages ($sialoadhesin^+$, **h**), and reticular cell subset ($ED14^+$, **i**) are depicted as blue cells.



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entrance gate, the HEV. The exit gate for both the lymph nodes and Peyer's patches is from the lymphatic sinuses to the efferent lymphatics.

In contrast, the spleen lacks both the HEVs and the afferent lymphatics. Furthermore, both entrance and exit gates in the spleen for the migration of blood immune cells through the white pulp are still unsettled. The candidates are the boundary between the marginal zone and the outer PALS, marginal zone bridging channel, and the deep splenic lymphatics (Fig. 2, 6), which will be discussed below in this section.

High endothelial venules (HEVs) in the secondary lymphoid organs

HEVs allow recirculating lymphocytes to enter the lymph nodes from the blood directly (Fig. 5a, 7a, b). HEVs possess trafficking molecules on their luminal surface, such as ICAM-1, selectin ligands (Tamatani *et al*., 1995), and chemokines (Miyasaka and Tanaka, 2004) to meet corresponding receptors on the surface of migrating cells (Fig. 5b, c). In addition, HEVs of the mesenteric lymph nodes and Peyer's patches, but not those of the peripheral lymph nodes, specifically possess MAdCAM-1 (Fig. 5d, e), whose ligand, $\alpha 4\,\beta 7$ integrin, is known as the lymphocyte homing receptor destined for the gut-associated lymphoid tissues (Iizuka *et al*., 2000).

Afferent lymphatic and marginal sinus in the lymph nodes

In the lymph nodes, the marginal sinus covering the interfollicular area is the actual gate for lymphocytes and DCs in the afferent lymph to the superficial cortex (Fig. 5f). This area contains a conduit for migrating cells and small molecules, such as chemokines: they are transported into the superficial cortex and then to the subfollicular area (Sainte-Marie and Peng, 1980; Katakai *et al*., 2004). Because large molecules do not enter this conduit, the presence of a certain gate is suggested (Gretz *et al*., 2000). The endothelia (littoral cells) of lymphatic sinuses as well as those of lymphatic vessels possess specific surface molecules, such as LYVE-1 (Fig. 5g) and podoplanin. The endothelia are internally lined by sialoadhesin$^+$ marginal metallophilic macrophages (Fig. 5h) and reticular cells scatter in the interfollicular area (Fig. 5i). It is of note that the processes of the macrophages project into the sinus lumen as if they are scanning the antigens or cells in the sinus derived from the afferent lymph (Fig. 5h)(Matsuno and Ushiki, 1997).

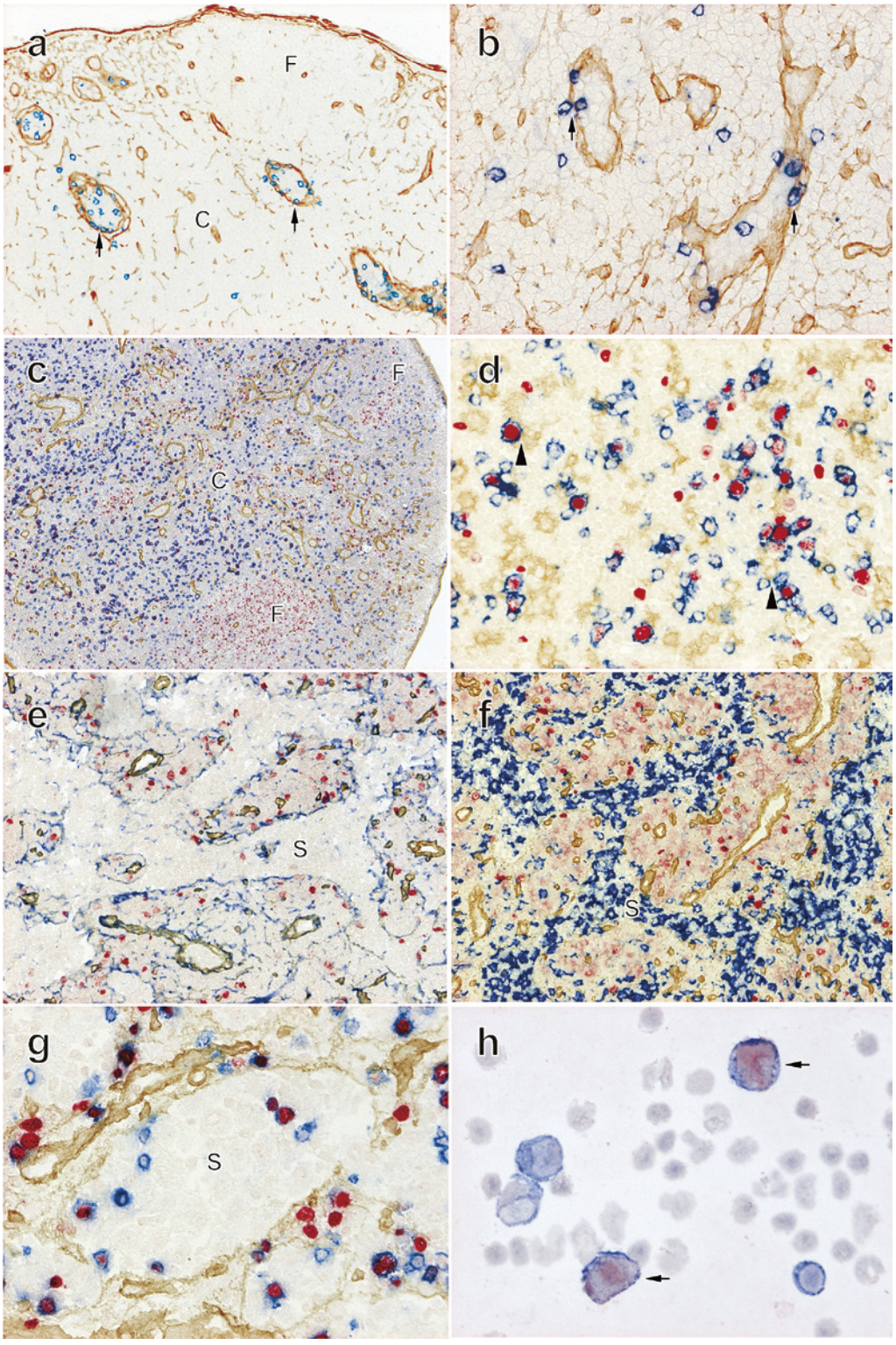
Lymphatic sinus and Efferent lymphatic in the lymph nodes and Peyer's patches

Cells in transit in the secondary lymphoid organs stay there for 12 h or longer (Smith and Ford, 1983; Xu *et al*., 2008) and then egress from there via the lymphatic sinus (Fig. 7e. f) connecting with the efferent lymphatics in case of the lymph nodes and Peyer's patches. Reticular cells outline the sinus (Fig. 7e) and signaling between sphingosine-1-phosphate (S1P), and its receptor-1 ($S1P_1$) is considered to be crucial for cells to enter the sinus from the parenchyma of lymph nodes and Peyer's patches (Cyster *et al*., 2005). In fact, naive T and B lymphocytes express high amounts of S1P1, and S1P is abundant in the lymph (Cyster *et al*., 2005).

Marginal zone-bridging channel in the spleen

In rats and mice, protrusions of the white pulp area across the marginal zone into the red pulp are called the marginal zone bridging channel (Mitchell, 1973). As will be described later, transferred congeneic lymphocytes were first observed in the marginal zone-bridging channel (Fig.6a) and later found in the more central part of the PALS in rats. Together with other reports (van Ewijk and

Fig. 6. Specialized structures of the spleen for immune cell migration. **a–e:** 15 min (**a**), 30 min (**b, d**), 3 h (**b**), and 24 h (**e**) after intravenous transfer of congeneic lymphocytes ($RT7^b$,blue). Brown stainings are type IV collagen (**a, b, g, h**), sialoadhesin (**c, d**), and recipient MHCII (**e, f**), respectively. These cells first appear in and around (**d,** arrows) the bridging channel (B) and in the outer margin of the white pulp where the marginal metallophilic macrophages (brown) are positioned. Note the migrating cells in contact with these macrophages (**c**, arrows). In the PALS, almost all the migrated congeneic donor cells constantly cluster with recipient MHCII$^+$ DCs (brown, **e**) even under such an unstimulated condition. **f, g, h:** One-way GvH reaction 24 h (**f**), 2 days (**g**), and 3 days (**h**) after parental lymphocyte transfer to F_1 hybrid rats. Note that most donor cells (blue) are clustering (arrowheads) with recipient MHCII$^+$ DCs in the PALS (brown, **f**) and some are actively proliferating ($BrdU^+$ red nucleus) there (**f, g**). Activated donor cells appear in the bridging channel (B) and in the neighboring red pulp (arrows) by day 3 after transfer (**h**).



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Nieuwenhuis, 1985; Pellas and Weiss, 1990a,b; Balazs *et al*., 2002), we consider this marginal zone-bridging channel the first possible gate of entry. The problem is that no specific accumulation of stromal cells or adhesion molecules has been detected so far in rats. This area is also a candidate for an exit gate for recirculating lymphocytes (Mitchell, 1973) or activated T cells to the red pulp (Fig. 6h) .

Splenic marginal sinus and marginal metallophilic macrophages

The splenic marginal sinus is the second possible gate of entry for migrating cells in the splenic white pulp (Fig. 6c). No specific molecule has been detected so far in rats, although MAdCAM-1 is expressed in the marginal sinus endothelia in mice (Mebius and Kraal, 2005). Interestingly, sialoadhesin$^+$ marginal metallophilic macrophages project their cytoprocesses into the sinus lumen as in the lymph nodes (Matsuno and Ushiki, 1997). This analogy leads us to speculate that marginal metallophilic macrophages may guide the entrance of migrating cells in the afferent lymph into the interfollicular area in the lymph nodes or that in the marginal zone into the PALS in the spleen through adhesion with their sialoadhesin$^+$ cytoplasmic processes.

Deep splenic lymphatics

In mice, distinctive lymphatics are present in the PALS running along the central artery to the splenic hilum and then to the splenic lymph nodes (Fig. 2)(Shimizu *et al*., 2009). Transferred mouse lymphocytes are suggested to enter this vessel and exit the white pulp (Pellas and Weiss, 1990a, b; Shimizu *et al*., 2009).

Trafficking of immune cells within the secondary lymphoid organs

T cell trafficking under a steady state

Naive T cells and T_{CM} continuously recirculate between the blood and lymph in a steady state for immunosurveillance (Sparshott and Bell, 1998; Westermann *et al*., 1997, 2005). In fact, these cells can be selectively collected in central lymph by cannulating the thoracic duct in rats (Smith and Ford, 1983; Matsuno *et al*., 1995). Rat thoracic duct lymphocytes (TDLs) are highly viable and up to 2×10^8 cells are yielded by overnight collection. Normal TDLs of ACI rat strain comprise approximately 65% of CD4$^+$, 15% of CD8$^+$, and 20% of B cells. Among T cells, approximately >90% are naive T cells. Accordingly, TDLs represent an ideal cell source for analyzing the *in vivo* trafficking of migrating T cells by adoptive transfer. Although technically difficult, mouse TDLs can be collected as well (Ionac, 2003).

When normal TDLs are intravenously transferred to recipient rats in a congeneic combination without an antigen challenge, migrated donor cells attach to the HEV of the lymph nodes and Peyer's patches, and instantly transmigrate through its wall to enter the paracortex (Fig. 7a, b) or the interfollicular area. In the spleen, they appear in and around the marginal zone-bridging channel or in the boundary between the marginal zone and the outer PALS in 10–15 min and quickly enter the PALS (Fig. 7a, c, d). Donor T cells accumulate in the T cell area of the secondary lymphoid organs by 3 h after transfer (Fig. 7b).

When T cells enter the lymph nodes via the afferent lymph, they readily migrate from the marginal sinus into the superficial cortex at the interfollicular region (Fig. 5f) (Sainte-Marie and Peng, 1980; Xu *et al*., 2008).

To note, most of the migrating T cells in the T cell area have contact with the resident interdigitating DCs and form a DC-T cell cluster there to survey the antigen

Fig. 7. Specialized structures of the lymph nodes for immune cell migration [2]. **a, b:** 15 and 30 min after intravenous transfer of congeneic lymphocytes (blue). Brown stainings are type IV collagen (**a, b, c, e, f**) and recipient MHCII (**d**), respectively. While most donor cells are confined within the high endothelial venules in **a** (arrows), many cells are transmigrated into the parenchyma in **b** (arrows). **c, d:** One-way GvH reaction. 2 days after the parental lymphocyte transfer to F_1 hybrid rats. Many donor cells are proliferating (blue cells with BrdU$^+$ red nucleus, **c**). Note most donor cells (blue) clustering (arrowheads) with recipient MHCII$^+$ DCs (brown, **d**). **e–f:** The exit route for migrating cells from lymph nodes. Lymph sinuses are outlined by reticular cells (ED14$^+$, **e**) and populated by many sialoadhesin$^+$ macrophages (ED3$^+$, **f**). Three days after induction of a lethal GvH disease, exiting donor cells (blue) are seen in the sinus lumen (**g**) and appear in the blood (**h**). Arrows indicate proliferating donor lymphoblasts (BrdU$^+$ red nucleus).

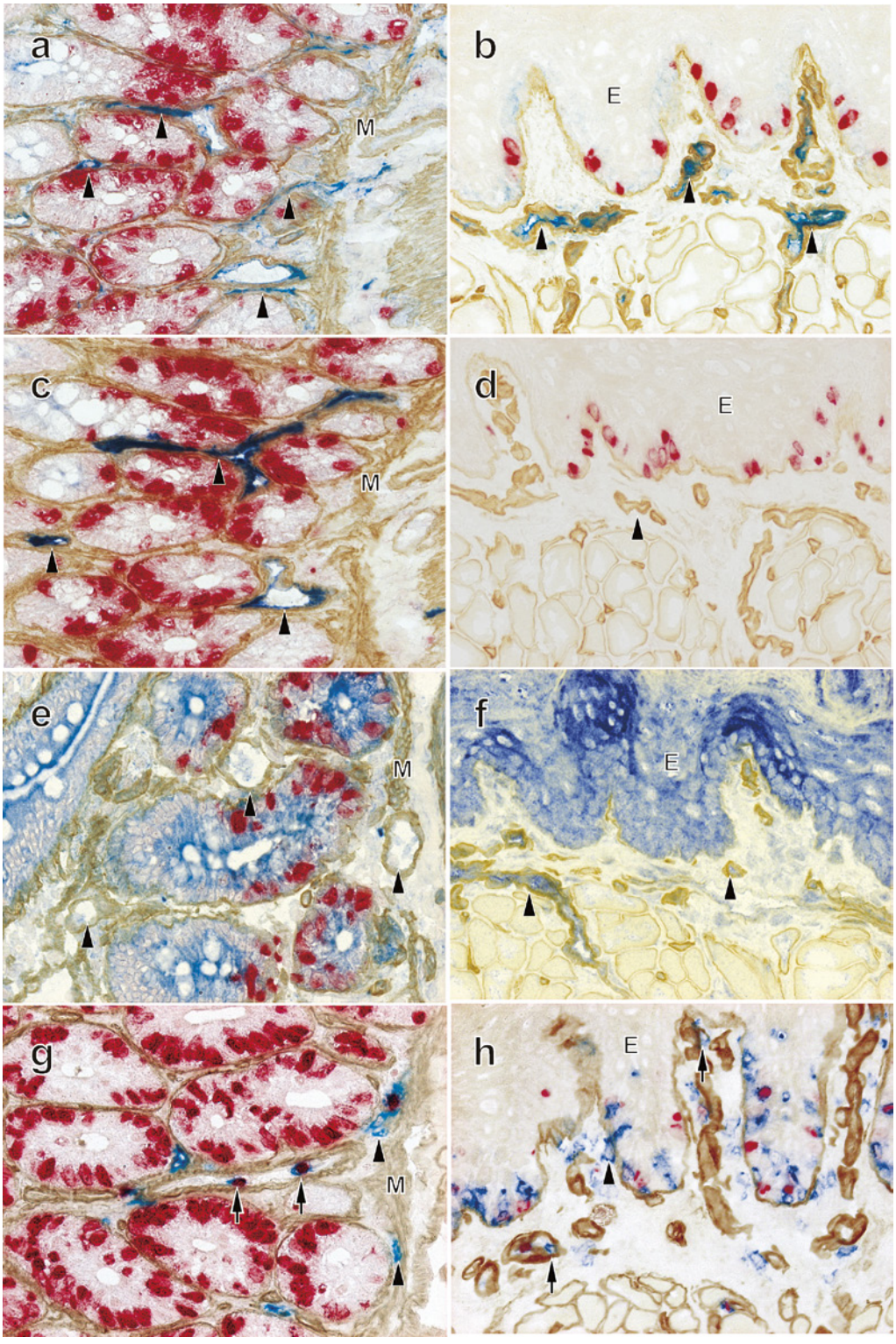

Fig. 8. Legend on the opposite page.

even in a steady state (Fig. 6e). Because their antigen is not mounted in the steady state, no activation occurs and bound donor cells soon leave the cluster.

Recirculating lymphocytes also constantly migrate to peripheral non-lymphoid organs for patrolling the whole body (Smith, McIntosh, Morris, 1970; Smith and Ford, 1983). In fact, TDLs, when intravenously transferred to the congeneic recipient, readily migrate to the liver (Xu *et al*., 2008). They accumulate in the portal area and quickly translocate into the draining lymphatics (Fig. 5f) in a fashion similar to DC transfer study (Kudo *et al*., 1997; Saiki *et al*., 2001b). Surprisingly, the minimal transit time in the liver is 3–4 h. This rapid transit might enable an efficient surveillance of the liver portal area by the recirculating lymphocytes.

T cell trafficking in the secondary lymphoid organs under emergency

When antigen reaches a lymph node, recirculating lymphocytes are massively recruited via the HEV and a dramatic fall in the output of cells in the efferent

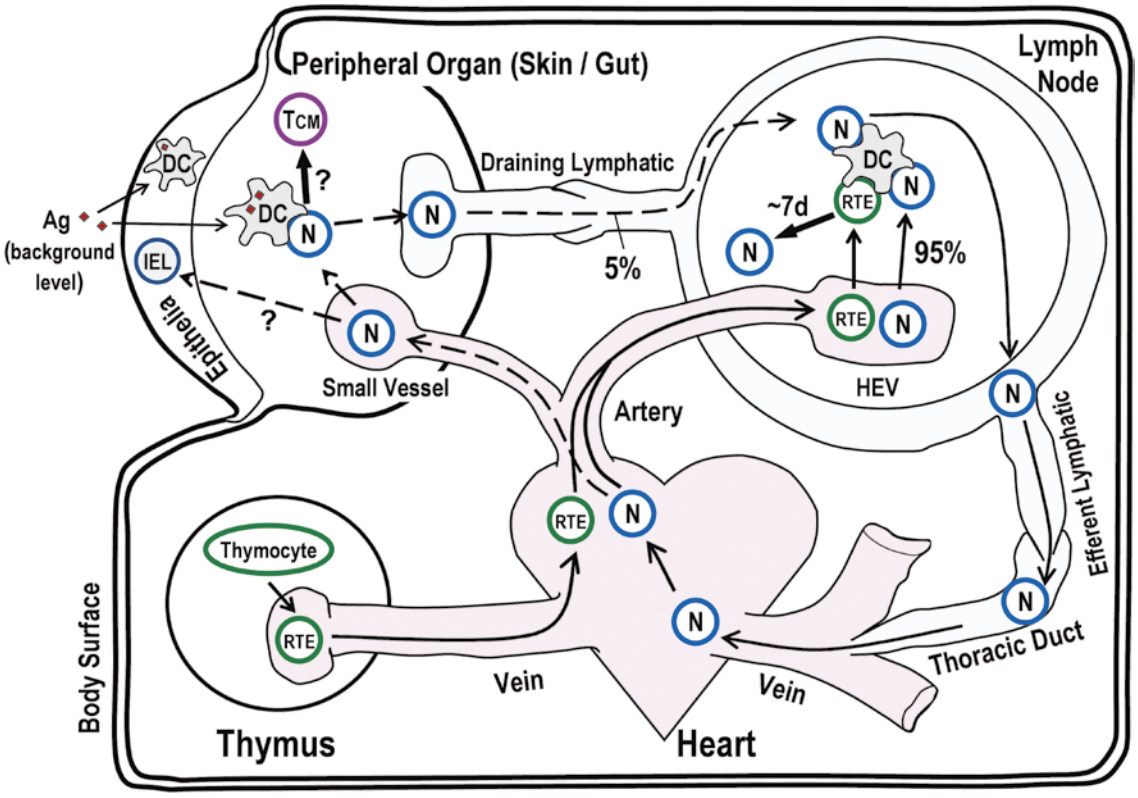

Fig. 9. A diagram depicting the trafficking of naive T cells (N) and recent thymic emigrants (RTE) under a normal steady state. Dotted lines indicate the minor route for naive T cells. The origin of intraepithelial lymphocyte (IEL) and the differentiation site for central memory T cells are unsettled. Blood circulation is colored pink and the lymph pathway light blue. TCM: central memory T cell.

Fig. 8. Entrance route for migrating effector cells in the lamina propria of the small intestine (**a, c, e, g**) and the skin dermis (**b, d, f, h**). Adhesion molecules such as ICAM-1 (**a, b**) are expressed on the small vessels of both organs, but MAdCAM-1 (**c, d**) and E-selectin (**e, f**) are selectively expressed on those in the small intestine (**c**) and skin (**f**), respectively (arrowheads). Transmigrating T cells (blue) are seen in and around the small vessels (arrows) 3 days and 4 days after induction of systemic GvH disease (**g, h**), respectively. Arrowheads show transmigrated cells (**g**) and a cell further entering the epidermis (**h**).

lymphatics is observed. The latter phenomenon is known as '1ymphocyte trapping' (Kotani *et al*., 1980) and is thought to result from the antigen-induced release of chemokines from DCs and T cells. As a consequence, antigen-reactive cells disappear from the recirculating pool of lymphocytes within 24 h of antigen challenge (Wilson *et al*., 1976).

When parental TDLs are intravenously injected into F_1 hybrid recipient rats, one-way graft-versus-host reaction occurs. In the early stage, donor T cells behave in the same manner as congeneic T cells. They quickly transmigrate to the HEV or enter the splenic PALS, accumulate in the T cell areas, and have contact with the recipient interdigitating DCs (Fig. 6f, 7d). The difference is that some T cells in the cluster stay there and start to synthesize DNA (i.e., become $BrdU^+$) and proliferate (Fig. 6f, g, 7c, d). Therefore, this cluster is an actual site for the alloantigen presentation where T cells become activated and start to expand and differentiate to TEFF (Saiki *et al*., 2001b; Ueta *et al*., 2008). After massive expansion in the T cell area of the whole secondary lymphoid organs, TEFF egress from there either via the medullary sinus (Fig. 7g) and efferent lymphatics of the lymph nodes (Fig. 1), *via* efferent lymphatics of the Peyer's patches (Fig. 3), or *via* the bridging channel (Fig. 6h) and the red pulp sinus of the spleen (Fig. 2). TEFF appear in the recirculating pool (i.e., the thoracic duct lymph and the blood) (Fig. 7h) on their way to target organs.

TEFF and TEM down-regulate CCR7 and L-selectin and, instead, acquire tropism to either the gut or skin *via* tissue-specific homing receptors (Mora and von Andrian, 2006). In mice, gut-tropic TEFF and TEM migrate preferentially to the lamina propria of the small intestine and express high levels of the integrin $\alpha 4\beta 7$ and the chemokine receptor CCR9. The principal $\alpha 4\beta 7$ ligand, MAdCAM-1, is expressed in venules of the gut lamina

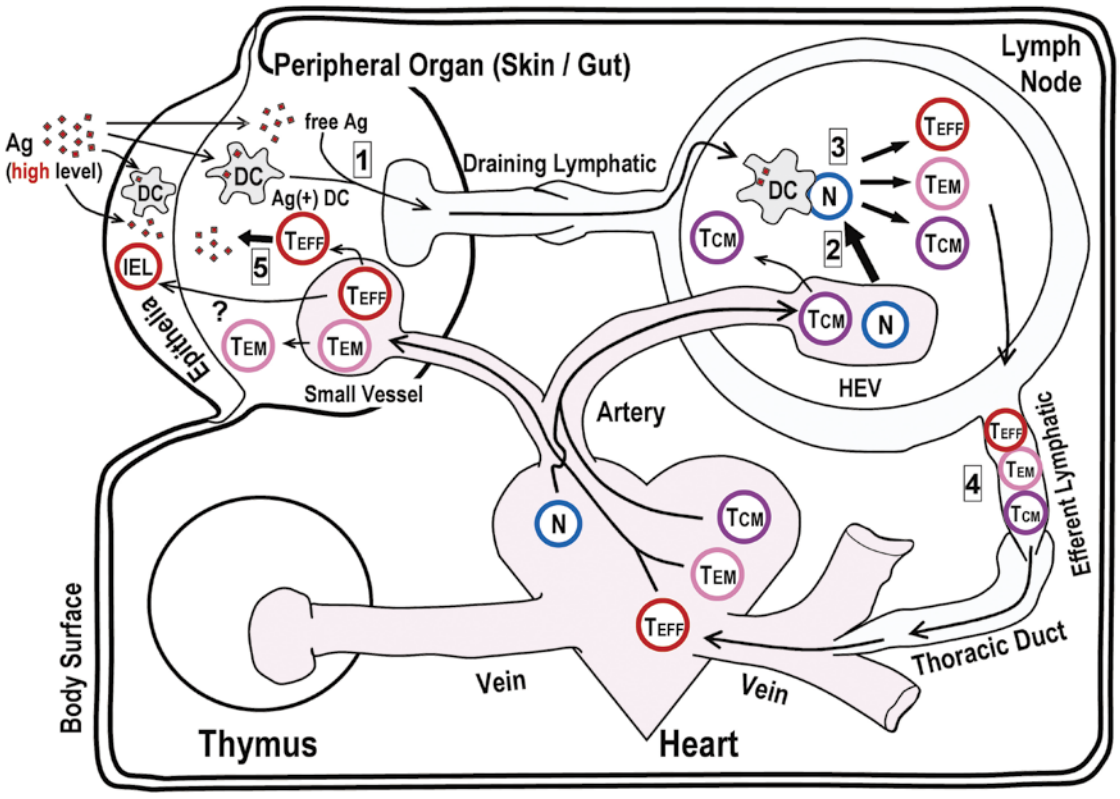

Fig. 10. A diagram depicting the trafficking of effector T cells (TEFF), effector memory T cells (TEM), central memory T cells (TCM), and antigen-transporting dendritic cells (DCs) in emergency states with high levels of antigen entry. Numbers in the square indicate sequential events from antigen entry to migration of TEFF and TEM to the target organs. Blood circulation is colored pink and the lymph pathway light blue. IEL: intraepithelial lymphocyte.

propria (Iizuka *et al*., 2000). The CCR9 ligand CCL25 is strongly expressed by epithelial cells in the small intestine and in lamina propria venules. Mice skin-tropic T_{EFF} and T_{EM} express E- and P-selectin ligands (cutaneous lymphocyte antigen) and the chemokine receptors CCR4 and/or CCR10. Skin venules also express functional E- and P-selectin constitutively and the ligands for CCR4 and CCR10 (Mora and von Andrian, 2006). In rats, the venules of the gut lamina propria also selectively express MAdCAM-1 (Fig. 5d, e, 8c, d) and skin venules E-selectin (Fig. 8f, e) and P-selectin. In addition, the HEV in the Peyer's patches and mesenteric lymph nodes of rats and mice express MAdCAM-1. These findings suggest the presence of a T cell subset in a steady state that constantly recirculates through the gastrointestinal tract and associated secondary lymphoid organs.

The existence of donor T_{EFF} subsets responsible for the graft-versus-host disease (GvHD) of either the gut (Fig. 8g) or skin (Fig. 8h) is still undetermined. We found that $CD103^+CD8^+$ donor T cells predominantly infiltrated into the gut epithelium and were responsible for the manifestations of intestinal GvHD. Their precursors, $CD8^+\ \alpha4\ integrin^+CD103^-$, are selectively produced in the mesenteric lymph nodes, indicating that this pathology is dependent on the gut lymph nodes (Zhou *et al*., 2008). Furthermore, $CCR9^+$ is upregulated in these lymph nodes (unpublished result). Further studies may facilitate clinical intervention to prevent a serious intestinal complication of GvHD selectively without suppressing the graft-versus-leukemia effectors.

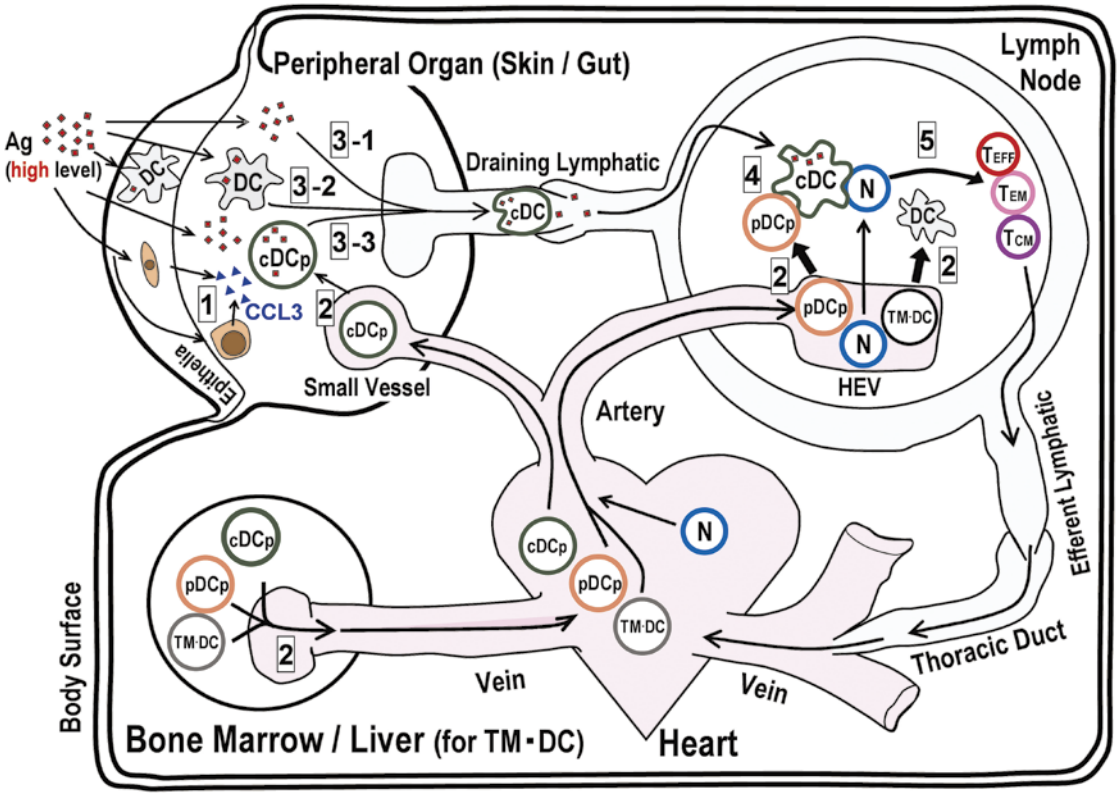

Fig. 11. A diagram depicting the danger signal-induced recruitment and trafficking of conventional and plasmacytoid dendritic cell precursors (cDCp and pDCp) and semimature transmigrating DC (TM-DC). Numbers in the square indicate sequential events from the induction of recruitment to the immune response. Number 3 indicates 3 possibilities of antigen transport: in a free form (3-1), by resident DCs (3-2), and by recruited and remobilized cDC precursors (3-3). N: naive T cell, T_{CM}: central memory T cell, T_{EFF}: effector T cell, T_{EM}: effector memory T cell.

DC trafficking under steady state

Even in the absence of invading pathogens, sentinel DCs constantly leave peripheral organs and enter the draining lymph (Matsuno and Ezaki, 2000). The hepatic lymph of normal rats contains the antigen-transporting DCs that constantly migrate from the liver to regional lymph nodes (Matsuno *et al*., 1995). After entering the lymph nodes, these DCs migrate from the marginal sinus into the superficial cortex at the interfollicular region, which is the same route as that of migrating lymphocytes. These DCs then accumulate in the area beneath the lymph follicle and are considered to become the interdigitating DCs (Matsuno and Ezaki, 2000). This subfollicular area is suggested to be a main site for the immune response in the lymph nodes and proposed as the immune platform (Katakai *et al*., 2004). In the Peyer's patches, DCs are located in the dome area and within the follicle-associated epithelia (Fig.4I). They may also migrate to the interfollicular area (Wilders *et al*., 1983; Hase *et al*., 2009) and then enter the draining lymph and accumulate in the mesenteric lymph nodes (Steer, 1980). These DCs may present environmental antigens to T and B cells (Hase *et al*., 2009).

DC trafficking in response to danger signals

Danger signals induce the accelerated trafficking of DC lineages, resulting in a timely and appropriate immune response. In skin inflammation models, a role of interstitial DCs in the dermis has become increasingly important. A recent report indicates that contact hypersensitivity is induced not by Langerhans cells but by the dermal interstitial DCs, which express macrophage galactose type C-type lectin 2 (Kumamoto *et al*., 2009).

In infection models, conventional DC precursors are recruited to the liver sinusoid in response to intravascular particulates in rats (Matsuno *et al*., 1996) and *Propionibacterium acnes* bacilli in mice, both via CCL3 (MIP1 α)-CCR1/5 chemokine signaling. Kupffer cells produce CCL3 to recruit these cells (Matsuno *et al*., 2002). These precursors capture antigens and migrate to the portal area, and then on to the regional hepatic lymph nodes via lymph, where they induce immune response (Fig.11)(Yoneyama *et al*., 2002).

Bacterial and viral infections in mice also induce a significant number of plasmacytoid DC precursors in the circulation. These cells further transmigrate the HEV and accumulate in the inflamed lymph nodes in a CXCL9- and E-selectin-dependent manner (Fig.11). Tumor necrosis factor- α induces systemic CCL3 secretion but also chemokine up-regulation on HEVs of the lymph nodes (Yoneyama *et al*., 2004). Furthermore, lymph node-recruited plasmacytoid DC precursors have helped DCs in the lymph nodes to induce cytotoxic lymphocytes in a model of cutaneous herpes simplex virus infection (Yoneyama *et al*., 2005).

In rat transplantation models, sentinel DCs in the heart allografts undergo reverse transmigration *via* the graft vessels into the recipient circulation (Saiki *et al*., 2001a). Of note, DCs go to not only the spleen but also the liver and translocate from the liver sinusoid to the hepatic lymph. This is also the case for the lymph DCs at the antigen transporting stage when intravenously transferred (Kudo *et al*., 1997). In the sinusoid, these cells selectively bind to Kupffer cells by using macrophage GalNAc lectin-like receptors (Uwatoku *et al*., 2001a, b). After being released from Kupffer cells, these DCs may enter the Disse's space and migrate to the portal area and finally enter the lymphatics.

Recently, we have reported that the rat liver as well as bone marrow contains a semimature DC population that can systemically transmigrate through blood vessel walls of the recipient secondary lymphoid organs—not only the spleen but also lymph nodes and Peyer's patches (Fig.11). There they quickly mature, and induce the diffuse intrahost $CD8^+$ T cell responses, which may promote graft rejection (Ueta *et al*., 2008). These DCs are distinct from the lymph DCs or sentinel DCs described above because the latter do not transmigrate to the lymph nodes or Peyer's patches from the blood. As blood-borne seeding of DC precursors to the secondary lymphoid organs has been recently suggested (Bonasio *et al*., 2006), this population might correspond to precursors of the interdigitating DCs.

Unsolved questions and future prospects

There are several crucial questions to be solved in this research area. First) can lymphocytes enter the blood *via* the HEVs in the reverse direction? So far, this reverse transmigration has not been reported (Mora and von Andrian, 2006). If yes, this route may become a short cut for the lymphocyte homing to target organs. Second) what are the trafficking molecules in the marginal sinus and metallophilic macrophages of both lymph node and spleen, and those also in the marginal zone bridging channel? This will provide us with crucial information concerning a whole view of trafficking molecules. The third question is which molecules can decide the homing property of effector T cells to non-epithelial organs such as the bone marrow? Further findings on organ-specific trafficking molecules will enable us to treat organ-specific diseases such as ulcerative colitis by the regulation of

pathogenic T_{EFF} and T_{EM} trafficking through blocking of these molecules. Finally, is the role of the Peyer's patches different from that of the mesenteric lymph nodes? This answer will provide important information concerning the mechanism of the oral tolerance and B cell differentiation and the role of each organ for these topics.

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