Lymph Node Stromal Cells: Diverse Meshwork Structures Weave Functionally Subdivided Niches

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Abstract Lymph nodes (LNs) are secondary lymphoid organs that function as the first line of defense against invasive foreign substances. Within the LNs, different types of immune cells are strategically localized to induce immune responses efficiently. Such a sophisticated tissue structure is a complex of functionally specialized niches, constructed by a variety of fibroblastic stromal cells. Elucidating the characteristics and functions of the niches and stromal cells will facilitate comprehension of the immune response induced in the LNs. Three recent studies offered novel insights into specialized stromal cells. In our discussion of these surprisingly diverse stromal cells, we will integrate information from these studies to improve knowledge about the structure and niches of LN.

1 Introduction

Lymph nodes (LNs) are situated at critical positions in the lymphatic vascular system and widely present throughout the body to filter lymph fluid exudate. They are also secondary lymphoid organs that function as the first line of defense against invasive pathogens or foreign substances. LNs continuously monitor the lymph fluid and, if antigens are detected, rapidly induce adaptive immune responses. The sophisticated tissue structure in LN is suitable for this purpose; it comprises several distinct areas in which different immune cell types are strategically localized (Qi et al. 2014). The "functional segregation" of immune cells is believed to optimize spatiotemporal regulation of motile behaviors, which induce efficient responses.

Individual LN has one or more basic structures called "compartments (or lobules)," which comprise two major parts, the cortex and medulla (Belisle and Sainte-Marie

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1981; Gretz et al. 1997; Willard-Mack 2006; Sainte-Marie 2010). The cortex is characterized by a dense lymphocyte accumulation in the hemisphere of afferent lymphatic entry. B cells and T cells are localized in different cortical areas; B cells in the superficial cortex forming multiple follicles, and T cells in the deeper layer called the T cell zone (or paracortex). Upon immune response induction, activated B cells start to construct a specialized structure called the germinal center (GC) in the follicle via the help of follicular helper T (Tfh) cells, which plays a vital role in generating humoral immunity (Victora and Nussenzweig 2012). In a single compartment, individual functional areas occupying a certain spatial expanse with the localization of specific immune cells are classified as "subcompartments" (Takeuchi et al. 2018). Subcompartments are tightly associated with unique non-hematopoietic fibroblastic stromal cells (FSCs) that exhibit specialized roles in organizing niches. Each FSC subset produces specific chemokines and cytokines for immune cells' localization and function (Cyster 1999; Mueller and Germain 2009; Katakai 2016). Importantly, the construction and maintenance of subcompartments require continuous interaction between immune cells and stromal cells (Roozendaal and Mebius 2011). For instance, follicular dendritic cells (FDCs), which are FSC subsets in the follicular center or GC light zone, organize subcompartments via the production of CXCL13 that attracts B cells and also Tfh cells (Allen and Cyster 2008; McHeyzer-Williams et al. 2011). Conversely, lymphotoxin (LT)-$\alpha 1\beta 2$ and TNFα produced from B cells is required for the maintenance of FDC function and tissue structure (Matsumoto et al. 1997).

As of 2016, four major FSC subsets associated with cortical subcompartments have been identified, including FDC, TRC (T zone reticular cell), MRC (marginal reticular cell), and CRC (CXCL12-expressing reticular cells) (Chen et al. 1978; Link et al. 2007; Katakai et al. 2008; Bannard et al. 2013; Katakai 2016). Several additional subsets that do not correlate with a particular subcompartment were also proposed (Malhotra et al. 2012; Mionnet et al. 2013; Sitnik et al. 2016). Such a growing heterogeneity of LN stromal cells led to slight confusion in the field. Meanwhile, previous reports show a tendency of bias toward the structure and function of cortical areas due to their noticeable anatomy and dedication to lymphocyte-centered responses. Stromal cell studies have also focused on cortical subsets, and information in other regions such as the medullary region of LN has been limited until recently.

In 2018, three independent studies showed extensive evidence that expands our knowledge on the diversity of LN stromal cells with findings of novel subsets (Table 1). Rodda et al. identified nine clusters in a single-cell RNA sequencing (scRNAseq) of $CD45^{-}CD31^{-}$ non-endothelial stromal cells (Rodda et al. 2018). Huang et al. proposed seven different populations of fibroblastic reticular cells (FRCs) with multiparameter flow cytometry-based clustering, primarily focusing on the medulla (Huang et al. 2018). Based on anatomical examinations using multiple gene reporter mice, Takeuchi et al. reported two novel FSC subsets in the cortex-medulla boundary and medullary cords, and proposed at least six FSC subsets existing in LNs (Takeuchi et al. 2018). Since these studies took different approaches in terms of methodologies and experimental conditions, the proposals of stromal subsets could contain conflicting classifications.

Table 1 Comparative summarization of recent three reports regarding FSC subsets and tissue subcompartments in LN

Subcompartment	2018 Rodda *et al.*	2018 Huang *et al.*	2018 Takeuchi *et al.* + reanalysis	Markers
B cell follicle (B cell zone) SCS-lining, Outer follicle / Follicular mantle	MRC	$Cxcl13^{+}Ccl21^{-}$ MRC	MRC	Madcam1, Tnfsf11, Cxcl13
		$Cxcl13^{-}Ccl21^{+}$ MRC		
Follicular center / GC light zone	FDC	FDC	FDC	Cr1, Cr2, Cxcl13
Inner follicle / GC dark zone	CRC		CRC	Cxcl12
T cell zone (Paracortex) T zone center	$Ccl19^{hi}$ TRC	$Cxcl13^{-}Ccl21^{+}$ TRC	$Ccl19^{hi}$ TRC	Ccl19, Ccl21, Pdpn
	$Cxcl9^{+}$ TRC			Cxcl9, Cxcl10 Activated?
FTI & IFR (CR)	$Ccl19^{lo}$ TRC	$Cxcl13^{+}Ccl21^{-}$ TRC (BRC)	$Ccl19^{lo}$ TRC	Ch25h, Ccl21
DCP		$Cxcl13^{-}Ccl21^{-}$ TRC	DRC	Il7, Cxcl12, Ccl21
Medulla Medullary Cord	$Inmt1^{+}$ SC	$Cxcl13^{-}Ccl21^{+}$ MedRC	$Lepr^{lo}$ MCRC	Cxcl12
			$Lepr^{hi}$ MCRC	Lepr, Cxcl12, Ccl19
	$Nr4a1^{+}$ SC	$Cxcl13^{-}Ccl21^{-}$ MedRC		Nr4a1 Activated?

Here, we comparatively reviewed these reports with a reevaluation of the scRNAseq dataset, and also histochemical confirmation, to enhance knowledge of the structural and functional significance of LN stromal cells. We defined a "stromal cell subset" as a cell population that constructs a subcompartment, i.e., niche for immune cells. We emphasized anatomical viewpoints to identify the stromal subset. Although single-cell and flow cytometry analyses are powerful approaches, positional information was entirely lost during cell isolation, possibly leading to excess subfractions or inseparable closely related subsets. Therefore, careful confirmation of actual microanatomy and cell/molecular distributions was indispensable.

2 Reevaluating a Single-Cell Analysis of LN Stromal Cells

To resolve discrepancies between the "original" single-cell analysis and other reports, we tried to reanalyze the datasets deposited in a database (Rodda et al. 2018). The results could also be incongruous owing to the use of the latest versions of programs, different from that used for the original analysis (Stuart et al. 2019). From the reanalysis, we successfully identified nine clusters (Fig. 1a); each cluster was assigned to the most probable stromal subset based on marker gene expression. Consequently, we obtained similar results for the majority of clusters as the original report, including FDC, MRC, Ccl19^{hi} TRC, Ccl19^{lo} TRC, Cd34^{+} stromal cell (SC), and perivascular cell (PvC) (Fig. 1b, c, and data not shown). Of note, the reanalysis improved some crucial points compared to the original report; (1) MRC fraction was enriched for Madcam1 expression, and (2) Ch25h expression was detected in Ccl19^{lo} TRC fraction (Fig. 1c; described later).

Reanalysis provided a few clusters with clearly different features from the original report (Figs. 1 and 2). The Cxcl9^{+} TRC cluster, proposed as one of the three TRC subsets (Rodda et al. 2018), became part of Ccl19^{hi} TRC fraction, and another Ccl19^{lo}Ccl21^{+} TRC-like cluster emerged in addition to the Ccl19^{lo}Ch25h^{+} TRCs. As the new Ccl19^{lo} cluster appeared to show the characteristics of a specific stromal cell subset in the cortex-medulla boundary (Takeuchi et al. 2018), we assigned this subset to a new cluster (Fig. 1a; described later). Two clusters differentially expressing Nr4a1 and Inmt were initially designated as stromal cells in the medullary cord (MC) (Rodda et al. 2018). However, in our reanalysis, the expression of these genes was comparable in two candidate medullary clusters (Fig. 1a, b). In particular, Inmt expression corresponded well with these clusters. It was also reported that the leptin receptor (Lepr) is highly expressed in medullary cord reticular cells (MCRCs) (Takeuchi et al. 2018). As the two clusters showed enrichment for Lepr expression, we assigned these as MCRCs (Fig. 1a, b). We also noticed that MCRC clusters could be distinguished by the expression level of Lepr, which was well correlated with the histological examinations of LNs (Fig. 2c). Accordingly, we named these Lepr^{hi} MCRC and Lepr^{lo} MCRC, respectively (Fig. 1a; described later). We will not provide details about CD34^{+} SC and PvCs, since these stromal cells are observed in the capsule or surroundings of blood vessels that we do not consider to be subcompartments. Taken together, we propose a current model for subcompartments associated with stromal cell subsets in LN (Fig. 3, Table 1). We will introduce each subset one by one below.

3 B Cell Follicle

Follicles are B cell aggregates located in the outer cortex below the capsule. In this area, three stromal subsets, MRCs, FDCs, and CRCs, provide specialized niches in the outer, center, and inner follicle, respectively. After antigen-specific activation, B

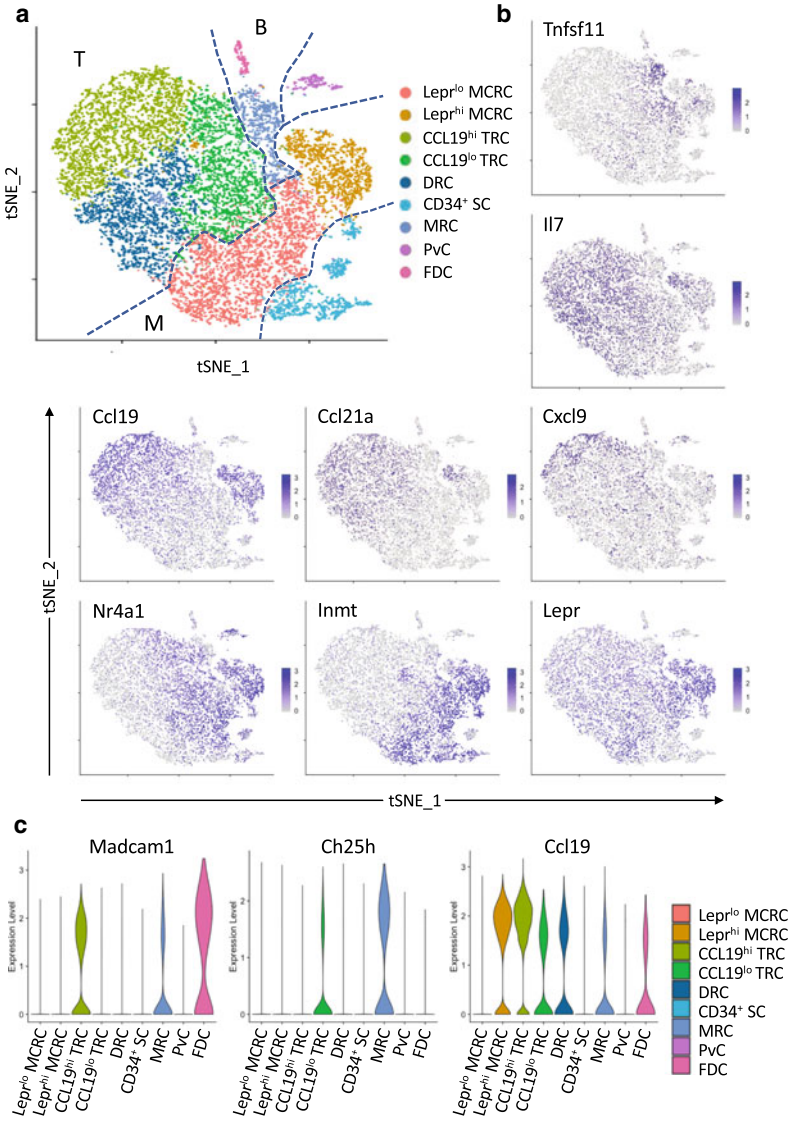

Fig. 1 Identification of 9 clusters by reanalyzing single-cell datasets reported by Rodda et al. 2018. **a** Clustering reanalysis visualized with tSNE. Each dot indicates a single cell that is colored by each cluster categorized. B: B cell zone, T: T cell zone, and M: Medullary cord, respectively. **b** Feature plot of indicated genes. Distribution of gene expression levels is projected on to tSNE plots. **c** Violin plots of Madcam1, Ch25h, and Ccl19 expression in each cluster. Reanalysis of scRNAseq datasets deposited in the GEO database (ID code: GSE112903) was performed using R (version 3.6.2) and Seurat package (version 3.1.0) (Stuart et al. 2019). Default parameters using 10 principle components (PCs) that were determined by the results of JackStrawPlot and ElbowPlot programs set for the analysis. The FindClusters program (resolution = 0.4) finally led to nine clusters, as shown in (**a**)

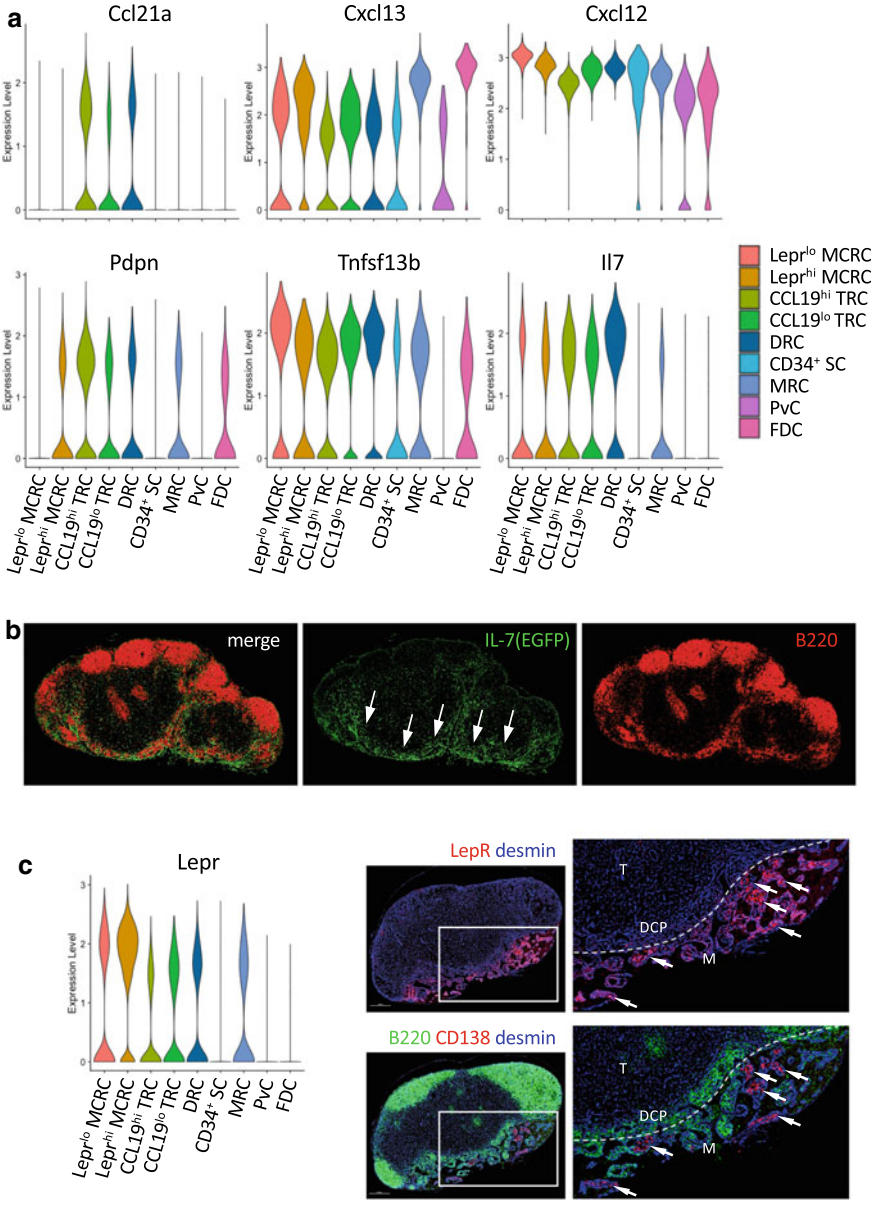

Fig. 2 Novel characteristics in TRC and MCRC subsets. **a** Violin plot of gene expression related to TRC subsets, **b** DRC is a high producer of IL-7. Section of a brachial LN from IL-7 GFP knock-in mouse (Hara et al. 2012) was stained for B220 and desmin. Arrows indicate the DCP area. **c** Expression pattern of Lepr in LN. Violin plot of Lepr expression (left) and immunohistochemistry of LN (right). Section of a cervical LN from C57BL/6 wt mouse was stained for Lepr and desmin (upper right) or B220, CD138, and desmin (lower right). Arrows in higher magnification views indicate the MCs with high Lepr staining colocalized with $CD138^+$ plasma cells

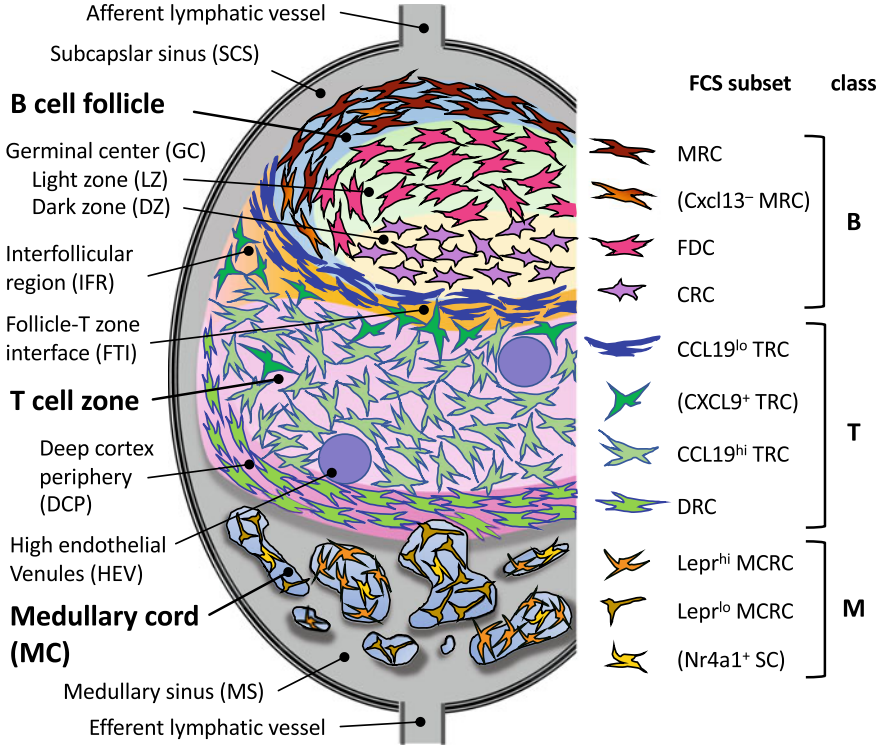

Fig. 3 A proposed model of tissue structure and FSC subsets in LN. Various types of stromal cells construct unique subcompartments, which are highly organized and functionally specialized niches for localized immune cells. The representative basic unit of a single "compartment (lobule)" composed of a single B cell follicle with GC, T cell zone, and medulla is shown. B: B cell zone, T: T cell zone, M: medullary cord

cells start to proliferate, giving rise to the GC in the follicle (Victora and Nussenzweig 2012). Functionally, GC is subdivided into two areas: the dark zone (DZ) where B cells undergo hyperproliferation and somatic hypermutation (SHM) in the B cell receptor (BCR) gene, and the light zone (LZ) in which B cells expressing high-affinity BCR are selected and differentiated into antibody-producing cells (Allen et al. 2007). GC development leads to the production of FDCs and CRCs to support the LZ and DZ, respectively, for the organization of highly specialized niches.

3.1 Marginal Reticular Cell (MRC)

MRCs are situated in the outermost part of follicles just below the subcapsular sinus (SCS), where afferent lymphatic inflow occurs in LNs (Katakai et al. 2008). MRCs express CXCL13, MAdCAM-1, and RANKL; expressions of these genes are

enriched in the corresponding cluster in single-cell reanalysis (Figs. 1b, c, and 2a). This subset is likely to descend directly from lymphoid tissue organizer (LTo) stromal cells (Hoorweg et al. 2015; Katakai 2012) and have the potential to differentiate into FDCs after proliferation (Jarjour et al. 2014). The specific localization of MRCs at the front line of antigen entry suggests a non-redundant role in forming a niche for a defensive barrier.

Several new findings of MRC function were recently reported; RANKL production in MRC is involved in the differentiation of sinusoidal macrophages (SMs) (Camara et al. 2019). Two types of SMs are positioned in the lymphatic sinuses of LNs; SCS macrophages (SSMs) capture lymph-borne antigens in a non-degradative way and transfer them into follicles across the SCS floor (Carrasco and Batista 2007; Phan et al. 2007), while medullary sinus macrophages (MSMs) exhibit robust phagocytosis and release inflammatory cytokines upon immune responses (Chatziandreou et al. 2017). Most SMs, in particular SSMs, disappeared in FRC-specific RANKL deficiency, suggesting the critical role of RANKL signaling in SM differentiation (Camara et al. 2019). Given that antigens, transferred by SSMs into follicular parenchyma, are further transported by migrating B cells up to FDCs (Phan et al. 2009), MRC production of CXCL13 is likely to be important in this process via facilitating B cell motility.

From anatomical viewpoints, Huang et al. classified FRCs into three subpopulations; MRCs ($BP3^+MAdCAM\text{-}1^+$), TRCs ($BP3^+MAdCAM\text{-}1^-$), and medullary reticular cells (MedRCs; $BP3^-MAdCAM\text{-}1^-$). Using multiparameter flow cytometry-based clustering, they further subdivided MRCs into two populations; $Ccl21^-Cxcl13^+$ MRC and $Ccl21^+Cxcl13^-$ MRC (Huang et al. 2018) (Table 1). However, the $Ccl21^+Cxcl13^-$ subset was undetectable in single-cell reanalysis because almost all the cells in the MRC cluster highly expressed CXCL13. Additionally, MAdCAM-1 expression was detected only in a small fraction of cells (Fig. 1c), even though most MRCs express MAdCAM-1 in immunohistochemistry. As the discrepancies may arise from a difference in the definition of subsets or methodology, it would be essential to confirm the correlation of actual microanatomy and molecular expression.

3.2 Follicular Dendritic Cell (FDC)

FDCs form a dense meshwork in the follicular center or GC-LZ with a unique antigen-presenting property for B cell affinity maturation. They show a distinct transcriptional profile among all FSC subsets in LNs (Rodda et al. 2018). These cells are characterized by the expression of CR1/CD35, CR2/CD21, FcγRIIB/CD32, FcεRII/CD23, MFGE8, MAdCAM-1, and deposition of complement components (Allen and Cyster 2008; Humphrey and Grennan 1982; Tew et al. 1982).

FDCs have been focused on crucial roles in antigen-specific antibody production, presenting immune complexes (IC) to B cells via complement- and Fc- receptors (Papamichail et al. 1975; Tew et al. 2001). Mice deficient in the Cr2 gene show impairment in antigen uptake and reduced responses to low dose or adjuvant-fee

antigens, despite retaining GC formation and affinity maturation (Roozendaal and Carroll 2007). They are also deficient in producing serum antibodies and long-lived antibody-forming cells (Barrington et al. 2002). Since internalization via CR1/CR2 keeps antigens intact in FDCs owing to non-degradative recycling and displaying with periodic intervals, this process is assumed to play a crucial role in long-term antigen presentation. However, analysis of FcγRIIB-deficient FDCs demonstrated that this Fc receptor is dispensable for antigen retention (Barrington et al. 2002; Roozendaal and Carroll 2007; Victora and Nussenzweig 2012). Instead, FcγRIIB may be critical in modulating FDC activities associated with trapping IgG-antigen ICs to maintain humoral immunity, recall responses, or both (Barrington et al. 2002; Carroll and Isenman 2012).

Chemokines and cytokines produced by FDCs are necessary to control the GC microenvironment. CXCL13 functions as a chemoattractant for B cells and Tfh cells expressing CXCR5 (Allen and Cyster 2008; McHeyzer-Williams et al. 2011). This chemokine induces the production of LTαβ and TNFα from B cells, which in turn facilitates CXCL13 production by FDCs, driving a positive feedback loop in maintaining follicular structure (Ansel et al. 2000; Cyster et al. 2000). IC-binding onto FDCs enhances IL-6 production, which further promotes B cell SHM and IgG production as well as GC development (Wu et al. 2009). FDCs are also the source of BAFF that is required for the development and survival of B cells (Schneider et al. 1999). Mice deficient in BAFF could not sustain initial GC response due to defects in FDC maturation and lack of the ability to trap and retain ICs (Rahman et al. 2003; Vora et al. 2003). Thus, the engagement of B cells and FDCs induces mutual activation to make a niche for antigen-specific antibody production.

3.3 CXCL12-Expressing Reticular Cells (CRC)

The DZ in GC is a niche for hyperproliferation and SHM of GC-B cells, so-called "centroblasts." Centroblasts express CXCR4, while the ligand CXCL12 is abundant in the DZ (Allen et al. 2004, 2007). CRC was identified as a stromal subset expressing CXCL12 and forming a network in DZ (Bannard et al. 2013). In the primary follicles, CRCs are positioned in close vicinity to the T cell zone suitable for the early stages of GC polarization (Bannard et al. 2013). Although CRCs and FDCs are morphologically similar, CRCs show undetectable or low expression levels in some FDC markers, including CD35 and distinct profile of chemokine in terms of CXCL12 and CXCL13, suggesting that two subsets could provide distinct niches within GCs. CXCL13 production in LZ controls the positioning of GC-B cells, whereas CXCL12 production in DZ is important for attracting CG-B cells from LZ. CXCR4-deficient B cells are unable to access to the DZ, leading to reduced BCR mutation. Interestingly, the proliferation of centroblasts and differentiation into centrocytes do not require DZ access; however, spatial segregation of two niches provides an optimal environment for effective GC responses (Bannard et al. 2013; Rodda et al. 2015).

In single-cell analyses, CRCs could not be detected as an individual cluster (Rodda et al. 2018) (Fig. 1a). FDCs and CRCs are presumably more similar to each other compared to other stromal subsets, even though some of the gene expressions are different. A large number of cells are required to distinguish these subsets in cluster analysis.

4 T Cell Zone

The T cell zone is situated in the deeper part of the cortex, where T cells and DCs accumulate. Naïve T cells actively migrate in this area for scanning antigens presented by DCs and are activated when they detect cognate antigens (Bajenoff et al. 2006; Miller et al. 2002). Therefore, the T cell zone is the center of immune surveillance mediated by T cells. The hallmark of FSCs in this area is CCL21 (Ccl21a) expression that attracts T cells and DCs via CCR7 (Luther et al. 2000; Link et al. 2007; Woolf et al. 2007). These stromal cells construct an elaborate network of extracellular matrix fiber bundle ensheathed by them, the structure known as the "conduit." The conduit functions as a highway for transporting small molecules less than 70 kDa into the deep paracortex (Gretz et al. 1997, 2000; Sixt et al. 2005). Historically, stromal cells in the T cell zone have been termed FRCs due to their characteristic morphology. However, since stromal cells with reticular morphology are found in many other places besides the paracortex (Gretz et al. 1997), FRCs in the T cell zone are more specifically called TRCs (Link et al. 2007).

Recent reports independently suggested that TRCs can be further divided into several subpopulations. Based on single-cell analysis, Rodda et al. proposed three TRC subsets, CCL19^{hi}, CCL19^{lo}, and CXCL9^{+} TRCs (Rodda et al. 2018). Huang et al. also showed three subsets, CCL21^{-}CXCL13^{-}, CCL21^{-}CXCL13^{+}, and CCL21^{+}CXCL13^{-} TRCs (Huang et al. 2018). From histological observations, a unique stromal subset expressing CCL21 and CXCL12 was identified in the cortex-medulla boundary (Takeuchi et al. 2018). Here, we discuss the relevance of these reports, together with the reanalysis of the single-cell dataset.

4.1 CCL19^{hi} TRC

CCL19^{hi} TRCs are the most distinct subset expressing typical markers of T zone stromal cells (Rodda et al. 2018) (Figs. 1a and 2a). As CCL19^{hi} TRCs are the primary CCL21 producers, they are likely the equivalent of CCL21^{+}CXCL13^{-} TRC (Huang et al. 2018) (Table 1). CCL21 proteins bound on the surface of TRCs and high endothelial venule through glycosaminoglycans (GAGs) play crucial roles in recruitment and interstitial migration of T cells and DCs (Handel et al. 2005; Woolf et al. 2007). DC migration is also regulated by podoplanin (PDPN), in agreement with the enrichment of PDPN expression in CCL19^{hi} TRCs (Rodda et al. 2018)

(Figs. 1b and 2a). By using C-type lectin receptor CLEC-2 that binds to PDPN, DCs can spread and migrate along with stromal cells (Acton et al. 2014). Additionally, engagement of PDPN and CLEC-2 is necessary to control the TRC network as an optimal niche (Astarita et al. 2015). Under resting conditions, TRCs have less opportunity to encounter CLEC-2^{+} mature DCs, thereby PDPN keeps actomyosin contractility in TRCs. When immune response arises, CLEC-2 on DCs causes actomyosin relaxing via PDPN and allows the TRC network to stretch. CLEC-2–PDPN axis also controls ECM production and deposition by TRCs (Martinez et al. 2019).

CXCL9^{+} TRCs were identified as a cluster in the first single-cell and histological analysis observed in the T cell zone and interfollicular regions (IFRs) (Rodda et al. 2018). They express type 1 IFN, IFN-γ receptors, and some IFN inducible genes. It was also reported that IFN-γ induces CXCL9 production in stromal cells for recruiting memory CD8^{+} T cells to the periphery of the T cell zone (Sung et al. 2012). Accordingly, CXCL9^{+} TRCs are suggested to be an activated fraction of CCL19^{hi} TRCs (Rodda et al. 2018). In our reanalysis, CXCL9^{+} TRCs did not form an independent cluster, but instead, they were mostly included in the Ccl19^{hi} TRC fraction (Fig. 1a, b). Thus, it is still unclear whether CXCL9^{+} TRCs constitute an individual subset for constructing a specialized niche.

4.2 CCL19^{lo} TRC

CCL19^{lo} TRCs express several genes characteristics of both in the T cell zone and follicles, such as CCL21, IL-7, and CXCL13 (Rodda et al. 2018) (Figs. 1b and 2a). Additionally, CCL19^{lo} TRCs highly express BAFF (Tnfsf13b), a cytokine critical for B cell survival. Therefore, it is reasonable that CCL19^{lo} TRCs possibly reside in the IFR and follicle-T zone interface (FTI), making a niche for engaging B and T cells. Previous reports showed that activated B and T cells express EBI2 (Gpr183), a receptor involved in guiding B cells to the outer follicle (Li et al. 2016; Pereira et al. 2009). An EBI2 ligand, 7α,25-dihydroxycholesterol (7α,25-HC) is synthesized by cholesterol-25-hydroxylase (Ch25h) (Hannedouche et al. 2011; Liu et al. 2011; Yi et al. 2012; Li et al. 2016) that is detected in the surrounding of follicles; IFRs and FTI, as well as the SCS-lining (Rodda et al. 2018). Reanalysis of single-cell datasets successfully detected Ch25h expression in CCL19^{lo} TRCs as well as MRCs (Fig. 1c). These findings suggest the perifollicular distribution of CCL19^{lo} TRCs.

Huang et al. identified CXCL13-producing TRC (CCL21^{-}CXCL13^{+} TRC) (Huang et al. 2018). Several reports suggest that CXCL13^{+} FRCs reside in the outer follicle and co-express BAFF, Delta like-4 (DL-4), and Ch25h (Cremasco et al. 2014; Fasnacht et al. 2014; Malhotra et al. 2012; Mionnet et al. 2013; Yi et al. 2012). Huang et al., therefore, proposed to call them "B zone reticular cells (BRCs)" (Huang et al. 2018). However, the anatomical location and gene expression profiles suggest that this population is likely to be CCL19^{lo} TRCs (Table 1). The IFR and FTI correspond to a region named the cortical ridge (CR), where a dense reticular network supports

DC–T–B interactions (Bajenoff et al. 2003; Katakai et al. 2004). It will be interesting to determine if the CR has some relevance to CCL19^{lo} TRCs as a functional subcompartment.

4.3 Deep Cortex Periphery Reticular Cell (DRC)

Based on histological examinations, Sainte-Marie et al. reported a tissue layer with dense reticulum at the boundary of the cortex and medulla, named "deep cortex periphery (DCP)" (Belisle et al. 1982; Sainte-Marie 2010). We recently found that B cells accumulate in the DCP in a CCL21- and CXCL12-dependent manner (Takeuchi et al. 2018). Stromal cells in the DCP showed unique properties to form a dense network expressing both CCL21 and CXCL12, allowing us to define them as a new subset named "DCP reticular cells (DRCs)." Interestingly, B and T cells are intermingled in this area, in marked contrast to the follicles. B cells in the DCP migrate at a slower velocity and higher turning angle than that in the follicles, suggesting that DRCs provide a specialized microenvironment. The optimal development of DCP involves B cells, because DCP formation was attenuated in B cell-deficient mice but restored by bone marrow transfer. These results suggest that DRCs require signals from B cells to construct the DCP (Takeuchi et al. 2018).

In the first single-cell analysis, no prominent cluster corresponding to DRCs was detected (Rodda et al. 2018). However, in our reanalysis, another CCL19^{lo} cluster was found in addition to CCL19^{lo}Ch25h^{+} TRCs (Fig. 1a, c). We considered that the new cluster represented DRCs because of high CXCL12 and CCL21 expression (Fig. 2a). Meanwhile, this cluster also showed the highest IL-7 expression in all stromal clusters (Figs. 1b and 2a). Immunohistochemical analysis using IL-7 reporter mice demonstrated that IL-7 expression was prominent in the DCP compared with other parts of LN (Fig. 2b), which convinced us that this cluster could be assigned as DRCs. DRC cluster shared some features with CCL19^{lo} TRCs, such as the expression of BAFF (Fig. 2a). These suggest that the DCP area supported by DRCs is a niche suitable for lymphocyte survival. Contrarily, Ch25h expression was weak in DRCs (Fig. 1c), suggesting that DRCs and CCL19^{lo} TRCs are likely to differ in function during immune responses.

5 Medullary Cord

Lymphocytes are highly concentrated in the cortex, whereas the medulla appears to have relatively low cell density due to the abundance of lymphatic sinus called the medullary sinus (MS). In the medulla, mesenchymal cells are mostly assembled to form the medullary cords (MCs); these are sheath-like parenchymal structures surrounding blood vessels (Ohtani and Ohtani 2008). It was considered that medullary FSCs were distinct from other subsets as this region harbors completely

different types of immune cells, such as macrophages, NK cells, and plasma cells (Gray and Cyster 2012; Hargreaves et al. 2001; Kastenmuller et al. 2012; Katakai et al. 2004). However, their precise nature had remained unclear until recently.

5.1 Medullary Cord Reticular Cell (MCRC)

Huang et al. intensively characterized reticular stromal cells in the MCs (MedRCs) as a new subset with the ability to regulate plasma cell homeostasis (Huang et al. 2018). MedRCs differ from TRCs in terms of lower expressions of CCL21, CXCL13, CCL19, and IL-7, but a higher IL-6. MedRCs are the primary source of IL-6, BAFF, and CXCL12, besides also producing APRIL (Cyster 2005; Mohr et al. 2009; Abe et al. 2012). Among these, IL-6 is especially important for the differentiation and survival of plasma cells, since the inhibition of IL-6, but not BAFF, APRIL, and CXCL12, block those events. On the other hand, homing and localization of plasma cells in the MCs depends on the CXCL12-CXCR4 axis (Hargreaves et al. 2001; Cyster 2005). MedRCs express a higher CXCL12 but lower CCL21 and CXCL13 than other subsets (Fig. 2a). Such a profile of chemokine expression is likely to attract plasma cells and macrophages expressing high CXCR4 but low CCR7 and CXCR5.

Huang et al. suggested that MedRCs are composed of two subpopulations, $CCL21^{+}CXCL13^{-}$ and $CCX21^{-}CXCL13^{+}$ cells (Huang et al. 2018). From a single-cell analysis, Rodda et al. also showed two subsets of medullary stromal cells; $Inmt^{+}$ and $Nr4a1^{+}$ SCs. They suggested a possibility that $Nr4a1^{+}$ SCs are the activated phenotype of other subsets, including $Inmt^{+}$ SCs (Rodda et al. 2018). Interestingly, in the reanalysis of a single-cell dataset, Inmt and Nr4a1 were broadly expressed in two medullary clusters rather than enriched in either of the clusters (Fig. 1a, b). We have independently identified an FSC subset in the MC; the MCRCs categorized as $CXCL12^{hi}CCL21^{low}Lepr^{+}$ cells (Takeuchi et al. 2018). Of note, the two medullary clusters corresponded with $Lepr^{hi}$ and $Lepr^{lo}$ fractions (Figs. 1b and 2c). Histological observations indicated that the MCs could be roughly separated into $Lepr^{hi}$ and $Lepr^{lo}$ areas, which appeared to correlate with the localization of plasma cells and B cells, respectively (Fig. 2c). Therefore, two types of MCRCs possibly represent different niches within the MCs.

6 Concluding Remarks

Integrating the three core reports and other previous knowledge led to a comprehensive picture of LN architecture founded upon stromal cell diversity. We thereby propose the latest model of the stromal structure, in which at least eight FSC subsets tightly associated with subcompartments construct a functionally organized “niche complex” (Table 1, Fig. 3). Additionally, closely related subsets in major areas of

LN can be classified into three upper categories; classes “B (B cell follicle),” “T (T cell zone),” and “M (medulla).”

Within the B cell follicles, the “class B” members MRCs, FDCs, and CRCs support distinct follicular niches. The functions of MRCs are gradually uncovered recently, in particular, the control of niches in the antigenic entry site via RANKL expression. FDCs constitute a prominent structure in the follicular center and GC-LZ, which is reflected by a discrete cluster expressing a variety of specific genes in single-cell analysis. In contrast, even though CRCs are histologically apparent in the inner follicle or GC-DZ, the corresponding cluster is currently undetected in single-cell analysis, probably due to the limited cell number of the datasets.

The “class T” includes FSC subsets in the T cell zone, CCL19^{hi} TRCs, CCL19^{lo} TRCs, and DRCs. The hallmark of these subsets is CCL21 expression. CCL19^{hi} TRCs constitute the center body of the T cell zone by highly producing CCL19 as well as CCL21. In concert with CLEC-2^{+} DCs, PDPN highly expressed in this subset is suggested to control the scale of the stromal network. CCL19^{lo} TRCs are likely the stromal component of the IFR and FTI (CR), providing a niche for B and T cell engagement. DRCs support the DCP area associated with B cell accumulation at the periphery of the deep T cell zone. Although DRCs highly express various survival factors for lymphocytes, it is still unclear why B cells need to be attracted to the opposite side of the follicles near the medulla.

Stromal cells of the medulla could be categorized as “class M” subsets providing a niche for plasma cells and medullary macrophages. Two types of FSCs possibly constitute distinct areas within the MC; Lepr^{hi}CCL19^{+} MCRCs and Lepr^{lo} MCRCs appear to correlate with the accumulations of plasma cells and B cells, respectively. There are some discrepancies between reports; however, the MCs are presumably composed of more than one FSC subset.

The subset composition of LN stromal cells in its entirety is surprisingly complicated and diverse to form functionally different subcompartments. Such a stromal cell diversity is probably indispensable to control elaborate and flexible immune responses. We speculate that the feature of each subset is not uniform nor stable but has overlaps and interchangeable property with neighboring populations. We, therefore, assume that it is possible to discover more FSC types or subsets with intermediate property, especially in the boundary of subcompartments. It is also possible that the condition of stromal cells, subset composition, or both, could be readily changed during immune responses or local stimuli. Overall, further in-depth analysis will be necessary to obtain a universal picture of the diversity and plasticity of LN FSCs.

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